A Novel Model of Acute Murine Hind Limb Ischemia

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Abstract

The McGivney Hemorrhoidal Ligator (MHL), a band designed to cause tissue necrosis, is the preferred experimental tool to create hind-limb ischemia-reperfusion (IR) injury in rodents. This report defines and compares the *ex vivo* band tension exerted by MHL and orthodontic rubber bands (ORB) along with select *in vivo* characteristics of IR.

**Methods:** *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 (LDI) and measurement of wet to dry ratio. Neuromuscular scoring and histologic extent of muscle fiber injury after IR with MHL and ORB were also compared. A dose response curve between the duration of ORB induced IR with both mitochondrial activity (Methyl-thiazol-tetrazolium-MTT) or tail perfusion (LDI) was generated.

**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 to dry ratio. After IR caused by ORB, there was significantly less neuromuscular dysfunction. Histologic assessment confirmed similar degrees of muscle fiber injury after IR 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 hours of ischemia.

**Conclusions:** ORB produce similar levels of tissue ischemia in murine models of limb IR with less levels non-specific injury. ORB may be the preferred model for selected studies of limb IR.

**Keywords:** Ischemia, Reperfusion, Tourniquets, Skeletal Muscle
Introduction

Acute limb ischemia and reperfusion (IR) 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 IR 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 hind-limb IR injury in mice (17-21, 27, 28). Tourniquet models are frequently used for murine studies of IR 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 non-specific neuromuscular damage due to the crushing force of the rubber band on the underlying tissue. This problem may be reduced, if complete hind-limb ischemia could be induced with minimal tension. To address this issue, our laboratory previously created a model of acute hind limb ischemia which 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, 11). Using the MHL and controlled tension tourniquet, this laboratory and others have identified local and systemic alterations in genes and their respective transcription factors
during ischemia and reperfusion (6, 10). 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 pro-inflammatory factors common to limb ischemia and reperfusion may be non-invasively imaged using molecular bio-imaging techniques (12). The CTT is designed to study single limb IR, 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 5. 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 in order to reproducibly create hind limb ischemia reperfusion in mice using minimal artifact.

Orthodontic rubber bands (ORB) 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 as compared to ORB’s of different diameters over a 90 minute period; (2) Determine whether MHL and ORB create similar grades of in vivo limb ischemia as measured by Laser Doppler Imaging (3); correlate the tension produced by the MHL and ORB with neuromuscular assessment of murine hind limbs; and (4) determine whether limb IR created by ORB can provide biochemical and histological evidence of muscle damage in response to stepwise increases in ischemia followed by reperfusion. 5). The overall goal of these experiments was to determine whether the ORB provides an alternate, specific model of complete hind-limb ischemia by generating significantly less force and non-specific neuromuscular injury than previously established models.

Methods:
**Ex-vivo measurements. Force measurements.** Orthodontic Rubber Bands (ORB) of different strengths (1/8” internal diameter- 3.0 oz, 4.0 oz, 6.0 oz- *Masel Corporation, Bristol PA*, and 4.5 oz- *American Orthodontics, Sheboygan, WI*) were compared against latex rubber bands provided by the distributors of the McGivney Hemorrhoidal Ligator (*George Percy McGown, Brooklyn, New York*). Rubber bands were mounted on metal dowels of varying diameters (0.73, 0.86, 1.05, 1.19 cm). These diameters were chosen based on measurements of the thigh of C57BL6 (22-28 g) mice, obese aged Apo E-/- and 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, 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 zeroing the tensiometer, the rubber band was then displaced 1 mm and a measurement (in kg) was recorded. Four different bands were tested in duplicate (at one minute intervals) for each ORB and MHL. To assess the presence of force decay over time, select bands were left on the 0.73cm rods for 90 minutes 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 86–23, 1985). C57BL6 mice (22-28g) (Jackson Laboratory, Bar Harbor, ME) were initially anesthetized by intraperitoneal (IP) administration of pentobarbital 60 to 90 mg/kg in a bolus of 0.4 ml NS. Additional boluses of NS (0.3 ml) were given just prior to
ischemia, prior to reperfusion and 3hr into the reperfusion period. During the pre- ischemic, ischemic and initial 3 hours of reperfusion intervals, animals were placed on a heating pad to maintain the body temperature at 37°C. For 24 hr. 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 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 (Figure 1A) for periods of hind limb ischemia for either experiments designed to study ischemia alone or ischemia followed by 4 or 24 hr. of reperfusion. The ORB or MHL were placed in the proximal thigh, and ischemia was confirmed with Laser Doppler Imaging (Figure 1B, see methods next paragraph). Mice remained anesthetized throughout the duration of ischemia with supplemental pentobarbital anesthesia as needed. At the end of the experiment, the animals were euthanized (200 mg/kg pentobarbital, IP), and both limbs were harvested and the skin removed. For tissue viability, animals were allowed to recover from anesthesia after the ischemic period, and after 24 hr of reperfusion, the animals were euthanized and tissue harvested. Sham 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 Inc.*, *Wilmington, DE*) was used to assess limb perfusion (8, 22). For experiments involving Laser Doppler Imaging (LDI), the fur was completely removed from both hind limbs 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 are restrained on the warming table. The laser beam (780 nm), reflected from moving red blood cells in nutritional capillaries, arterioles and venules is detected and processed to provide a computerized, color-coded image. Using
image analysis software (Laser Doppler Perfusion Measure, V3.08, Moor Instruments Inc.), mean flux values representing tissue perfusion were calculated from the relative flux units/cm² in the areas corresponding to the plantar aspect of the hind limb 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 minutes into the procedure to assess for limb ischemia (Figure 1B). Data were expressed as percent basal perfusion in the limbs. *Laser Assessment of Systemic Perfusion*: Mouse tail flux (1380±41 units/cm2) was no different (p=.18 ANOVA, n=6) from upper extremity (1258 ± 30 units/cm2) or hind limb flux (1307 ± 55 units/cm2) and therefore it was used as a surrogate for systemic tissue perfusion. Mouse tails were scanned at 4 intervals.

1). 30 minutes after induction of pentobarbital anesthesia (baseline),
2). Completion of ischemia with no reperfusion under pentobarbital anesthesia (0R),
3). After 4 hours reperfusion (4R) and
4). After 24 hours reperfusion (24R).

Sham mice were scanned at the identical conditions except without placement of ORB. All mice received additional boluses of NS (0.3 ml) just prior to ischemia, prior to reperfusion and 3 hr into the reperfusion period. At 4 and 24 hours reperfusion, mouse tails from sham and IR mice were scanned under isofluorane anesthesia. Isoflurane was delivered by facemask, 2% for induction and 1% for maintenance, along with continuous oxygen at 2 L/min. After 4 hours 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. 3 oz, 4oz, 4.5oz, 6oz and MHL rubber bands were
applied to murine hind limbs for 90 minutes 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, tissue samples were blotted, weighed, and placed in a drying oven at 55°C until a constant weight was obtained (usually 36-48hrs). Muscle edema was quantitated using the wet weight to dry weight ratio (W/D). The W/D of the ischemic limbs was compared to the W/D observed in sham 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).**

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, paw extension and flexion are intact

2 = diminished flexion and extension of the knee, paw flexion preserved, no paw extension,

3 = no flexion of the calf, no flexion or extension of the paw (paw drop) dragging.

Functional recovery at 24 hours of reperfusion was compared between the 4.0 oz, 4.5 oz. and 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 conditions, mouse limbs were harvested, the skin removed, and the tissue (distal thigh and calf) cut into 3 pieces to increase
surface area and uptake of the tetrazolium salt as previously described (5, 11). Each piece was weighed and placed in a small tube with 3 ml of PBS (pH 7.4) supplemented with 300 µl of 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium, MTT) (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. 200 µl aliquots 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 percent of the normalized OD570 in reperfused tissue vs. the OD570 detected in sham non-ischemic non-reperfused muscle.

**Histology.** Limbs from mice subjected to 1.5 hours of ischemia followed by 24 hours reperfusion using either the 4.5 oz ORB or the MHL were fixed in 4% paraformaldehyde for at least 4 hours. The gastrocnemius muscle was dissected out, rinsed in Dulbecco’s Phosphate Buffered Saline (PBS) for 1 hour and dehydrated. The samples were embedded in Jb-4 glycomethylmetacrylate, cut cross sectionally at 2 micron thickness and stained with Masson Trichrome. Stained slides were examined under microscopy at 20x magnification (NikonE600 Upright Microscope). Fifteen random fields were photographed using a Cool SNAP Color Camera and RS Image software program, (Roper Scientific, 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, 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 ± standard error of the mean. Comparisons were made using ANOVA and student’s t-test. A p value <0.05 was considered significant. Post Hoc Tests included, Dunn’s, Tukey-Kramer and Mann Whitney tests.

**Results:**

**Ex-Vivo Circumferential Tension Measurements (Figure 2).** Within each diameter tested, the MHL bands exerted significantly greater tension (*, p<0.001) compared to the 3.0, 4.0, 4.5 and 6.0 oz ORB. This tension was as much as 3 times the tension exerted by the 4.0 oz ORB. The 6.0 oz ORB exerted an intermediate level of tension, which was significantly less (*, p<0.001) than that exerted by the MHL, but significantly greater (+, p<0.01) than the tension exerted by the 4.0, 4.5 and 3.0 oz ORB. The 4.0 and 4.5 oz ORB, exerted similar levels of tension at all diameters tested.

**Effect of Diameter on Band Tension (Figure 2)-** 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 oz. and 6.0 oz. ORB (p= 0.16, 0.49, and 0.41 via ANOVA respectively). At 1.19cm, MHL exerted less tension than measured at 1.05 diameter (0.25±0.011 vs. 0.29 ± 0.008 kg; *** p<0.05). At 1.05cm, MHL exerted significantly greater tension than levels detected at 0.73cm (0.29±0.008 vs. 0.24±0.001 kg, **p<0.01). At 1.05 and 1.19cm, the tension 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 that measured at 0.73cm (0.05±0.002 vs 0.06+0.002 kg, ^p<0.01) and 0.86cm(0.05+0.002 vs 0.07+0.001 kg, ^p<0.01).
**Tension Decay** (Figure 3): The 4.0 oz. and 4.5 oz. ORB’s showed no significant decline in the tension after 90 minutes. 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 minutes (p=0.008).

**In Vivo Responses to Hind Limb Ischemia Reperfusion**

**Tissue edema** (Figure 4A): Mice subjected to 90 minutes of ischemia with 2.0 and 3.0 oz ORB 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 for the 4.0 (p=0.17, n=4), 4.5 (p=0.13, n=4), 6.0 oz. ORB (p=0.92, n=4) or the MHL (p=0.70, n=4).

**Limb Ischemia** (Figure 4B ) -The hind-limbs of mice with the 4.0, 4.5 oz. and the MHL all showed a significant decrease in absolute flux units when compared to their baseline (p<0.0001, n=10). There was no significant difference in the degree of tissue ischemia caused by these ORB and MHL.

**Neuromuscular Score** (Figure 5). Mice subjected to 90 minutes of ischemia followed by 24 hours reperfusion with the 4 and 4.5 oz bands had equivalent levels of neuromuscular compromise. Mice subjected to I/R with the MHL had significantly worse neuromuscular score as compared to the 4 and 4.5 oz mice (p<0.01, n=10-14). Despite similar levels of tissue ischemia, the MHL causes greater neuromuscular deficits at 24 hours reperfusion. The neuromuscular dysfunction observed following ischemia reperfusion with the MHL may be caused by a combination of crush and ischemic injury.

**Histology** (Figure 6): After 1.5 hours ischemia and 24 hours reperfusion with either the MHL or 4.5 oz ORB, the number of injured fibers were not different- (24.1±1.3 (ORB) vs. 18±5.3(MHL) injured fibers/high powered field, p>0.05).
Mitochondrial activity (Figure 7). A graded response to IR induced by the 4.5oz ORB was demonstrated using mitochondrial activity as a measure of viability. After 1.5hr or 2.0hrs of ischemia followed by reperfusion, there was a drop to 78.8±3.6 and 76.2±1.5 percent of sham mitochondrial activity respectively. As the duration of ischemia increased, there was a gradual decrease in mitochondrial activity by 24 hours of reperfusion. Significant decreases in mitochondrial activity were observed when comparing the 2.5 hr (63.7+1.8% sham, ** p<0.05, n=5-7 vs. 1.5 and 2 hrs ischemia) and 3.0 hr ischemia (47.3+2.9% sham, *p<0.01, n=5-7 vs. 1.5, 2 and 2.5 hrs ischemia) followed by 24 hours reperfusion.

Systemic Effect of IR:

Mortality: Mortality was 1/12 (8%) animal in the 3.0/24 hr. IR group. No animals died in the sham (0/8), 1.5/4 IR group (0/10), 1.5/24 hr. IR (0/12) or 2.5 hr. IR (0/12) groups.

Representative Tail Scans (Figure 8A): Representative LDI scans from baseline, End Ischemia, No Reperfusion (0R), 4 hrs Reperfusion (4R), and 24 hrs Reperfusion (24R) are demonstrated.

Tail Perfusion (Figure 8B)-. At No reperfusion (0R), there was no significant difference in tail perfusion in mice subjected to sham, 1.5hrs, 2.5 or 3 hrs ischemia. There was no significant difference in tail perfusion within the sham and 1.5/24-hr IR groups at 0R, 4R and 24R. At 4R, 2.5 hr mice showed significant decrease in perfusion vs. sham (53.2±3.0 vs. 101.1±4.0 percent baseline, *p<0.01) and mice subjected to 1.5hr ischemia (53.2±3.0 vs 91.0±9.0 percent baseline, *p<0.01) and sham. At 4R, 3.0 hr mice showed significant decrease in perfusion vs sham (46.5±5.3 vs 101.1±4.0 percent baseline, *p<0.01) and mice subjected to 1.5 hr ischemia (46.5±5.3 vs 91.0±9.0 percent baseline, *p<0.01). At 24R, tail perfusion in 2.5 hr mice was not less than 1.5 hr mice (69.0±8.9 vs 97.2+3.8 percent baseline, p>0.05). In contrast, at 24R, tail
perfusion in 3.0 hr mice remained significantly less that 1.5hr mice (48.2±7.9 vs. 97.2±3.8 percent baseline, **p<0.001).

**Discussion**

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

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

To determine whether ORB might be applicable to use in studies of hindlimb ischemia reperfusion, experiments were designed to compare the ORB tension with that of the popular McGivney Hemorrhoidal Ligator 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 ± 0.15cm, n=10). Based on this information, the ORB 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 ORB tested (Figure 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. ORB 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 (Figure 3). While the tension in the MHL deteriorated, the tension in the 4 and 4.5 ORB was stable over 90 minutes of ischemia. These finding indicate a consistent and reliable tension exerted by the ORB.

Once the ex vivo force measurements indicated that the ORB provided consistent circumferential tensions, studies were designed to evaluate the ability of the ORB to maintain limb ischemia reperfusion 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 prior to reperfusion (5). When the 3.0 oz. ORB was applied to mouse limbs for 90 minutes, 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, 4.5, 6, and MHL bands were applied. Objective wet weight to dry weight measurements indicated there was no evidence of tissue edema as compared to sham animals and one another (Figure 4).

Since the Wet/dry weight ratios for the 4.0, 4.5 and 6.0 oz ORB’s were equivalent to MHL, experiments were undertaken to determine whether the 4.0 and 4.5 oz ORB could create an
acceptable level of ischemia as compared to MHL and measured by laser Doppler imaging. The 4 and 4.5 oz ORB 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 and 4.5 ORB produced tissue ischemia equivalent to the MHL in vivo (Figure 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 vs. MHL, a group of mice were subjected to 90 minutes of ischemia followed by 24 hours reperfusion (Figure 4B). By 24 hours reperfusion, the ORB mice experienced substantial recovery, but the MHL mice did not. The mice that had the 4.0 and 4.5 oz ORB’s retained substantial use of their hind-limbs, while 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 hours 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’s 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. Histologic assessment of gastrocnemius muscle fiber injury showed no significant difference between muscle made ischemic with the MHL or ORB (Figure 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 IR 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 IR are well documented in other models (18). 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 IR 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, 18). One potential reason for this is the high mortality associated with severe limb ischemia in those models. Yassin et al, in a rat model of bilateral IR, reported 50% and 60% mortality at 24 and 48 hours after bilateral limb ischemia and reperfusion (29). Using the ORB model, the hemodynamic effects of bilateral lower extremity IR are documented using tail laser doppler imaging as a surrogate for systemic perfusion and possibly blood pressure. The decrease in systemic perfusion is consistent and dependent on the duration of ischemia. This decrease in perfusion persists even at 24 hours of reperfusion. Interestingly, the mortality associated with hind limb IR 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 anesthesia. There was near 100% mortality under pentobarbital anesthesia at 4R and 24R mice subjected to 2.5 and 3 hrs ischemia. Because of these events, we repeated all of the tail perfusion scanning (in ischemic and sham 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 ischemia and reperfusion influenced this decrease in observed mortality.
Conclusion:

Orthodontic rubber bands (ORB’s) were tested and proven to be effective in producing complete ischemic injury in a reproducible manner creating a minimal amount of non-specific artifact. This system is inexpensive, portable and provides the capacity to evaluate the effects of bilateral and unilateral hindlimb ischemia reperfusion. Since ORB 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 which mimic many of the inflammatory features of adult onset type 2 diabetes mellitus and atherosclerosis in humans.
Acknowledgments.

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References


Legends

Figure 1. **A. McGiveny Ligator Applicator.** The Orthodontic rubber band and the hemorrhoidal ligator fit on the application in identical fashion. **B.** Representative position of the Orthodontic Rubber Band and the MHL on a mouse limb with their respective laser Doppler Images. An Arrow indicates the position of the knee.

Figure 2. **Ex-Vivo Circumferential Tension Measurements vs. Diameter.** Within each diameter tested, the MHL bands exerted significantly greater tension than all other bands (*, p<0.001). 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 ORB and less than that MHL. The 4.0 and 4.5 oz ORB exerted similar levels of tension at all diameters tested. Both the MHL (**p< 0.01, ***p< 0.05) and the 3.0 oz ORB’s (^p<0.05) exhibited significant differences in tension between columns of different diameters. (^, p<0.01).

Figure 3. **Force decay over 90 minutes using 1.05 cm diameter rod.** This graph shows the decay in tension of two different ORB’s (4 and 4.5 oz.), compared to the MHL. After 90 minutes, there is a significant decrease in tension using the MHL(*p=0.008), whereas the 4 and 4.5 oz. ORB’s provide a constant amount of force during the same period.

Figure 4. **In Vivo Ischemia- A: Wet/Dry Ratio-** There was no increase in wet to dry ratio after 90 minutes of ischemia for the ORB’s and MHL, indicating no arterial inflow and outflow during ischemia. **B: Laser Doppler Imaging.** Measured hind limb perfusion in flux units after 30 minutes of ischemia with the 4 oz, 4.5 oz ORB’s and MHL. Showed no flow and no differences in the degree of perfusion in mouse limbs made ischemia with MHL vs. the 4 and 4.5 oz.ORB.
**Figure 5. Neuromuscular Score:** The MHL produced a more compromised (*, p< 0.01) neuromuscular function after 90 minutes of ischemia and 24 hours of reperfusion when compared to both the 4.0 and 4.5 oz. ORB.

**Figure 6. Histological Assessment of Skeletal Muscle Fiber Injury.** Histological evidence of injury, expressed as % injured fibers, showed no statistical difference when comparing injury induced with the 4.5 ORB vs. the MHL post bilateral 90 minutes of ischemia and 24 hours of reperfusion.

**Figure 7. Effect of 4.5oz ORB IR on Mitochondrial Activity.** As the duration of ischemia increased, the skeletal muscle mitochondrial activity decreased. *p<0.01 vs 1.5, 2.0, 2.5 hrs ischemia and 24 hrs reperfusion. **p<0.05 vs. 1.5, 2.0 and 3.0 hrs ischemia and 24 hrs reperfusion.

**Figure 8. Effect of IR on Tail Perfusion.** A. Representative LDI images during baseline, ischemia, 4hrs reperfusion, 24hrs reperfusion. B. Quantitative assessment of tail perfusion. Tail flux was expressed as percent sham. In mice subjected to 2.5 and 3.0 hrs ischemia, tail flux at 4 hrs reperfusion was significantly (*p<0.01) less than sham and mice subjected to 1.5 hrs ischemia. By 24 hrs reperfusion, only the mice subjected to 3.0 hrs of ischemia had less perfusion than sham and mice subjected to 1.5 hrs ischemia (**p< 0.001).
Figure 1. A. McGiveny Ligator Applicator. The Orthodontic rubber band and the hemorhoidal ligator fit on the application in identical fashion. B. Representative position of the Orthodontic Rubber Band and the MHL on a mouse limb with there respective laser Doppler Images. An Arrow indicates the position of the knee.
Figure 2. Ex-Vivo Circumferential Tension Measurements vs. Diameter. Within each diameter tested, the MHL bands exerted significantly greater tension than all other bands (*, p<0.001). 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 ORB and less than that MHL. The 4.0 and 4.5 oz ORB exerted similar levels of tension at all diameters tested. Both the MHL (**p< 0.01,***p< 0.05) and the 3.0 oz ORB’s (^p<0.05) exhibited significant differences in tension between columns of different diameters. (^, p<0.01).
Figure 3. Force decay over 90 minutes using 1.05 cm diameter rod. This graph shows the decay in tension of two different ORB’s (4 and 4.5 oz.), compared to the MHL. After 90 minutes, there is a significant decrease in tension using the MHL(*p=0.008), whereas the 4 and 4.5 oz. ORB’s provide a constant amount of force during the same period.
Figure 4. In Vivo Ischemia- A: Wet/Dry Ratio- There was no increase in wet to dry ratio after 90 minutes of ischemia for the ORB’s and MHL, indicating no arterial inflow and outflow during ischemia. B: Laser Doppler Imaging. Measured hind limb perfusion in flux units after 30 minutes of ischemia with the 4 oz, 4.5 oz ORB’s and MHL. Showed no flow and no differences in the degree of perfusion in mouse limbs made ischemia with MHL vs. the 4 and 4.5 oz ORB.
Figure 5. Neuromuscular Score: The MHL produced a more compromised (*, p< 0.01) neuromuscular function after 90 minutes of ischemia and 24 hours of reperfusion when compared to both the 4.0 and 4.5 oz. ORB.
Figure 6. Histological Assessment of Skeletal Muscle Fiber Injury. Histological evidence of injury, expressed as % injured fibers, showed no statistical difference when comparing injury induced with the 4.5 ORB vs. the MHL post bilateral 90 minutes of ischemia and 24 hours of reperfusion.
Figure 7. Effect of 4.5oz ORB IR on Mitochondrial Activity. As the duration of ischemia increased, the skeletal muscle mitochondrial activity decreased. *p<0.01 vs 1.5, 2.0, 2.5 hrs ischemia and 24 hrs reperfusion. **p<0.05 vs. 1.5, 2.0 and 3.0 hrs ischemia and 24 hrs reperfusion.
Figure 8. Effect of IR on Tail Perfusion. A. Representative LDI images during baseline, ischemia, 4hrs reperfusion, 24hrs reperfusion. B. Quantitative assessment of tail perfusion. Tail flux was expressed as percent sham. In mice subjected to 2.5 and 3.0 hrs ischemia, tail flux at 4 hrs reperfusion was significantly (*p<0.01) less than sham and mice subjected to 1.5 hrs ischemia. By 24 hrs reperfusion, only the mice subjected to 3.0 hrs of ischemia had less perfusion than sham and mice subjected to 1.5 hrs ischemia (**p< 0.001).