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Defining the Nonreturn Time for Intestinal Ischemia Reperfusion Injury in Mice

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ABSTRACT

Among the abdominal organs, the intestine is probably the most sensitive to ischemia reperfusion injury (IRI), a phenomenon that occurs in many intestinal disorders. Few studies have reported in detail the impact of intestinal ischemia time in mice. We evaluated the effect of various warm intestinal ischemia times in an intestinal IRI model in mice. Adult male Balb/c mice were divided into 4 groups that differed in intestinal ischemia time: G1, 30; minutes; G2, 35 minutes; G3, 40 minutes; and G4, 45 minutes. Histological evaluation showed average Park scores as follows: G1 0.6 ± 0.55; G2 1.8 ± 0.45; G3 4.8 ± 2.25; and G4 5 ± 1.79. All animals from G1 survived 30 hours. G2 animals showed intermediate behavior with all succumbing between 18 and 30 hours postprocedure. G3 and G4 displayed similar survival results with animals succumbing before 6 hours after intestinal reperfusion. These data showed that Park index scores of 3 or higher were related to early death. We concluded that the 5 minutes between 35 and 40 minutes is the critical limit, after which all mice die after reperfusion. This result may represent a valuable tool for future research in mice.

THE INTESTINE is probably the most sensitive among the organs to ischemia reperfusion injury (IRI), a phenomenon that occurs in many disorders such as strangulated hernia, volvulus, necrotizing enterocolitis, mesenteric embolic, procoagulant activation, and intestinal transplantation.[1]

During ischemia, the organ deprived of oxygen consumes and depletes adenosine triphosphate (ATP) and other metabolic energy products. Simultaneously, active transmembrane ion transport mechanisms operate at reduced rates, causing ion imbalance within cells.[2] The ATP depletion that occurs during ischemia causes accumulation of hypoxanthine, the substrate of Xanthine Oxidase, which is responsible for the production of reactive oxygen species during reperfusion.[3] Therefore, although a blood supply is essential for tissue survival, restoration of blood flow to the ischemic intestine may paradoxically exacerbate the tissue injury.

The reperfusion phase is characterized by increased production of oxygen and nitrogen free radicals by activation of the Xanthine Oxidase system among others, contributing to cell and tissue damage.[4]

The intestine is composed of labile cells that are easily damaged during episodes of ischemia followed by reperfusion. Enterocytes located at the tip of the villi are more sensitive to ischemic insults than those residing at the crypt base.[5] Therefore, IRI may alter the integrity of the mucosal enteric barrier, facilitating bacterial translocation and sepsis. In addition during reperfusion, proinflammatory factors such as tumor necrosis factor (TNFα) and other cytokines are synthesized, contributing to local and systemic inflammatory responses leading to distant damage to organs such

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as the liver and lungs,[6] predisposing to multiorgan failure and death.[7]

It has been reported that length of ischemia is reflected in the severity of intestinal damage. However, in murine models, few studies have detailed the impact of intestinal ischemia followed by reperfusion. Despite disadvantages in surgical management, mice offer several advantages in terms of availability of genetically defined lines and selective reagents for further research.

The aim of the present study was to evaluate the effects of various warm intestinal ischemia times on the local and remote target tissue damage during immediate reperfusion as well as on host survival.

MATERIALS AND METHODS

Animal Use and Care

Fifty adult male Balb/c mice of average weight 30 ± 3 g were housed in a climate-controlled room with 12-hour light-dark cycles. They were fed standard laboratory chow and allowed water ad libitum. All the experiments were performed according to the guidelines set by the National Institutes of Health (NIH publication, Vol 25, No 28, revised 1996).

Surgical Procedure

The mouse model of intestinal IRI used occlusion of the superior mesenteric artery (SMA) using a microvascular clamp.[8] Mice anesthetized with intraperitoneal injection of Ketamine (100 mg/kg), Diazepam (5 mg/kg), and Atropine (0.04 mg/kg) were placed in the supine position. The surgical area was prepared by placing towels, and the corneas protected with eye ointment. We administered 50 IU of Heparin diluted in 1 mL normal saline subcutaneously and Lidocaine (10 mg/kg) into the skin and subcutaneous tissue. Antisepsis used 70° alcohol. Animals were subjected to a celiotomy with intestinal loops lateralized to the left flank using wet swabs and protected with gauze moistened with warm saline solution. Microsurgical instruments and a surgical microscope were used to magnify the field (×10). The SMA was isolated and occluded with a vascular clamp for various periods. We verified correct placement of the clamp by discoloration and pallor of the jejunum and ileum. Dissection of the SMA must be adequate, bearing in mind that it is closely associated with other blood vessels such as the celiac trunk.

After the stipulated time, the clamp was removed; the intestine returned immediately to its normal color. The abdomen was closed with 5-0 nylon. Finally, subcutaneous warm saline solution (2 mL) was administered and topical Lidocaine applied to the musculocutaneous incision for pain control.

Mice recovered until sampling on a thermal blanket to prevent hypothermia, one of the leading causes of death in mice subjected to surgical procedures.

Experimental Groups

Animals were divided into 5 groups that differed in intestinal ischemia time: Group 1 (N = 11) had the SMA occluded for 30 minutes; group 2 (N = 11), 35 minutes; groups 3 (N = 11) and 4 (N = 11), 40 and 45 minutes, respectively. Once the stipulated ischemia time in each group was over, the clamp was removed. Thirty minutes postreperfusion we obtained samples of liver, lung, and intestine from 5 animals in each group. We obtained samples from the distal jejunum and proximal ileum of the gastrointestinal tract because of their high sensitivity to IRI. Samples fixed, in 10% formaldehyde were dehydrated, and embedded in paraffin. Sections stained with hematoxylin-eosin were evaluated using Park's score.[9] Microscopic observations of liver and lung were performed to evaluate remote tissue damage. The remaining animals in each group were returned to the facility for observation at 3, 6, 12, 18, 24, and 30 hours after reperfusion to measure host survival. According to the criteria used in other published studies, we considered long-term survivors to be animals that reached 16 hours after reperfusion.[10] The final group (N = 6) was a sham cohort in which anesthesia was followed by celiotomy but no vascular occlusion. Three of 6 animals were sampled at 75 minutes after laparotomy (simulating 45 minutes of ischemia plus 30 minutes of reperfusion); survival was analyzed in the remaining 3 hosts.

Statistics

Descriptive statistics for the degree of IRI were reported as mean values ± standard deviation (SD). The Kaplan-Meier method was used for actuarial survivals with comparisons using the log-rank test, which was considered significant when $P \leq .05$.

RESULTS

Histopathology

Intestinal damage was scored according to Park's classification. The sham group showed normal histology of the jejunum and ileum. In group 1, the histological evaluation showed an average score of 0.6 ± 0.55, (median, 0; range, 0–1) corresponding to 75% of the cases analyzed. In this group the most common observation was extracellular edema located at the tip of the villi. The second group showed a median value of grade 2 injury (average, 1.8 ± 0.45). Although they displayed extracellular edema that involved the entire length of the villi below the enterocyte layer, there was preserved crypt-villi architecture. Group 3 showed a much greater degree of injury compared with the previous groups (Park score, 4.8 ± 2.25 [median, 3]), with erosions of enterocytes in 3/5 samples. The remaining 2 samples showed even greater degrees of damage with complete loss of villi and infarction of the crypts. Group 4 revealed even more compromised histology (average, 5 ± 1.79; median, 4), with 75% of samples in the range of 3 to 8. The injury included areas of extensive denudated villi and mucosal atrophy (Fig 1).

Despite the histological intestinal injury, there were no liver abnormalities. However, moderate lung injuries were observed in all groups subjected to SMA occlusion for at least 30 minutes postreperfusion, namely, infiltration of polymorphonuclear leukocytes into the alveolar septal wall, which was increased in thickness. The degree of lung injury was similar in all groups except the sham hosts, which showed normal lung histology (Fig 2).

Survival

Differences in survival were observed according to the procedure. All sham and group 1 animals survived for 30 hours. Group 2 animals showed an intermediate behavior,

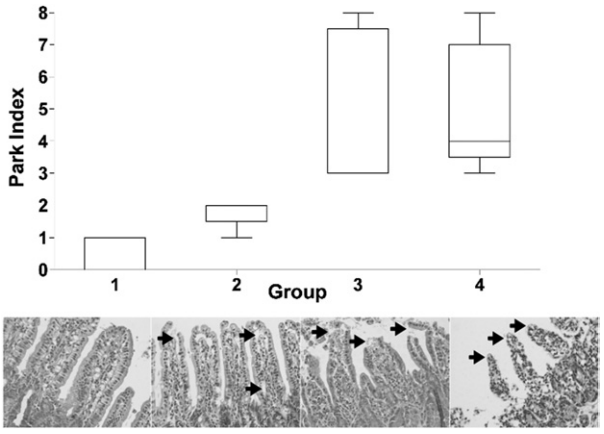

Fig 1. Analysis of Park Index according to ischemia time. Groups 1 to 4 correspond to 30, 35, 40, and 45 minutes of ischemia, respectively, as defined in Matherials and Methods. Median, 50th percentile, and 75th percentile are represented as a box-plot. Histological aspects of jejunum and ileum of mice subjected to IRI after hematoxylin-eosin staining (original magnification ×10). Arrowheads indicate characteristic lesions observed such as extended subepithelial space (group 2), loss and detachment of enterocytes (group 3), and denuded villi without enterocytes (group 4).

all succumbing between 18 and 30 hours postsurgery: 4 between 18 and 24 hours, and 2 between 24 and 30 hours. Groups 3 and 4 displayed similar survival results with the animals succumbing before 6 hours of intestinal reperfusion: 2 died between 0 and 3 h and the remaining hosts between 3 and 6 hours. No animal among these groups was a long-term survivor (Fig 3). There was a significant difference observed among animals with less than 35 minutes of ischemia.

DISCUSSION

IRI causes morbidity and mortality in various intestinal diseases. During intestinal transplantation the graft is necessarily subjected to IRI, a step that cannot be avoided.[11] It has been reported that the ischemic period either by itself or when followed by reperfusion is decisive in terms of intestinal functional and structural damage.[12] However, the time to cause lethal intestinal damage, affecting postreperfusion survival, is unclear.

The model used in the present study demonstrated that the 5 minutes between 35 and 40 minutes of ischemia represent a critical limit after which all mice will die upon reperfusion. Park's evaluations of groups 1 and 2 showed edema but no erosion of enterocytes. This level of injury allowed animals to achieve prolonged survival. Histopathologic analyses performed in groups 3 and 4 that experienced more than 35 minutes of ischemia showed epithelial detachment and villus loss that could allow passage of endotoxin and bacteria into the systemic compartment causing remote damage and subsequent death. No animals among these groups achieved prolonged survival. These data showed that Park index scores of 3 or higher were related to early death. Data previously published by others and previous not published results suggested that endotoxemia was the cause of postreperfusion death.[13]

Using C3H/HeJ mice, which have a spontaneous mutation in Toll-like receptor 4 leading to resistance towards membrane lipopolysacaride (LPS),[14] we observed a 100% survival rate at 30 hours after surgery despite 35 minutes of intestinal ischemia. These results supported the importance of LPS translocation as a cause of death among mice that underwent intestinal IRI events.

All groups subjected to intestinal ischemia showed the same degree of lung injury after immediate reperfusion. To evaluate whether ischemia time affects lung damage, it would be appropriate to obtain samples further after the onset of reperfusion.

When comparing our results with those obtained using Balb/c mice or rats, mice are more susceptible to IRI. Sileri et al[15] obtained an 85% survival rate at 24 hours after reperfusion in rats that underwent 45 minutes of intestinal ischemia. These data show a significant difference between species.

Despite the technical challenge of performing this procedure in mice, the proposed model was reproducible and appropriate for future investigations to improve our understanding of strategies to reduce and to develop IRI phenomena,[16–18] using transgenic or knock-out animals.

The present study showed that ischemia time was a crucial factor associated with the histopathologic damage

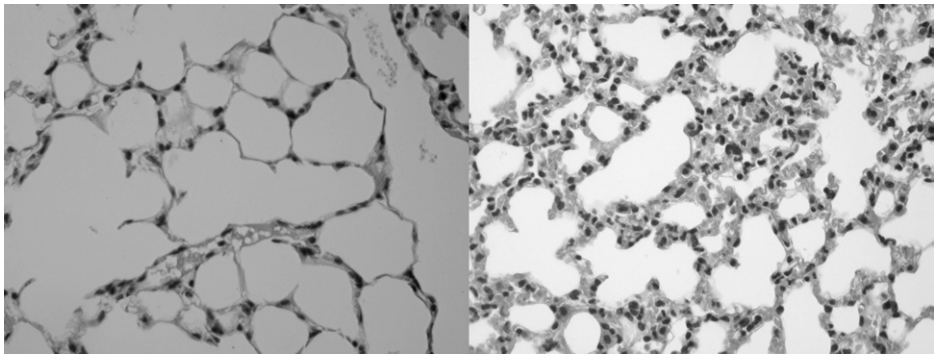

Fig 2. Morphological studies of lungs (hematoxylin-eosin; ×40). Sham-operated mice demonstrating no lung injury (left image). Alveolar walls increased in thickness and neutrophil infiltration (right image).

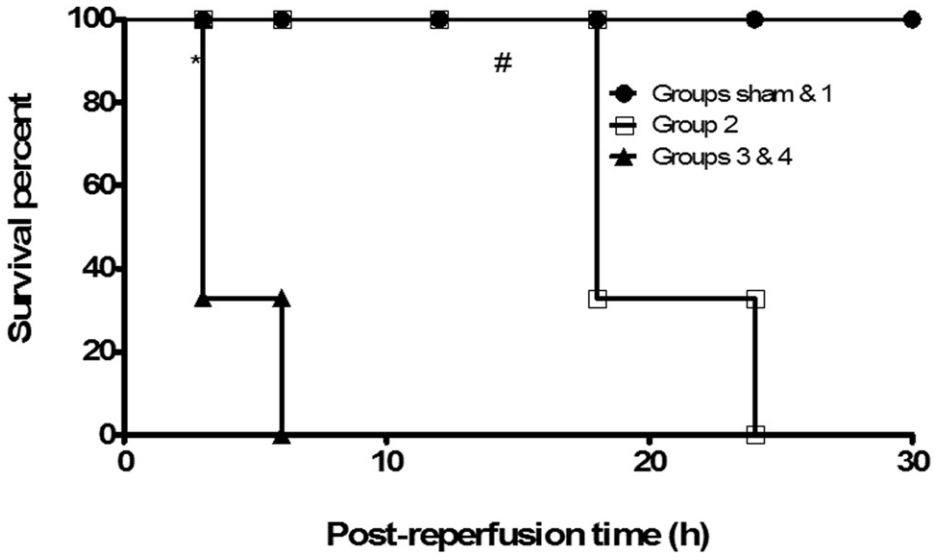

Fig 3. Survival in groups subjected to different times of intestinal ischemia. Log-rank test for survival analysis. (*) $P < .001$ vs group 1 and 2. (#) $P < .001$ vs sham and group 1.

and degree of survival observed immediately after reperfusion. We showed that 30 or more minutes of intestinal ischemia triggered an inflammatory response and caused histological lung damage. Establishing strategies to mitigate IRI and thereby increase postsurgical survival are major objectives of basic and translational research in intestinal transplantation.

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