A novel model of acute murine hindlimb ischemia

Robert S. Crawford,1 Faraz F. Hashmi,1 John E. Jones,1 Hassan Albadawi,1 Michael McCormack,2 Kyle Eberlin,2 Fateh Entabi,1 Marvin D. Atkins,1 Mark F. Conrad,1 W. Gerald Austen, Jr,2 and Michael T. Watkins1

1Department of Surgery, Division of Vascular and Endovascular Surgery; and 2Division of Plastic Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts

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Crawford RS, Hashmi FF, Jones JE, Albadawi H, McCormack M, Eberlin K, Entabi F, Atkins MD, Conrad MF, Austen WG, Jr, Watkins MT: A novel model of acute murine hindlimb ischemia. Am J Physiol Heart Circ Physiol 292: H830 –H837, 2007. First published September 29, 2006; doi:10.1152/ajpheart.00581.2006.— The McGivney hemorrhoidal ligator (MHL), a band designed to cause tissue necrosis, is the preferred experimental tool to create hindlimb ischemia-reperfusion (I/R) injury in rodents. This report defines and compares the ex vivo band tension exerted by MHL and orthodontic rubber bands (ORBs) along with select in vivo characteristics of I/R. As to method, ex vivo band tension was measured over relevant diameters using a tensiometer. In vivo assessment of murine limb perfusion during ischemia with ORB and MHL was compared using laser Doppler imaging and measurement of wet weight-to-dry weight ratio. Neuromuscular scoring and histological extent of muscle fiber injury after I/R with MHL and ORB were also compared. A dose-response curve, between the duration of ORB-induced I/R with both mitochondrial activity (mitochondrial-thiazol-tetrazolium) or tail perfusion [laser Doppler imaging (LDI)], was generated. As a results, ex vivo measurements showed that ORB exerted significantly less force than the MHL. Despite less tension in ORB, in vivo testing of the ORB confirmed complete ischemia by both LDI and wet weight-to-dry weight ratio. After I/R, caused by ORB, there was significantly less neuromuscular dysfunction. Histological assessment confirmed similar degrees of muscle fiber injury after I/R with either the MHL or ORB. Increasing durations of ischemia created by the ORB followed by reperfusion significantly decreased mitochondrial activity and tail perfusion after 24 h of ischemia. In conclusions, ORB produced similar levels of tissue ischemia in murine models of limb I/R with fewer levels of nonspecific injury. ORB may be the preferred model for selected studies of limb I/R.

ACUTE LIMB ISCHEMIA-REPERFUSION (I/R) injury continues to be a prevalent clinical problem extensively studied in experimental models. Several large- and medium-sized animal models (canine, rabbit, and rat) have been used to study this phenomenon (1–4, 9, 16, 24, 25). The experimental variables employed in these studies are designed to study both the local consequences of I/R as well as the systemic consequences of severe bilateral limb ischemia (29–31).

The McGivney hemorrhoidal ligator band (MHL) is an established model of tourniquet-induced hindlimb I/R injury in mice (17–21, 27, 28). Tourniquet models are frequently used for murine studies of I/R models because experimental models of angiogenesis in mice have demonstrated the need to simultaneously occlude several branches of the femoral and iliac arteries to achieve reliable levels of limb ischemia. The rich collateral blood supply surrounding the murine pelvic girdle provides substantial collateral flow usually from iliac and tail branches, which perfuse the posterior thigh. While popular and widely used, the MHL model has been criticized for its inability to control for nonspecific neuromuscular damage due to the crushing force of the rubber band on the underlying tissue. This problem may be reduced if complete hindlimb ischemia could be induced with minimal tension. To address this issue, our laboratory previously created a model of acute hindlimb ischemia that used a tourniquet attached to strain gauge and a winch (5). This device, the controlled tension tourniquet (CTT), allowed application of minimal amounts of reproducible circumferential tension to create complete hindlimb ischemia. Reperfusion could also be easily achieved by loosening the tension on the winch (5, 6, 10). Using the MHL and controlled-tension tourniquet, we and others have identified local and systemic alterations in genes and their respective transcription factors during I/R (6, 11). Despite the defined reproducible nature of limb ischemia created by the CTT, there are drawbacks to its use. The bulky nature of the CTT, which contains winches, strain gauges, and pulleys, precludes its use for molecular imaging. Many proinflammatory factors common to limb I/R may be noninvasively imaged using molecular bioimaging techniques (12). The CTT is designed to study single-limb I/R, which has not been associated with substantial systemic effects during reperfusion. The CTT limits the number of mice that can be studied at one time to five. Finally, since the tourniquets are not elastic, the tension must be applied manually. For these reasons, the need exists for a compact minimally traumatic device to reproducibly create hindlimb I/R in mice using minimal artifact.

Orthodontic rubber bands (ORBs) are commercially available in a variety of sizes and strengths. They are inexpensive, effective, easily available, and can be applied to the mouse limb using a standard MHL applicator. The experiments in this report were designed to 1) define and compare the ex vivo force generated by the MHL compared with ORBs of different diameters over a 90-min period; 2) determine whether MHL and ORB create similar grades of in vivo limb ischemia as measured by laser Doppler imaging (LDI); 3) correlate the tension produced by the MHL and ORB with neuromuscular assessment of murine hindlimbs; and 4) determine whether limb I/R created by ORB can provide biochemical and histological evidence of muscle damage in response to stepwise increases in ischemia followed by reperfusion. The overall goal

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of these experiments was to determine whether the ORB provides an alternate, specific model of complete hindlimb ischemia by generating significantly less force and nonspecific neuromuscular injury than previously established models.

METHODS

Ex Vivo Force Measurements

ORBs of different strengths [1/8 in. ID; 3.0, 4.0, and 6.0 oz (Masel, Bristol, PA) and 4.5 oz (American Orthodontics, Sheboygan, WI)] were compared against latex rubber bands provided by the distributors of the MHL (George Percy McGown, Brooklyn, NY). Rubber bands were mounted on metal dowels of varying diameters (0.73, 0.86, 1.05, and 1.19 cm). These diameters were chosen based on measurements of the thigh of C57BL6 (22–28 g) mice, aged Apo E\(^{-/-}\) mice, and obese leptin-deficient mice that exhibit many of the inflammatory aspects of atherosclerosis and diabetes in humans. The metal dowels were mounted on a micromanipulator device (World Precision Instruments, Sarasota, FL). The position of rubber bands mounted on metal dowels in the manipulator was controlled by a stage micrometer that allowed movement of the dowel in 1-mm increments. To measure tension, the micromanipulator was secured onto a metal base by a magnetic arm. A tensiometer (Mark-10, C. S. C. Force Measurement, North Agowan, MA) was connected to the rubber band using a hook in a straight line with the mounted rubber bands. After the tensiometer was zeroed, the rubber band was then displaced 1 mm and a measurement (in kg) was recorded. Four different bands were tested in duplicate (at 1-min intervals) for each ORB and MHL. To assess the presence of force decay over time, select bands were left on the 0.73-cm rods for 90 min and remeasured in an identical fashion.

Animal Care Protocol

Animal care and experimental procedures complied with “Principles of Laboratory Animal Care” (Guide for the Care and Use of Laboratory Animals, National Institutes of Health Publication No. 86-23, Revised 1996) and were approved by the Massachusetts General Hospital’s Institutional Review Board. C57BL6 mice (22–28 g) (Jackson, Bar Harbor, ME) were initially anesthetized by intraperitoneal administration of pentobarbital sodium (60 to 90 mg/kg in a bolus of 0.4 ml normal saline). Additional boluses of normal saline (0.3 ml) were given just before ischemia, before reperfusion, and 3 h into the reperfusion period. During the preischemic, ischemic, and initial 3 h of reperfusion intervals, animals were placed on a heating pad to maintain the body temperature at 37°C. For 24-h reperfusion experiments, mice were returned to their cages in the vivarium and were allowed access to water and chow ad libitum. Mice were kept in a 12-h:12-h light-dark cycle, and the room temperature was kept constant between 24–26°C.

In vivo limb ischemia. Thirty minutes after the induction of anesthesia, the McGivney ligator applicator was used to apply the ORB or MHL (Fig. 1A) for periods of hindlimb ischemia for either experiments designed to study ischemia alone or ischemia followed by 4 or 24 h of reperfusion. The ORB or MHL was placed in the proximal thigh, and ischemia was confirmed with LDI (Fig. 1B, see Determination of Tissue Blood Flow). Mice remained anesthetized throughout the duration of ischemia with supplemental anesthesia (pentobarbital

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Fig. 1. A: McGivney hemorrhoidal ligator applicator (MHL). Orthodontic rubber band (ORB) and the MHL fit on the application in identical fashion. B: representative position of the ORB and the MHL on a mouse limb with their respective laser Doppler images (LDIs). Arrow indicates the position of the knee.
sodium) as needed. At the end of the experiment, the animals were euthanized (200 mg/kg pentobarbital sodium ip), and both limbs were harvested, and the skin was removed. For tissue viability, animals were allowed to recover from anesthesia after the ischemic period, and after 24 h of reperfusion, the animals were euthanized and the tissue was harvested. Sham-operated animals were treated exactly as the experimental animals, except that no rubber bands were applied.

**Determination of Tissue Blood Flow**

A laser Doppler imager (Moor Instruments, Wilmington, DE) was used to assess limb perfusion (8, 22). For experiments involving LDI, the fur was completely removed from both hindlimbs with an electric shaver after induction of anesthesia. The laser Doppler source was mounted on a movable rack exactly 10 cm above the mice limbs when the animals were restrained on the warming table. The laser beam (780 nm), reflected from moving red blood cells in nutritional capillaries, arterioles, and venules, was detected and processed to provide a computerized, color-coded image. With the use of image analysis software (laser Doppler perfusion measure, V3.08, Moor Instruments), mean flux values representing tissue perfusion were calculated from the relative flux (in U/cm²) in the areas corresponding to the plantar aspect of the hindlimb or tail.

**Limb ischemia.** Baseline images were obtained from each mouse 30 min after induction of anesthesia. Ischemia was induced with the rubber bands, and another laser Doppler image was obtained 30 min into the procedure to assess for limb ischemia (Fig. 1B). Data were expressed as percent basal perfusion in the limbs.

**Laser assessment of systemic perfusion.** Mouse tail flux (1,380 ± 41 U/cm²) was no different (P = 0.18, ANOVA, n = 6) from upper extremity (1,258 ± 30 U/cm²) or hindlimb flux (1,307 ± 55 U/cm²), and therefore it was used as a surrogate for systemic tissue perfusion. Mouse tails were scanned at four intervals: 1) at 30 min after induction of pentobarbital sodium anesthesia (baseline), 2) at completion of ischemia with no reperfusion under pentobarbital anesthesia (OR), 3) after 4 h of reperfusion (4R), and 4) after 24 h of reperfusion (24R). Sham-operated mice were scanned at the identical conditions, except without placement of the ORB. All mice received additional boluses of normal saline (0.3 ml) just before ischemia, before reperfusion, and 3 h into the reperfusion period. At 4 and 24 h of reperfusion, mouse tails from sham-operated and I/R mice were scanned under isoflurane anesthesia. Isotrufurane was delivered by face mask, 2% for induction, and 1% for maintenance, along with continuous oxygen at 2 l/min. After 4 h of reperfusion, mice were returned to the vivarium and were allowed access to water and chow ad libitum.

**Tissue edema During Ischemia**

Tissue edema during ischemia indicates ongoing arterial inflow in the setting of complete venous obstruction. MHL rubber bands (3.0, 4.0, 4.5, and 6 oz) were applied to murine hindlimbs for 90 min of ischemia without reperfusion. During the ischemic period, mice were examined for evidence of edema. At the end of the ischemic period, limbs exhibiting no gross evidence of edema were harvested from euthanized mice with the rubber bands remaining in place. The ischemic muscle (distal thigh and calf muscle) was isolated, and tissue samples were blotted, weighed, and placed in a drying oven at 55°C until a constant weight was obtained (usually 36–48 h). Muscle edema was quantitated by using the wet weight-to-dry weight ratio (W/D). The W/D of the ischemic limbs was compared with the W/D observed in sham-operated mice. Mice exhibiting gross evidence of tissue edema during this ischemic period were euthanized and not subjected to quantitative analysis.

**Neuromuscular Scores**

A neuromuscular score was assessed by two blinded observers using a modified clinical score previously described (15). Neuromuscular scoring was based on motion below the knee (i.e., the calf muscles) and below the ankle (paw-toe motion) as follows: 0 = full range of motion at the knee (flexion and extension) and paw level (flexion and extension); 1 = diminished flexion and extension of the calf, and paw extension and flexion are intact; 2 = diminished flexion and extension of the knee, paw flexion preserved, and no paw extension; 3 = no flexion of the calf, and no flexion or extension of the paw (paw drop) dragging. Functional recovery at 24 h of reperfusion was compared between the 4.0- and 4.5-oz MHL bands.

**Estimation of Tissue Viability**

Tissue viability was estimated by the reduction of a tetrazolium salt to water insoluble colored formazan crystals by electron carriers and oxidative enzymes in the mitochondria of viable tissue. After reperfusion or sham-operated conditions, mouse limbs were harvested, the skin was removed, and the tissue (distal thigh and calf) was cut into three pieces to piece the surface area and uptake of the tetrazolium salt as previously described (5, 10). Each piece was weighed and placed in a small tube with 3 ml PBS (pH 7.4) supplemented with 300 µl of 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (Sigma, St. Louis, MO). The samples were then incubated for 3 h at 37°C in the dark on a rotating mixer. The samples were then removed, washed with distilled water, and blotted dry. The water-insoluble formazan salt was extracted in 3 ml of 2-propanol for 6 h at 37°C in the dark on a rotating mixer. Aliquots (200 µl) were removed, and the absorbance was determined at 570 nm using a microplate reader. The tissue samples were then dried at 90°C for 24 h. The absorbance was normalized to the dry tissue weight. The viability index was expressed as the percentage of the normalized OD570 in reperfused tissue versus the OD570 detected in sham-operated, nonischemic, and nonreperfused muscle.

**Histology**

Limb sections from mice subjected to 1.5 h of ischemia followed by 24 h of reperfusion, using either the 4.5-oz ORB or the MHL, were fixed in 4% paraformaldehyde for at least 4 h. The gastrocnemius muscle was dissected out, rinsed in Dulbecco’s PBS for 1 h, and dehydrated. The samples were embedded in JB-4 glycomethylmetacrylate, cut cross-sectionally at 2-µm thickness, and stained with Masson trichrome. Stained slides were examined under microscopy at ×20 magnification (NikonE600 upright microscope). Fifteen random fields were photographed by using a cool SNAP color camera and RS image software program (RoperScientific, Tuscan, AZ). Muscle fibers were scored as uninjured or injured based on the morphology of the individual fibers. Uninjured fibers were characterized as having well-defined borders, consistency of texture, and uniformity throughout the fiber without holes or breaks. The satellite cells and pericellular nuclei were easily visualized in uninjured fibers. In contrast, injured fibers had broken or ragged borders and inconsistent texture, color, and holes in the cytoplasm. Nuclei detachment was commonly seen among injured fibers.

**Statistical Analysis**

Statistical analysis was performed with Instat (GraphPad, San Diego, CA). Data were expressed as means ± SE. Comparisons were made using ANOVA and Student’s t-test. A P value <0.05 was considered significant. Post hoc tests included Dunn, Tukey-Kramer, and Mann-Whitney tests.

**RESULTS**

**Ex Vivo Circumferential Tension Measurements**

Within each diameter tested, the MHL bands exerted significantly greater tension (P < 0.001) compared with the 3.0-, 4.0-, 4.5-, and 6.0-oz ORBs (Fig. 2). This tension was as much
as three times the tension exerted by the 4.0-oz ORB. The 6.0-oz ORB exerted an intermediate level of tension, which was significantly greater (+P < 0.01) than that produced by the 3.0-, 4.0-, and 4.5-oz ORBs and less than the MHL bands. The 4.0- and 4.5-oz ORBs exerted similar levels of tension at all diameters tested. Both the MHL bands (**P < 0.01; ***P < 0.05) and the 3.0-oz ORB (P < 0.05) exhibited significant differences in tension between columns of different diameters (P < 0.05). Number of rubber bands.

Effect of diameter on band tension. As the diameter increased from 0.73 to 1.19 cm, there was no significant difference in tension measured by the 4.0-, 4.5-, and 6.0-oz ORBs (P = 0.16, 0.49, and 0.41 via ANOVA, respectively; Fig. 2). At 1.19 cm, MHL exerted less tension than measured at 1.05-cm diameter (0.25 ± 0.006 vs. 0.29 ± 0.008 kg; P < 0.05). At 1.05 cm, MHL exerted significantly greater tension than levels detected at 0.73 cm (0.29 ± 0.008 vs. 0.24 ± 0.001 kg, P < 0.01). At 1.05 and 1.19 cm, the tensions measured in the 3-oz ORB were not different from one another (0.05 ± 0.002 vs. 0.052 ± 0.001, P > 0.05). At both diameters, the ORB exerted less tension than measured at 0.73 cm (0.05 ± 0.002 vs. 0.06 ± 0.002 kg, P < 0.01) and 0.86 cm (0.05 ± 0.002 vs. 0.07 ± 0.001 kg, P < 0.01).

Tension decay. The 4.0- and 4.5-oz ORBs showed no significant decline in the tension after 90 min (Fig. 3). Conversely, the tension generated by the MHL significantly decreased from 0.33 ± 0.006 kg at baseline to 0.28 ± 0.0008 kg after 90 min (P = 0.008).

In Vivo Responses to Hindlimb I/R

Tissue edema. Mice subjected to 90 min of ischemia with 2.0- and 3.0-oz ORBs exhibited gross evidence of tissue edema and thus were not subjected to quantitative analysis. No significant difference in tissue edema was detected between limbs subjected to ischemia alone (Fig. 4A) for the 4.0- (P = 0.17, n = 4), 4.5- (P = 0.13, n = 4), and 6.0-oz (P = 0.92, n = 4) ORBs or the MHLs (P = 0.70, n = 4).
Limb ischemia. The hindlimbs of mice with the 4.0- and 4.5-oz ORBs and the MHLs all showed a significant decrease in absolute flux units compared with their baseline ($P < 0.0001$, $n = 10$; Fig. 4B). There was no significant difference in the degree of tissue ischemia caused by these ORBs and MHLs.

Neuromuscular score. Mice subjected to 90 min of ischemia followed by 24 h of reperfusion with the 4.0- and 4.5-oz bands had equivalent levels of neuromuscular compromise (Fig. 5). Mice subjected to I/R with the MHL had a significantly worse neuromuscular score compared with the 4.0- and 4.5-oz ORB mice ($P < 0.01$, $n = 10–14$). Despite similar levels of tissue ischemia, the MHL causes greater neuromuscular deficits at 24 h of reperfusion. The neuromuscular dysfunction observed following I/R with the MHL may be caused by a combination of crush and ischemic injury.

Histology. After 1.5 h of ischemia and 24 h of reperfusion with either the MHL or 4.5-oz ORB, the number of injured fibers was not different (24.1 ± 1.3 (ORB) vs. 18 ± 5.3 (MHL) injured fibers/high powered field, $P > 0.05$; Fig. 6).

Mitochondrial activity. A graded response to I/R induced by the 4.5-oz ORB was demonstrated by using mitochondrial activity as a measure of viability (Fig. 7). After 1.5 or 2 h of ischemia followed by reperfusion, there was a drop to 78.8 ± 3.6% and 76.2 ± 1.5% of sham-operated mitochondrial activity, respectively. As the duration of ischemia increased, there was a gradual decrease in mitochondrial activity by 24 h of reperfusion. Significant decreases in mitochondrial activity were observed when comparing the 2.5 h (63.7 ± 1.8% sham, $P < 0.05$, $n = 5–7$ vs. 1.5 and 2 h ischemia) and 3 h of ischemia (47.3 ± 2.9% sham, $P < 0.01$, $n = 5–7$ vs. 1.5, 2, and 2.5 h of ischemia) followed by 24 h of reperfusion.

Systemic Effect Of I/R

Mortality. Mortality (number of dead mice/number of mice subjected to an experimental protocol) was 1/12 (8%) in the 3.0/24-h I/R group. No animals died in the sham-operated group (0/8), 1.5/40-I/R group (0/10), 1.5/24 h I/R group (0/12), or 2.5 h-I/R group (0/12).

Fig. 5. Neuromuscular score. The MHL band produced a more compromised (**$P < 0.01$) neuromuscular function after 90 min of ischemia and 24 h of reperfusion compared with both the 4.0- and 4.5-oz ORBs. $n$, Number of mice.

Fig. 6. Histological assessment of skeletal muscle fiber injury. Histological evidence of injury, expressed as percent injured fibers, showed no statistical difference when comparing injury induced with the 4.5-oz ORB vs. the MHL band postbilateral 90 min of ischemia and 24 h of reperfusion.

Representative tail scans. Representative LDI scans from baseline, end ischemia, no reperfusion (0R), 4 h reperfusion (4R), and 24 h reperfusion (24R) are demonstrated (Fig. 8A).

Tail perfusion. At no reperfusion (0R), there was no significant difference in tail perfusion in mice subjected to sham operation or 1.5, 2, or 3 h of ischemia (Fig. 8B). There was no significant difference in tail perfusion within the sham-operated mice and 1.5/24-h I/R groups at 0R, 4R, and 24R.

At 4R, 2.5-h mice showed significant decrease in perfusion versus sham operation (53.2 ± 3.0 vs. 101.1 ± 4.0% baseline, $P < 0.01$) and mice subjected to 1.5 h of ischemia (53.2 ± 3.0 vs. 91.0 ± 9.0% baseline, $P < 0.01$) and sham-operation. At 4R, 3-h mice showed significant decrease in perfusion versus sham operation (46.5 ± 5.3 vs. 101.1 ± 4.0% baseline, $P < 0.01$) and mice subjected to 1.5 h of ischemia (46.5 ± 5.3 vs. 91.0 ± 9.0% baseline, $P < 0.01$). At 24R, tail perfusion in 2.5-h mice was not less than 1.5-h mice (69.0 ± 8.9 vs. 97.2 ± 3.8% baseline, $P > 0.05$). In contrast, at 24R, tail perfusion in 3-h mice remained significantly less than 1.5-h mice (48.2 ± 7.9 vs. 97/2 ± 3.8% baseline, $P < 0.001$).

Fig. 7. Effect of 4.5-oz ORB ischemia-reperfusion on mitochondrial activity. As the duration of ischemia increased, the skeletal muscle mitochondrial activity decreased. $n$, Number of mice. *$P < 0.01$ vs. 1.5, 2, and 2.5 h of ischemia and 24 h of reperfusion; **$P < 0.01$ vs. 1.5, 2, and 3 h of ischemia and 24 h of reperfusion; $n$, number of mice. Values along x-axis represent hours of ischemia/hours of reperfusion.
DISCUSSION

These studies indicate that ORBs produce complete, consistent, and reproducible ischemia in a murine model of I/R using significantly less tension than MHL. In addition, these experiments demonstrate that at comparable degrees of tissue ischemia, ORBs cause less subjective neuromuscular injury than observed with the MHL. Since the ORBs have measurably less circumferential tension, it is possible that there is less nonspecific injury (i.e., crush to nerves and other structures) during the ischemia created with the ORBs. In addition, the ORBs provide similar degrees of mitochondrial dysfunction and systemic hypoperfusion previously described in models of I/R that utilized undefined tourniquet tension.

ORBs are manufactured by several companies for the purpose of providing orthodontic tension under physiological tissues for extended periods of time. ORBs are available with several internal diameters (1/8 – 3/8 in.) and can produce various levels of tension (2.0 – 8.0 oz). The tension generated by the ORBs under different conditions and their reliability have been well studied in the dental literature (13, 14). Because of their unique characteristics, ORBs have found their way into many other applications, including airplane model building.

To determine whether ORBs might be applicable to use in studies of hindlimb I/R, experiments were designed to compare the ORB tension with that of the popular MHL under anatomically relevant conditions. Measurements in our laboratory showed that the diameter of the mice limbs used for our experiments ranged from 0.8 to 1.1 cm (mean = 0.93 ± 0.15 cm; n = 10). Based on this information, the ORBs were mounted on metal dowels of comparable diameters (0.73 – 1.19 cm). These analyses indicated that MHL bands produced significantly more tension than all ORBs tested (Fig. 2). Unlike the ORB, the tension in the MHL varied at the upper end of the diameters tested. In contrast, both the 4.0- and 4.5-oz ORBs exhibited equivalent levels of tension throughout all the diameters tested. As a further ex vivo analysis of tension exerted by the ORB and MHL, the MHL demonstrated significant and consistent deterioration of tension at the 1.05-cm diameter (Fig. 3). Whereas the tension in the MHL deteriorated, the tension in the 4.0- and 4.5-oz ORBs was stable over 90 min of ischemia. These findings indicate a consistent and reliable tension exerted by the ORBs.

Once the ex vivo force measurements indicated that the ORBs provided consistent circumferential tensions, studies were designed to evaluate the ability of the ORBs to maintain limb I/R under experimentally relevant in vivo conditions. In previous experiments with the CTT, ongoing arterial perfusion in the setting of complete venous obstruction was characterized by increased tissue edema before reperfusion (5). When the 3.0-oz ORB was applied to mouse limbs for 90 min, the limbs
became grossly edematous. In contrast to experiments with the 3-oz ORB, there was no gross evidence of edema in the mouse limbs when the 4.0-, 4.5-, and 6.0-oz ORBs and MHL bands were applied. Objective W/D measurements indicated there was no evidence of tissue edema compared with sham-operated animals and with one another (Fig. 4).

Since the W/D values for the 4.0-, 4.5-, and 6.0-oz ORBs were equivalent to MHLs, experiments were undertaken to determine whether the 4.0- and 4.5-oz ORBs could create an acceptable level of ischemia compared with MHLs and were measured by LDI. The 4.0- and 4.5-oz ORBs were also chosen because the goal of these experiments was to create ischemia with the least artifact mediated by crush injury. Based on LDI, the 4.0- and 4.5-oz ORBs produced tissue ischemia equivalent to the MHL in vivo (Fig. 4A), even though the ORB exerts substantially greater tension ex vivo.

To determine whether there was a significant difference in subjective signs of neuromuscular injury in mice subjected to ischemia with ORB versus MHL, a group of mice were subjected to 90 min of ischemia followed by 24 h of reperfusion (Fig. 4B). By 24 h of reperfusion, the ORB mice experienced substantial recovery, but the MHL mice did not. The mice that had the 4.0- and 4.5-oz ORBs retained substantial use of their hindlimbs, whereas the mice whose ischemia was created by the MHL uniformly dragged their limbs and moved almost exclusively by pulling themselves with their front limbs. Thus, for similar levels of ischemia, the MHL mice sustained greater limb dysfunction even after 24 h of reperfusion. We cannot determine whether this ongoing limb dysfunction is related to direct nerve injury or focal crush injury at the site of tourniquet application, but it is unlikely to be related to ischemia since the tissues had similar levels of tissue ischemia. Evidence to indicate similar levels of specific ischemic injury induced by MHL and ORB is provided by microscopic assessment of skeletal muscle. Histological assessment of gastrocnemius muscle fiber injury showed no significant difference between muscle made ischemic with the MHL or ORB (Fig. 8).

Based on the combination of in vivo and ex vivo testing, the 4.5-oz ORB was selected for the assessment of graded durations of I/R injury. In response to a stepwise increase in the duration of ischemia, there was a stepwise decrease in mitochondrial activity, which is an accepted biochemical marker of tissue viability. These tests validate the ORB as a reliable and biologically relevant experimental tool to create limb ischemia with minimal artifact.

The systemic consequences of I/R are well documented in other models (17). Increased intestinal permeability (7, 30, 31), lung edema (23, 26), renal failure (32), and even mortality (29) are among the most prevalent. Relevant models to study the systemic effects of I/R on remote organs and also the effects of the systemic response in exacerbating extremity injury are useful. Most of the previous work has focused on the effect of severe ischemia and short reperfusion times on the systemic response (7, 17). One potential reason for this is the high mortality associated with severe limb ischemia in those models. Yassin et al. (29), in a rat model of bilateral I/R, reported 50% and 60% mortality at 24 and 48 h after bilateral limb I/R. With the use of the ORB model, the hemodynamic effects of bilateral lower extremity I/R are documented by using tail LDI as a surrogate for systemic perfusion and possibly blood pressure. The decrease in systemic perfusion is consistent with and dependent on the duration of ischemia. This decrease in perfusion persists even at 24 h of reperfusion. Interestingly, the mortality associated with hindlimb I/R in the murine model (8%) is less than that observed by Yassin et al. (29). Evidence to suggest that the mice are hemodynamically fragile at the 4R and 24R period was provided by our initial attempts to scan these mice under pentobarbital sodium anesthesia. There was a near 100% mortality under pentobarbital sodium anesthesia with 4R and 24R mice subjected to 2.5 and 3 h of ischemia. Because of these events, we repeated all of the tail perfusion scanning (in ischemic and sham-operated mice) at 4R and 24R under finely controlled inhalational anesthesia. It is possible that the specificity of the ischemic insult (i.e., less crush injury) and the administration of normal saline boluses during I/R influenced this decrease in observed mortality.

In conclusion, ORB were tested and proven to be effective in producing complete ischemic injury in a reproducible manner, creating a minimal amount of nonspecific artifact. This system is inexpensive, portable, and provides the capacity to evaluate the effects of bilateral and unilateral hindlimb I/R. Since ORBs are commercially available in different reproducible tensions, the kind of band used to create ischemia can be modified depending on the size of the mouse limb. Murine mouse limbs are often enlarged in the obese leptin-deficient and aged hypercholesterolemic ApoE/−/− mice that mimic many of the inflammatory features of adult-onset Type 2 diabetes mellitus and atherosclerosis in humans.

GRANTS

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