Vertebrate Animals

Aim 2 of this proposal requires *in vivo* experiments, and Aim 1C requires isolation of primary cells from vertebrate animals; all other aims will be accomplished using *in vitro* studies.

1. **Description of Procedures.** 6-7 week old Sprague Dawley rat pups will be used for the cell culture studies in the proposed aims. One-year-old male (550–650 g) and female (350-450 g) Sprague Dawley rats will be used for the animal studies.

   TOTAL RATS: <u>131 RATS</u>

   Sub-Aim 1C. Number of rats (6 rats): Primary cardiac myocytes will be isolated from 6-7 week old Sprague Dawley rats using the Worthington Cardiomyocyte Isolation System (Worthington Biochemical Corporation, Lakewood, NJ). Hearts will be excised from rats deeply anesthetized with isoflurane. 2 rats are required for each experiment. As three cell culture experiments are proposed, 6 total animals are required.

   Sub-Aim 2A. Number of rats (22 rats): One-year-old male (550-650 g) and female (350-450 g) Sprague Dawley rats will be used to assess the therapeutic benefit of the proposed approach. Power indicates that 11 animals will be required for each of the two treatment groups, treated vs. untreated. A left thoracotomy will be made at the level of the fourth intercostal space, and the pericardium will be gently pulled apart. Under a dissecting microscope, the left anterior descending (LAD) coronary artery will be visualized. A no. 4 ½ circle taper point needle will be used to pass 6–0 polypropylene suture underneath the LAD at a point 2 mm distal to the tip of the left auricle. A loose double knot will be tied, through which a short length of PE-10 tubing will be placed, and the loop will be tightened and secured with a slipknot. Occlusion will be verified by confirming pallor in the LAD's territory. Ischemia will be maintained and skin will be clamped together for 60 minutes. Afterwards, the knot will be untied and the tubing removed. The suture will be left in place for determination of infarct size and area-at-risk (AAR) by triphenyltetrazolium chloride (TTC) and Alcian blue staining. After 24 hours of reperfusion, the animals will be reanesthetized and the chest reopened. Analysis of the infarct will be completed after intracardiac injection of potassium chloride and excision of the heart.

   Sub-Aim 2B. Number of rats (38 rats): First, our fluorescent H_2O_2 probe (caged or active/uncaged) will be injected into in animals subjected to I/R injury or to sham surgery. After euthanasia, fluorescence will be quantified in organ homogenates. Power analysis indicates that 2 animals will be necessary for each of the 4 treatment groups. Second, in animals treated with either the active drug, caged compound, or saline and subjected to I/R injury or sham surgery, Erk2 and Stat3 phosphorylation will be compared between injured and uninjured tissues by Western blot. Based on power analysis, 5 animals will be needed for each of the 6 treatment groups. Overall, 38 rats will be needed.

   Sub-Aim 2C. Number of rats (65 rats): To determine the extent to which the proposed treatment approach reduces chronic injury after ischemia-reperfusion in Sub-Aim 2C, rats will be monitored for 28 days post-surgery. At five time points (pre-surgery baseline, 3 days, 7 days, 14 days, and 28 days), the rats will be anesthetized for echocardiography. After the final echocardiographic reading, anesthesia will be deepened, and hemodynamics will be further assessed by PV catherization. Based on power analysis, 9 animals per treatment group will be necessary. Based on the Wang group's past experience with a similar model, 13 animals will be used in each treatment group to compensate for attrition and to ensure a large enough sample size for adequate power at the end of the experiment. Five treatment groups (vehicle, acute treatment, acute and chronic treatment, chronic treatment, and vehicle/sham-operated) will be present, necessitating 65 animals total. The sham surgery will stop after dissection of the pericardium.

   Housing. Animals will be housed at the Division of Laboratory Animal Resources (DLAR) at the University of Pittsburgh. Rats will be housed in pairs with unrestricted access to food and water, with bedding provided in each cage. Staff veterinarians and veterinary technicians in addition to the PI will monitor the animals' health at least once per day; staff will recommend treatment with antibiotics and analgesics as necessary to minimize pain in the animals. Veterinary staff members are available during business hours, and are always on-call in case of emergencies. The staff can also intervene or suggest euthanasia for any animals showing signs of distress, which, for this model, include hunched, immobile posture; spiked coat; self-mutilation; aggressive behavior; vocalizations; and signs of excessive inflammation at the surgical site.

2. **Justifications.** Live organisms are necessary for the proposed work because cell culture experiments alone do not accurately recapitulate myocardial ischemia-reperfusion in a way that allows assessment of clinically-important parameters (infarct size/area-at-risk and hemodynamics). Additionally, cultured cells cannot be used to detect perturbations of cell signaling pathways in uninjured organs. Current mathematical models and computer simulations are insufficient for this purpose. Rats are a well-accepted myocardial ischemia model, and accepted models are not available in lower species (e.g., invertebrates). Sprague Dawley rats were chosen because they have been well-established as a model organism for myocardial ischemia-reperfusion, and are widely considered to be a docile, easy-to-handle strain.

3. **Minimization of Pain and Distress.** For surgical procedures, anesthesia with isoflurane will be accompanied by analgesia and sedation with ketamine and xylazine (100 mg/kg i.p. and 7.5 mg/kg i.p., respectively). Anesthesia will be induced using 2-5% isoflurane in oxygen and maintained using 1-3% isoflurane in oxygen. After surgery, EMLA cream will be applied to the surgical site as a local anesthetic. Post-operative rats will recover in an oxygen-rich chamber, and will not be returned to their housing until fully awake and mobile. Buprenorphine hydrochloride (0.1 mg/kg SC BID) and ketorolac (5 mg/kg SC BID) will be given for three days to minimize pain. Antibiotics will be given if the surgical site becomes infected. This surgery has a risk of pneumothorax, and animals will be euthanized if discomfort arises from this condition. Buprenorphine will be given as needed if any signs of pain or distress are observed in the animals. If any animals have a severe negative reaction to surgery, characterized by aggression, vocalization, and/or hunched posture, veterinary staff will be consulted, and animals will be euthanized if necessary.

4. **Euthanasia.** Euthanasia will be performed by cardiac injection of potassium chloride (KCl) in deeply anesthetized rats. This method is necessary to ensure that the heart is in diastole when it is excised, which is essential for the proposed experiments. The American Veterinary Medical Association (AVMA) *Guidelines for the Euthanasia of Animals* approves this method as a special case if the animal is fully anesthetized. Isoflurane will be used to ensure full anesthesia before KCl injections.