ASRM Scientific Paper Presentations: Basic Science

January 17, 2016 – 11:15 AM to 12:15 PM

11:15 AM - 11:20 AM

Effect of Electroacupuncture at Zusanli(ST36) on Dorsal Random-pattern Skin Flap Survival in A Rat Model

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Specific aims & objectives: Electroacupuncture at Zusanli(ST36) could accelerate angiogenesis by up-regulating HIF-1 protein, and enhance skin microcirculation blood perfusion. The paper aims to study the effects of electroacupuncture at Zuansanli(ST36) on random-pattern flap survival rate in a rat model. Materials and methods: Thirty female Sprague-Dawley rats were randomly divided into three groups: (1) control group(no postoperative electro- acupuncture) ; (2) study A group (postoperative electroacupuncture at non-acupoint areas, 10 Hz for 30 min per day) and (3) study B group (postoperative electroacupuncture at Zusanli , 10 Hz for 30 min per day). The ‘McFarlane flap' rat models were established on the rat dorsum. Observation：After 7days' electroacupuncture, the flap survival area ratio was measured .The tissue samples were taken for histological analysis. The vascular endothelial growth factor (VEGF), malondialdehyde (MDA) and Superoxide dismutase (SOD) expression were detected by immune- histochemistry. Results: 7 days after operation ,the flap necrotic areas ratio in control group （66.65±2.81）% and in study A（64.65±2.42）% were significantly larger compared with that of the study B（48.81±2.33）%（P <0.01）.Histological analysis demonstrated angiogenesis with mean vessel density per mm2 be lower in control group(15.4±4.4) and in study A (17.4±4.3) than in study B group(27.2±4.1) （P <0.05）. VEGF expression and SOD contents were significantly increased in study B Group compared with study A Group and control group ($p < 0 . 01$), whereas MDA level was reduced ($p < 0.05$). Discussion: Electroacupuncture at Zuansanli(ST36) may increase the expression of VEGF, which can effectively increase angiogenesis to provide inadequate vascular perfusion and block the release of ischemia reperfusion injury associated free radical, as well as the production of the superoxide anion. In addition, the up-regulated SOD and the down-regulated MDA are evidence for it. Conclusion: Electroacupuncture at Zuansanli(ST36) can improve random flap survival rate effectively.

11:20 AM - 11:25 AM

Tissue Monitoring with 3-wavelength Light Emitting Diode Based Near Infrared

Spectroscopy

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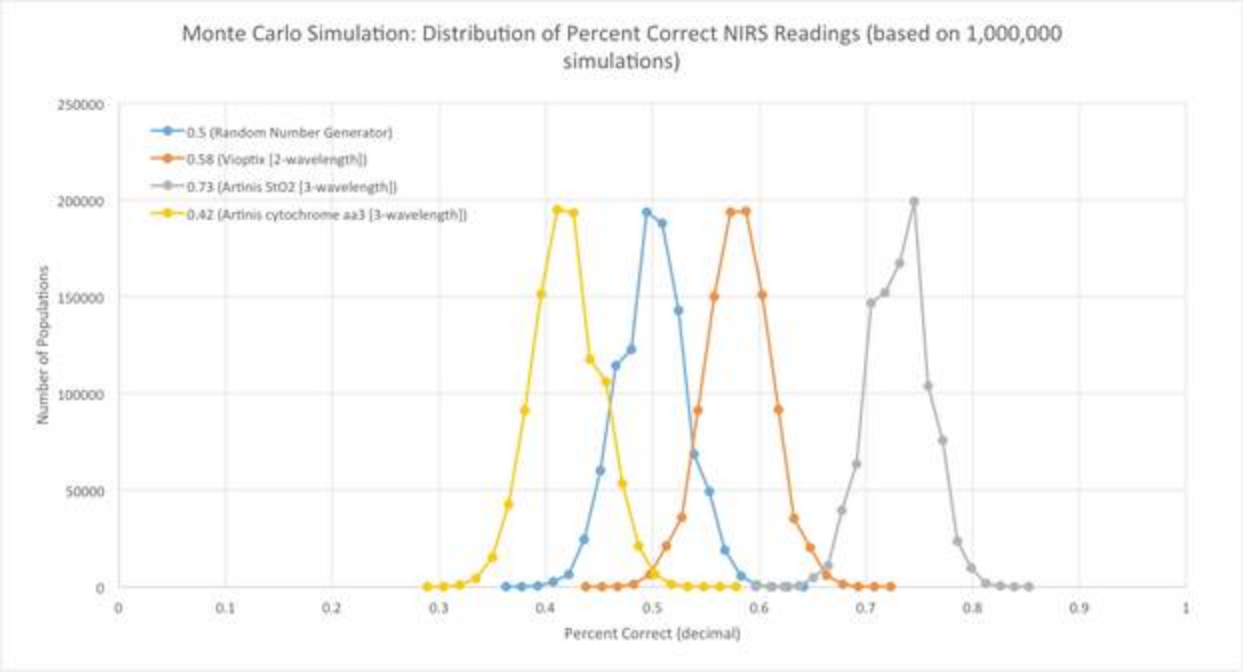
Background: Flap monitoring with a two-wavelength light emitting diode (LED)-based near infrared spectroscopy (NIRS) device, which measures deoxyhemoglobin and oxyhemoglobin to calculate tissue oxygenation (StO_2), facilitates early detection of vascular compromise and decreased flap failure. However, standard NIRS devices that employ two wavelengths of light to assess tissue oxygenation are susceptible to artifact from background noise and demonstrate significant variability in the clinical setting. As the number of wavelengths detected by a NIRS device is increased, the accuracy of tissue oxygenation measurements can be improved and additional chromophores other than oxyhemoglobin and deoxyhemoglobin can be measured. A three-wavelength NIRS device (Artinis) that also detects cytochrome aa_3, a measure of intra-cellular oxygen demand, was compared to the standard two-wavelength device commonly used for flap monitoring (ViOptix) to determine if there is an improvement in the accuracy of tissue oxygen measurements.

Methods: ViOptix and Artinis were applied to the volar forearms of human volunteers and a blood pressure cuff was placed proximally. Once stable baseline readings of StO_2 and cytochrome aa_3 were achieved the cuff was inflated above the systolic blood pressure to occlude arterial inflow and venous outflow. The protocol incorporated arterial and venous occlusion to produce the largest measurable effect. The inflation pressure was maintained until the readings plateaued and then the cuff was deflated. StO_2 readings were obtained from both devices. Artinis also yielded cytochrome aa_3 redox state measurements.

Results: StO_2 readings from both devices were proportionate during ischemia ($R^2 = 0.79$, $p<0.01$). During the ischemic event it was hypothesized that each measurement of StO_2 or cytochrome aa_3 would be less than the preceding measurement. StO_2 readings from the ViOptix and StO_2 and cytochrome aa_3 readings from the Artinis detected a correct trend in desaturation 58, 73, and 42% of the time, respectively (Figure). Statistical analysis showed that Artinis outperformed ViOptix ($p<0.01$) as a measure of change in StO_2 during ischemia. The Artinis cytochrome aa_3 algorithm did not effectively detect a significant change in cytochrome aa_3 reduction during ischemia.

Conclusions: The addition of a 3rd wavelength in LED-based NIRS monitoring improved the accuracy of trend monitoring of StO_2. However, the 3-wavelength device lacked the sensitivity to reliably measure changes in cytochrome aa_3. More work is needed to determine if broadband devices, which employ over 100 discreet wavelengths of light to detect cytochrome aa_3, provide clinically useful information that could be applied to monitoring free tissue transfer.

Figure:



11:25 AM - 11:30 AM

Remote Ischemic Perconditioning Attenuates Reperfusion Injury of Experimental Musculocutaneous Flaps

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BACKGROUND

Skeletal muscle tissue is at risk of necrosis after prolonged ischemia. In free flap reconstruction and replantation surgery, global tissue ischemia is obligatory until blood supply is restored by microvascular anastomoses. Anoxia and ischemia cause tissue damage, and the restoration of blood supply causes further cellular damage known as reperfusion injury.

Brief episodes of ischemia and reperfusion in one tissue, has been shown to confer a global protection of anatomically remote tissues and organs subjected to ischemia-reperfusion injury. This treatment has been termed remote ischemic conditioning.

The aim of the study was to investigate the effect of remote ischemic perconditioning (RIPER) on acute inflammation in porcine muscle flaps subjected to ischemia-reperfusion injury.

METHODS AND MATERIALS

In 16 pigs, one musculocutaneous latissimus dorsi flap was dissected on its vascular pedicle and subjected to four hours of normothermic ischemia and seven hours of reperfusion. The animals were allocated into a control group without RIPER (n = 8) and an experimental group with RIPER (n = 8).

RIPER was performed by inducing lower extremity ischemia with a tourniquet inflated to 200 mmHg in the experimental group. Three cycles of 10 minutes ischemia followed by 10 minutes reperfusion were applied during flap ischemia starting 60 minutes prior to flap reperfusion.

Acute inflammation was described by pro-inflammatory cytokine secretion (IL-6 and IL-12p40) from the flap during reperfusion, calculated as the cytokine concentration of flap venous effluent blood minus the cytokine concentration of systemic venous blood.

Furthermore, flap biopsies were taken following seven hours of reperfusion for quantitative determination of macrophages by immunohistochemistry.

RESULTS

The IL-6 and IL-12p40 secretions were significantly reduced in the experimental group compared with the control group at five hours of reperfusion ($p = 0.036$ and $p = 0.005$ respectively). Furthermore, the IL-6 secretion peaked earlier in the experimental group compared to the control group (at three hours of reperfusion compared with five hours of reperfusion respectively).

The macrophage area fraction was reduced in skeletal muscle samples from the experimental group compared with the control group, but not significantly ($p = 0.55$).

CONCLUSIONS

The main finding is that RIPER attenuated IL-6 and IL-12p40 mediated inflammation of musculocutaneous flaps subjected to ischemia-reperfusion injury. RIPER is safe, non-invasive, and inexpensive; and the treatment has potential clinical applicability in free flap reconstruction and replantation surgery.

11:30 AM - 11:33 AM
Discussion

11:33 AM - 11:38 AM
Remote Ischemic Preconditioning of both donor adipose and recipient wound bed greatly improves fat graft viability
UT Southwestern, Dallas, TX, USA
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Introduction:

Fat grafting has become a useful adjunct in the reconstructive surgeon’s treatment armamentarium, but local ischemia prior to the development of recipient circulation may contribute to highly variable long-term results. Remote Ischemic Preconditioning (RIPC) is a cheap non-invasive technique that has been used in several animal models and multicenter clinical trials to induce cell protection. The specific aim of this project was to analyze the volume retention of lipoaspirate transferred in the setting of either donor or recipient RIPC.

Methods:

We obtained subcutaneous adipose tissue from FVB mice transgenically engineered to express GFP and Luciferase. These samples were obtained either with or without the use of temporary hindlimb tourniquet time prior to harvest. The treatment and control fat was injected into the dorsal skin folds of genetically identical FVB mice that did not express GFP or Luciferase. These samples were placed in either recipients who did or did not undergo the use of temporary hindlimb tourniquet time prior to placement. The viability and volume of the transferred tissue was examined over a 28-day time period by bioluminescence after intraperitoneal injection of Luciferin using a Maestro IVIS optical small animal scanner. Additionally, after experimental completion the tissue transferred was explanted and examined histologically. The specimens were stained with H&E and GFP.

Results:

There was a significant difference in bioluminescence at Day 0, 14, and 28. The RIPC donor or recipient mouse alone groups demonstrated approximately 2 to 3-fold increase over control at each time point. However the use of RIPC in both the donor and recipient mice had a 9-fold increase in bioluminescence over the course of the experiment. Histological analysis at 28 days confirmed the presence of donor adipocytes, and that they were gradually replaced by recipient inflammation and scar tissue. However the amount of interstitial fibrosis was substantially less in the RIPC groups. These findings were particularly more pronounced when RIPC was use for both donor and recipient mice.

Conclusions:

This work demonstrates that RIPC, a free and widely available therapy, has the ability to increase the viability of donor adipocytes when transferred via liposuction cannula. The transferred RIPC tissue is less likely to undergo interstitial fibrosis. More specifically, RIPC treatment of both donated adipose tissue and recipient wound beds, as would be the case in clinical practice, demonstrates the greatest overall adipose cellular viability and native architecture.

11:38 AM - 11:43 AM

Direct Transplant Intra-Arterial Delivery of Limited Quantities of Adipose Derived Stem Cells Allows Their Efficient and Safe Targeting

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Background: Mesenchymal stem cells (MSCs) are adult multipotent cells that possess regenerative and immunosuppressant properties. Homing of MSCs to target organ/tissue remains a major challenge as intravenous (IV) delivery results in intravascular entrapment of MSCs in the lungs and other vascularized organs. Recently, intra-arterial (IA) administration of MSCs demonstrated improved delivery of cells to target organs although entrapment of some cells in the lungs was still observed. Additionally, IA administration often resulted in vessels obstruction. We designed a novel method for local MSCs IA delivery directly into the transplanted graft, improving cell targeting with minimal cell dosing.

Methods: A syngeneic groin free flap transplant between Lewis rats was performed. Decreasing quantities of adipose derived MSCs (ASCs) were administered via the femoral artery and allowed to settle in the vasculature for 10 minutes prior to the final reperfusion. *In vivo*real time fluorescence imaging and intravital microscopy were used to define ASCs IA movement after transplantation.

Results: High concentrations above 10^5 ASCs per injection resulted in flap necrosis. At $5X10^4$ASCs, long-term flap viability was observed. Whole-body imaging of labeled ASCs demonstrated significant targeting of cells into the viable flap even at such a low cell quantity. ASCs were observed in proximity to small blood vessels within the flap and did not disrupt blood flow as demonstrated by intravital Doppler images.

Conclusion: Taking the IA delivery method advantages into account, we proposed an IA injection of cells directly into a free flap transplant, essentially delivering the cells into an isolated organ, thus minimizing systemic spread and lung entrapment.

Local IA administration of ASCs into a vascularized transplant/flap is feasible and allows high local cell concentrations with minimal cell dosing. Targeted delivery of MSCs into a transplant/free flap may be used to increase the therapeutic efficacy of the cells for various purposes.

11:43 AM - 11:48 AM

Generation of Donor-specific Antibody Abolished Donor-specific Tolerance to Vascularized Composite Allotransplants in Brown Norway Rats

Chang Gung Memorial Hospital, Taipei, Taiwan
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Introduction: Vascularized composite allotransplantation has brought about a new era for reconstructive microsurgery. To circumvent rejection due to allogenecity, various protocols for induction of donor-specific tolerance has been designed in animal models with success. However, it is not well established that the applicability of a given protocol or the allograft outcome are influenced by the genetic background of the recipients.

Materials and Methods: A novel model comprised of combined groin flap with whole femur osteomyocutaneous flap was transplanted under the following regiment: antilymphocyte serum (ALS) at day -3, +1; cyclosporine (CsA 16mg/kg) at day 0-7; and rapamycin at day 8-28. Wild

type Brown-Norway (BN) rats and Lewis (LEW) were used as *VCA recipients and donors, respectively*. *The femur bone in VCA was removed from tolerant group at day 90 for some recipients with survived VCA*. Different hematopoietic cell lineages in recipients' peripheral blood were assessed by flow cytometry. Secondary Lewis and F344 antigen challenge in the form of skin grafting was performed on BN recipients with long-term survived VCA.

Results: Overall graft survival rate, with rejection-free, was *71.4 %*. *No fibrosis or rejection in the bony component of the flap in all tolerant animals was detected at day 90*. All tolerant animals demonstrated donor-specific tolerance with acceptance of Lewis skin graft and rejection of F344 skin graft at day 100. *The femur bone was successfully removed without compromising flap survival and without inducing rejection up to day 200*. Donor skin tolerance was maintained after bone removal, as confirmed by skin graft challenge. Peripheral lymphocyte panel of the tolerant recipients showed higher level of CD4 T cells and lower level of B cells compared to the rejection counterparts. On longer follow-up, however, rejection developed in some tolerant animals including those who had the bone removed, and donor-specific antibodies were detected. Of importance, rejection was detected later in bone-removed animals.

Conclusion: The VBM is capable under short-term immunosuppressant treatment to achieve tolerance via donor cells engraftment. Once engrafted donor cells are well-established, the vascularized bone marrow may become expendable. However, donor-specific antibodies can develop later or reach significant level in old grafts which will result in late graft failure.

11:48 AM - 11:51 AM
Discussion

11:51 AM - 11:56 AM
In Vitro Characterization of the Phenotype, Genotype and Clonogenic Properties of ex vivo Created Human Hematopoietic Chimeric Cells - a Novel Approach for Tolerance Induction in Vca Transplantation
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Background: Cell-based therapies represent a new promising approach for tolerance induction in transplantation. One of the methods for immune response modulation in solid organ and vascularized composite allograft (VCA) recipients is donor bone marrow (BM) transplantation. We propose a new cellular therapy based on the *ex vivo* created donor-recipient chimeric cells as an alternative approach to BM-based therapies in support of solid organ and VCA transplantation. The aim of this study was creation and preliminary characterization of the fused human BM-derived CD34+ hematopoietic chimeric cells.

Materials and Methods: Seventeen *ex vivo* fusions were performed to create human BM-derived $CD34^+$ hematopoietic chimeric cells. Briefly, mononuclear cells (MNCs) isolated from two unrelated donors (female and male) were sorted out by MACS technology to obtain $CD34^+$ cells. Next, $CD34^+$ cells from each donor were stained separately by PKH26 (red) and PKH67 (green) and fused in a ratio 1:1 with polyethylene glycol (PEG). Double PKH26 and PKH67 stained cells were sorted out and subjected to further assessments. Flow cytometry (FC), (CD34,

CD133, CD117, CD90, CD4, CD8, CD25, CD5, CD19, CD14 markers, viability tests), confocal microscopy (CM), genotype HLA typing via PCR-rSSOP, STR, LTC-IC and CFU assays were used to characterize the properties of created human hematopoietic chimeric cells.

Results: FC and CM analysis confirmed $CD34^+$ cell fusion and creation of human hematopoietic chimeric cells (HHCC). Using PCR-rSSOP we determined that HHCC share HLA class I and class II antigens specific for both BM fusion donors. The presence of genetic material from both BM donors in HHCC was also confirmed by STR. After fusion ~99% of HHCC were viable and had low level of apoptosis (2.7% and 1.2% of HHCC in early and late stages of apoptosis, respectively). Phenotype characterization showed expression of all assessed markers on the surface of HHCC. LTC-IC and CFU assays showed that HHCC have clonogenic potential and can differentiate into all classes of myeloid and erythroid progenitor cells.

Conclusions: We successfully confirmed feasibility of *ex vivo* fusion of human BM-derived $CD34^+$ hematopoietic cells leading to creation of HHCC. We characterized the viability, phenotype, genotype and clonogenic properties of HHCC. This unique concept of HHCC-based cell therapy introduces new applications in transplant surgery. **The ultimate goal is to induce tolerance in solid organ and VCA transplants with application of HHCC as a supportive therapy.**

12:01 PM - 12:06 PM

A Novel Microsurgical Approach to Fetal Craniofacial Repair

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BACKGROUND Cleft lip with or without palate (CL/P) affects approximately 1 in 500-700 live births. Current surgical options for this deformity offer patients exceptional transformations but are not without complications. Alternatively, intrauterine repair has been a topic of interest since the discovery of scarless fetal wound healing and growth factors that contribute to this phenomenon. Previously, we demonstrated that Pbx-Wnt signaling at the embryonic lambdoidal junction mediates midfacial morphogenesis via localized apoptotic programs and results in orofacial clefting if interrupted. In addition, we not only developed a unique compound Pbx-deficient murine model with fully penetrant CL/P, but also demonstrated genetic rescue strategies to reconstitute Wnt signaling and correct midface clefting. We now seek to identify novel avenues for tissue repair of this facial abnormality by using *Wnt*-soaked collagen microspheres to restore craniofacial developmental programs in our *Pbx*-deficient embryonic murine model *ex utero.*

METHODS *Wildtype* and *Pbx*-deficient murine embryos were dissected from the uterus and placed in a 37°C modified Whole Embryo Culture (WEC) system. At gestation day E11.5, *Wnt9b*-soaked collagen microspheres were microsurgically implanted at the midface lambda junction. Correction of CL/P was assessed by gross morphology, histology, and evaluation of the restoration of apoptotic programs. In addition, titration assays were conducted to optimize

the dose of *Wnt* by assessing protein content and release kinetics. *In vitro* studies were also done to determine optimal formulations of collagen microspheres.

RESULTS We have demonstrated that embryos continue to develop normally in our modified WEC system for about 24 hours, evidenced by normal facial development in the cultured embryos when compared to embryos fixed at immediate removal from the uterus. Type I collagen microspheres, approximately 50-70 microns, were fabricated and revealed to have appropriate release kinetics. Targeted delivery of *Wnt9b* to the lambdoidal junction resulted in augmented expression of *Wnt* when compared to the normal endogenous signaling of *Wnt*, under X-gal staining. The implanted microspheres did not effect normal development of the lambdoidal junction in *wildtype* embryos or alter cleft development in *Pbx* knockouts.

CONCLUSION We have successfully established the viability of our ex vivo WEC system as well as the feasibility of implanting collagen microspheres as targeted drug delivery vehicles. We believe that our research will open new avenues towards unconventional and innovative prenatal interventions for patients with CL/P as well as provide future applicable repair approaches for congenital head and neck disorders.

12:06 PM - 12:11 PM

Feasibility and Viability of Vascularized Lymph Node Transfers in a Rat Model

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Background:

Vascularized lymph node transfer (VLNT) has recently received attention as a potential surgical treatment for lymphedema. Despite good results in some series, the real benefits of VLNT are difficult to ascertain due to the limited number of patients who have undergone the procedure. In order to scientifically investigate the feasibility, viability and limitations of VLNT, we are using rats as an animal model to study VLNT. The aims of the current study are threefold: 1) determine the technical feasibility of VLNT in a rat, 2) determine the viability of the transferred nodes over time, and 3) assess the re-establishment of disrupted lymphatic channels after VLNT.

Methods:

To study the technical feasibility of VLNT in the lower extremity of Sprague-Dawley rats, we are performing end-to-end microvascular anastomoses of superficial epigastric vessels on which the groin lymph node basin has been harvested as a free flap **(Figure 1).** We then investigate the viability of transferred lymph nodes as well as assess for the re-establishment of lymphatic channels in the transplanted node basin. Anastomosis patency is assessed visually and with a microvascular flow probe at 1 hour and 2 weeks postop. Rats are sacrificed at 2 weeks postop and lymph node viability assessed through histological examination. In addition, one rat has been followed for five months postop and 3 other rats for 1 month postop, and reestablishment of

severed lymphatic channels has been assessed non-invasively through examination with fluorescent indocyanine green (ICG).

Results:

VLNT was performed in 8 rats to date (and results of additional planned VLNTs will be reported at the time of the meeting). Anastomoses were patent in all 8 rats at 1 hour postop. Two rats were sacrificed at 2 weeks postop, and transplanted lymph nodes were shown to be viable on H and E staining **(Figure 2).** In one rat with 5 months follow-up and 3 rats with one month follow-up there has been no ICG uptake in the transplanted inguinal lymph nodes, indicating a lack of lymphatic channel reestablishment in the transplanted node basin **(Figure 3).**

Conclusions:

VLNT can be successfully performed in the rat model. The procedure is technically feasible, and postop histologic studies show the transferred nodes to be viable. Additional studies are required to determine if lymphatic channels are re-established in the transferred node basins.

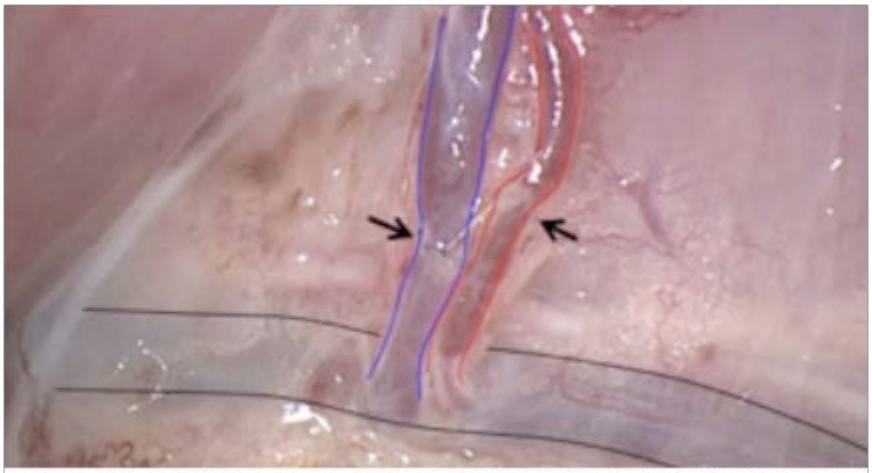

Figure 1: End-to-end rat superficial epigastric anastomosis at 24 hours. The superficial epigastric vein (blue) and artery (red) can be seen coming off of the femoral vessels. The black arrows point to the site of anastomosis.

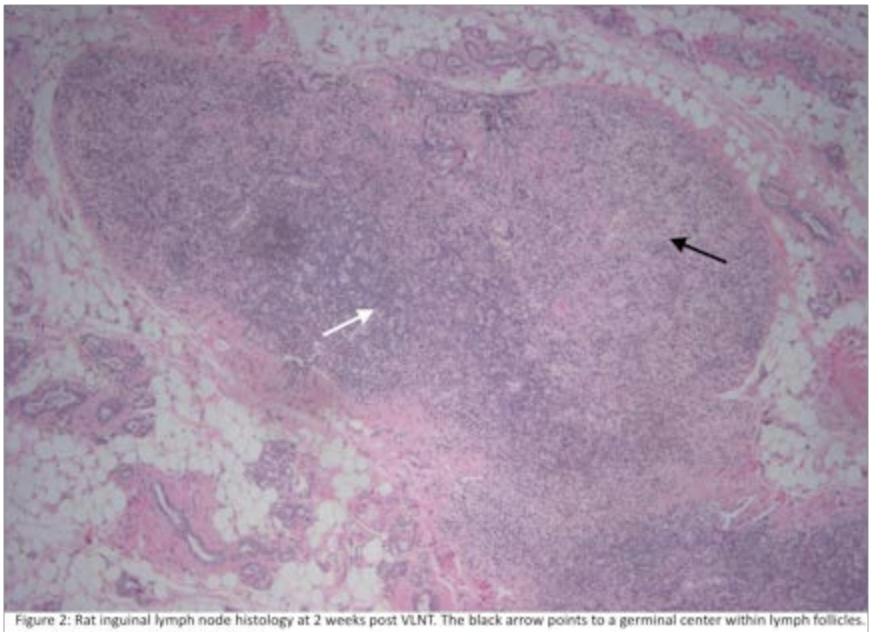

Figure 2: Rat inguinal lymph node histology at 2 weeks post VLNT. The black arrow points to a germinal center within lymph follicles. The white arrow points to the medulla.

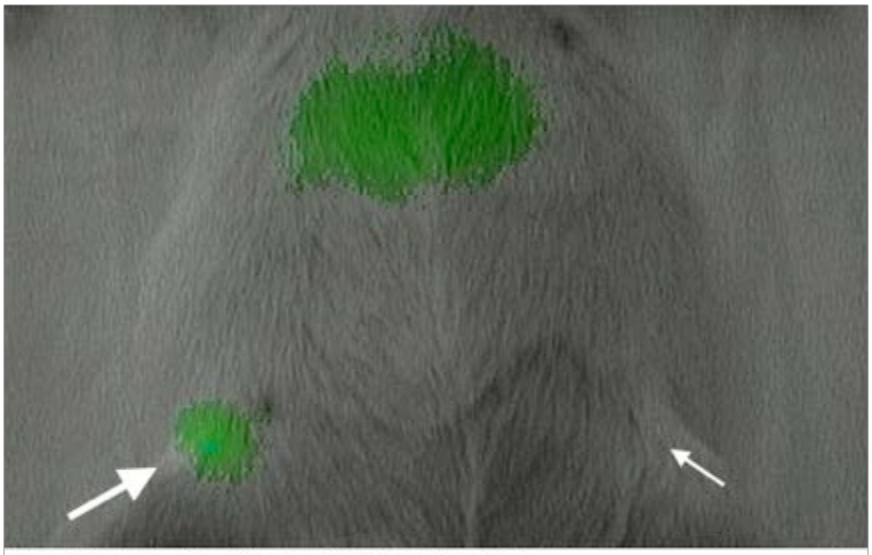

Figure 3: Lymphatic uptake of fluorescent ICG at 5 months post VLNT
The large arrow points to normal uptake in the non-operated right inguinal lymph node. Uptake is also seen in the liver as ICG is hepatically metabolized. The small arrow points to the operated left inguinal side with no ICG uptake in the VLNT

12:11 PM - 12:15 PM

Discussion