Quantification of Chronic Lymphedema in a Revised Mouse Model

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Published in:
Annals of Plastic Surgery

DOI:
10.1097/SAP.0000000000001537

Publication date:
2018

Document version:
Accepted manuscript

Citation for published version (APA):

Go to publication entry in University of Southern Denmark's Research Portal

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Download date: 03. Jun. 2021
**Introduction**

Lymphedema is a frequent side effect following cancer treatment with lymph node involvement and adjuvant radiotherapy[1]. The condition is a complex composition, characterized by chronic tissue swelling, inflammation and inhibited lymph flow, which makes the disease unsuitable for in vitro studies. Despite the importance and prevalence of the disorder, our knowledge about the underlying pathophysiology is sparse and development of treatment modalities are hampered by the lack of reliable animal models[2]. Rodents have always been a preclinical cost efficient platform for studying diseases, but several shortcomings of current lymphedema models are hindering progress into the clinic[3]. The rodent tail lymphedema model has been used by several authors due to the ease of the procedure[4]; however the rodent tail lacks lymph nodes and differs to the human limb in essential physiological and anatomical properties. Rodent models of limb lymphedema represent a closer representation and have previously been sought established by a combination of surgical lymph node ablation and a single high dose of irradiation[5,6] or a silicone splint emplacement[7]. However these methods have been subject to severe morbidity or spontaneous lymphedema resolution, which is not reflected in the disease seen in humans[3]. Irradiation therapy is a known inducer of lymphatic injury, but an optimal dose titration for induction of preclinical lymphedema has yet to be uncovered.

Thus, our aim was to develop a clinical relevant lymphedema hind limb model and methods for objective quantification. Built upon the limitations of previous models, we propose a refined lymphedema model established with combined lymphadenectomy and fractioned irradiation.
Methods

All experiments were conducted in accordance with national and institutional guidelines for the care and use of animals in research. This study was approved by The Danish National Animal Inspectorate (2016-15-0201-00909) and reported according to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines[8].

Study design

In this experimental study, lymphedema was sought induced in the right hind limbs of mice, while the left hind limb served as an internal control, figure 1. Eight treatment groups of 4 mice per group served in this study. All groups underwent surgical ablation of lymphatic drainage paths. Six of these groups received ionized radiation therapy in one or two fractions (range: 7.5-30Gy). One group received no irradiation, however a silicone splint was implanted between wound edges and finally one group received surgical lymphatic obstruction only. All mice underwent weekly \(\mu\)CT scans of the operated and unoperated limbs for repetitive volume assessments up until the 8\(^{th}\) week after the surgical procedure. At the 4\(^{th}\) week, two mice from each group underwent Technetium-99m-Human Serum Albumin (\(^{99m}\)Tc-HSA) lymphoscintigraphy. At the end of the study, all mice were euthanized and hind limbs from three mice in each group were dissected for immunohistochemistry and assessment of lymphatic vessel ectasia in the dorsal footpad.
Animals

In total, 32 9-week old female inbred C57BL/6 mice weighing between 19-23g were purchased from Taconic (Taconic Biosciences, Ejby, Denmark). All mice were acclimatized for 7 days in groups of four per cage after arrival. All animals were maintained on a normal 12 hours day/night cycle at 21°C with a humidity of 45-55%, and fed a standard diet with unrestricted access to water.

Experimental procedure

One investigator performed all experimental procedures. All animals were anesthetized by a subcutaneous injection of fentanyl 788µg/kg, fluanisone 25mg/kg and midazolam 12.5 mg/kg.

Surgery

A previous method of lymphatic tissue ablation in the mouse hind limb was adapted [5]. Briefly, a circumferential incision was made proximal on the mid-thigh area. After a subcutaneous injection of 0.1mL patent blue V to the right 2nd and 3rd toe, a postnodal lymphatic vessel was easily identified along the ischiatic vein. Then, the skin was bluntly dissected just distal to the knee, and a blue stained popliteal lymph node and two prenodal lymphatic vessels were macroscopically identified. Stained pre- and postnodal lymphatics were tied with 10-0 nylon suture and blockage was confirmed with a careful milking test, figure 2. During the procedure, care was taken not to injure the ischiatic vein. Afterwards, the popliteal and inguinal lymph nodes were resected along with their respective fat pads. Finally, the procedure was concluded with a circumferential suturing of the skin edges down to the muscle fascia with 6-0 nylon, leaving a wound-gap of 2mm to constrain the superficial lymph flow. Postoperatively, mice were housed individually and received subcutaneous injections with 0.02ml buprenorphine 3 times daily for 3 days.
Radiation

Radiation was emitted from a Gulmay D3100 x-ray machine (Xstrahl, Camberley, UK) at a dose rate of 5.11Gy/min (100kVp, 10mA, HVL 2.53 Al). Treatment was given at one or two fractions ranging from 7.5Gy to 30Gy depending on group allocation. The irradiated area (25mm Ø) was enclosed by 1.5mm thick lead-rubber.

Silicone splint

In the Silicone Splint group, a 3mm wide rectangular silicone splint was cut from a 1mm thick silicone sheet (Aalborg Gummivarefabrik A/S, Aalborg, Denmark). Mice allocated to the silicone group had the splint sutured to the inside of the inguinal wound and fixated between the skin edges and underlying muscle fascia during surgery[7].

Soft tissue and skin breakdown assessment

Operated hind limbs were scrutinized weekly for any signs of skin breakdown, non-healing wounds, wound necrosis and/or wound oozing.
Computed Tomography (CT) and Single-Photon Emission Computed Tomography (SPECT)

lymphoscintigraphy

Small-animal SPECT/CT was performed on a Siemens INVEON multimodality pre-clinical scanner (Siemens pre-clinical solutions, Tennessee, USA). During each imaging session, mice were anesthetized with a mixture of 1.5-2.0% isoflurane and 100% oxygen and placed in a prone position on a heated SPECT/CT animal bed (38mm).

To assess the lymphatic clearance, mice received a subcutaneous bolus injection of 0.02ml 99mTc-HSA between the 2nd and 3rd toe of each hind paw. Doses were approximately 25 MBq (Vasculocis; CIS Bio International, Paris, France) and administered using a 0.5ml 30G insulin syringe (Covidien, Medtronic, Minneapolis, USA). The tracer uptake was assumed to have reached steady state after 45min[9]. Mice underwent SPECT/CT at 45min and 4hours after injection of 99mTc-HSA and moved around freely between imaging sessions.

CT-scans were performed with the following settings; 360° rotation with 360 projections. The magnification was set at low-medium with a binding factor of 2x2, yielding an isotropic pixel size of 47.83µm and a transaxial field view of 49mm. Tube voltage was set to 80kV, current was 500µA and each projection was exposed for 950ms. CT scans were reconstructed using Feldkamp algorithm, with Sheep-Logan filter and slight noise reduction. SPECT images were acquired using mouse whole body pinhole collimators (5 pinholes). A full 360° rotation with 60 projections and fixed radius of 30mm yielded a reconstructed image of 38mm transaxial field of view. A 20% energy window centered on the energy peak of 99mTc at 140keV was used. Acquisition duration was set to 10sec/projection in the early scan and 30 sec/projection in the late scan. CT and SPECT images were co-registered using a transformation matrix and SPECT data was reconstructed using Siemens MAP3D algorithm (matrix 128x128, 0.5mm pixels, 16 iterations and 6 subsets).
Analysis

Data analysis of the SPECT/CT fused images was performed with the INVEON Research Workplace software, version 4.2 (IRW, Siemens Healthcare, Ballerup, Denmark). Volumes of interest were defined around each injection site. Care was taken to include all activity in the hind limb. The activity decline from the injection site was assumed to follow a mono exponential function $e^{-kt}$. The removal rate was calculated as 100% $k$ (%/min).

Volumetric measures of hind limbs were calculated from acquired CT images. The distal tibiofibular joint was localized in 3D axial images using a previously described method [10], and functioned as the upper volumetric boundary limit. The hind limb distally from the tibiofibular joint was then defined using the region of interest function in Siemens IRW software, figure 3.

Lymph vessel immunohistochemistry

Hind limbs were fixed in 4% paraformaldehyde for 48 hours. Afterwards the feet were cut 10mm distal to the heel and decalcified in 4.0M formic acid/0.5M sodium formate for 4 hours before paraffin embedding. To detect lymphatic vessels, 3µm thick sections were obtained from the cut surface and stained using anti-LYVE1 (ab33682; Abcam, Cambridge, UK) as primary antibody was used. Antigen retrieval was achieved by Tris-EDTA buffer (pH 9.0) microwaving for 15min at 440W. Specimens were incubated with primary antibody (1:1000) for 60min at room temperature. Envision+ HRP labelled polymer (K4003; Dako; Agilent, Glostrup, Denmark) was used as detection system.
Analysis

Sectioned images were obtained using NanoZoomer 2.0-HT digital Slide Scanner and analyzed in Visiopharm Vision software version 2017.1 (Visiopharm, Hoersholm, Denmark). For each sectioned image, the region of interest was within the dorsal footpad area bounded laterally by the three middle metatarsal. The area was enclosed manually in two dimensions using the image analysis region of interest function, and the lumen of all stained lymphatic vessels inside the area were manually enclosed using point counting[11]. The areas within the region of interest occupied by lymphatic vessels were for each foot automatically calculated by Visiopharm Vision software.

Statistical analysis

All data were analyzed using STATA14 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP, USA) and Prism 6.0 (Graphpad Software, California, USA). A paired design using two-way ANOVA test was used to compare operated and control hind limbs within each group with Sidak’s multiple comparisons test. Sidak’s multiple comparison was used for comparing operated and unoperated limbs of the animals in each experimental group at each timepoint. All values were reported as a mean± standard deviation. A two-tailed p-value less than 0.05 was considered significant.
Results

Skin breakdown and adverse events

Mice in the 1x20Gy group developed various degrees of soft tissue and skin breakdown after 3 weeks, and all mice in the 2x15Gy, 2x20Gy and 1x30Gy groups developed severe soft tissue and skin breakdown after 2 weeks, table 1. After consultation with the local laboratory of animal science veterinarian, all mice were kept alive and received daily analgesic (Carprofen, 2mg/kg), however no mice underwent spontaneous wound healing. No mice in the remaining groups (Surgery Only, Silicone Splint, 2x7.5Gy and 2x10Gy) developed any adverse complications. In groups without adverse complications, the skin gap healed uneventful by the 2nd week of follow up. In the Silicone group, the implanted silicone splint was separated from the wound in all mice at the 2nd week of follow up.

Limb volumes

All operated limbs presented with initial postoperative swelling, figure 4. Hind limbs in the Surgery Only group had a non-significant limb enlargement at week 1, which recovered spontaneously after the 2nd week. Operated limbs in the Silicone Splint and 2x7.5Gy group had significantly higher volumes than their control limbs during the first two weeks of follow up, but the effect was lost at the 3rd week. The 2x10Gy, 1x20Gy and 1x30Gy groups maintained a relatively constant excessive volume during the entire follow up. In the 2x10Gy group, the lymphedematous limbs retained a mean excessive volume of 28.7±3.8% at the 8th week follow up. Groups that received high
irradiation dosages in two fractions, 2x15Gy and 2x20Gy showed a progressive increase in excessive volume at each following week.

**Lymphoscintigraphy**

Lymphatic clearance was calculated for operated and unoperated limbs derived from lymphoscintigraphy scans taken 45min and 4hours after tracer injection, *figure 5*. Lymphatic imaging revealed that mice irradiated with 2x10Gy had severe lymphatic backflow with lymph stasis distally to the operated site. To a lesser extend, some backflow was noticiable in the Silicone group and groups irradiated with 2x7.5Gy and 1x20Gy. Mice in the 2x15Gy, 2x20Gy and 1x30Gy groups showed patterns of lymph stasis in the footpad, likely due to circumferential soft tissue necrosis proximal of the ankle. No lymph back flow was visible in the Surgery Only group or the unoperated left hindlimbs.

Quantification of lymphatic clearance showed a significantly inhibited lymph flow in the 2x10Gy, 1x20Gy, 2x15Gy, 2x20Gy and 1x30Gy irradiated groups, *figure 6*. Mice in the Silicone and 2x7.5Gy group had a statistically non-significant difference in lymph clearence. The Surgery Only group had no statistical difference between their operated and unoperated limbs.

**Immunohistochemistry**

Lymph vessel dilation was assessed with LYVE-1 immunohistochemistry of the dorsal footpad, in experimental groups with and without soft tissue breakdown *figure 7*. Lymph vessel lumens in the groups without soft tissue breakdown were significantly enlarged in the 2x10Gy group, while the cumulative lymphatic lumens in the Surgery Only, Silicone and 2x7.5Gy group matched the lumen in their respective internal control limbs, *figure 8A*. The ratio of lymph vessel lumen inside the region of interest was significantly enlarged in the 2x10Gy, *figure 8B*. In the Surgery Only, Silicone
and 2x7.5Gy group, the lymphatic lumen to soft tissue ratio were corresponding to their control limbs.

Experimental groups with adverse skin breakdown, group 2x15Gy, 2x20Gy and 1x30Gy, had grossly and statistically significant dilation in lymph vessel lumen compared to internal control limbs, while the difference in the 1x20Gy group was non-significant, *figure 9A*. In the lymphedemateous limbs, the enlarged lymphatic vessel lumen constituted a relative greater area of the total subcutaneous tissue compared to the control limbs in the 2x15Gy, 2x20Gy and 1x30Gy group, *figure 9B*. Following a similar pattern, the lymph vessel to soft tissue ratio was nonsignificant in the 1x20Gy group. Examination of the sections for inflammatory cells revealed no inflammation.

**Discussion**

In this study, we evaluated the efficacy of 8 experimental lymphedema mouse hind limb models over the course of 8 weeks. Lymphedema was induced by surgical ablation of lymphatics in combination with either irradiation or with a silicone splint. Low irradiation dosages provided no lasting lymphedema beyond the acute postoperative phase, and higher irradiation dosages resulted in lasting lymphedema but with adverse soft tissue necrosis. We were unable to reproduce lasting lymphedema using the silicone splinted model[7]. In this study, the balance between desired lymphedematous outcomes and adverse events was achieved by combining surgical lymph resection with pre- and postoperative irradiation of 10Gy. Thereby, a simple refinement of existing methods resulted in excessive volume, impaired lymphatic transport and ectatic lymph vessels over the course of 8 weeks, without any severe complications such as non-healing wounds, *table 2*. 
Irradiation therapy is known to inhibit wound healing and potentially also the surgically induced inflammatory response[12–14]. A single fraction of preoperative high-dose irradiation has resulted in extensive morbidity[5,15], and there has been a need for titration of adequate radiation dosage[16]. In this study, we combined a reduced fraction of pre- and postoperative irradiation, with the aim of compromising lymphatic revascularization, while maintaining soft tissue architecture. We conducted a series of experiments, to find the optimal irradiation dose and fraction to induce lasting lymphedema without adverse tissue breakdown. Our results confirm the previous findings, that surgery alone is insufficient in inducing lasting edema[6,17], and high dose irradiation causes unacceptable soft tissue morbidity[6,16,18].

Lymphedema is caused by reduced lymph drainage capacity and therefore previous models have stressed the importance of evaluating lymph drainage function. Previous lymphedema models have advocated the use of photodynamic eye and IndoCyanine Green (ICG) lymphography for identification of dermal backflow patterns. While ICG lymphography has a high sensitivity and specificity for lymphedema staging in the clinic, it does not provide a quantitative measure for the functional lymphatic flow[19]. In this study, we monitored lymphatic flow patterns using $^{99m}$Tc-HSA lymphoscintigraphy after the tracer reached steady state. In addition, we calculated a numeric measure for the lymphatic pumping system, by determining the mean tracer clearance between two time points.

Circumference measurements are routinely used for diagnosing lymphedema in the clinic[20], however any manual measurement is prone to bias, especially in the setting of small sized animals. In previous animal models, edema has usually been quantified using water displacement, clippers or a circumference assessment[3]. In this study, bias was minimized by quantifying the swelling using a predefined high-resolution 3D volumetry setup[10].
Immunohistochemical staining of lymph vessels is also commonly effectuated in preclinical lymphedema research, to demonstrate lymph vessel dilation in the subcutaneous tissue secondary to lymph stasis. Lymph vessel immunoreactivity is usually quantified by the sheer number or by a one dimensional lymph vessel diameter assessment prepared from cross-section samples of the footpad[7,15,16], but with no clear definition of the inspected cross-section area. In this study, we defined a region of interest with clear bone segment boundaries. Thus, lymphatic endothelia in two dimensions could reliably be identified and lumen quantified regardless of soft tissue edema. A two dimensional quantification of lymph vessels is further appropriate, to discriminate between collapsed and dilated vessels. In lymphedematous tissue sections, we found that lymph vessel lumen covered an exponentially larger ratio of the soft tissue area, than sections from controls and un-edematous groups. The ectatic lumens are likely caused by lymph stasis and not increased lymph ejection, as lymph vessel lumen and lymph clearance were not correlated. In lymphedema, inflammation has been found to facilitate fibrosis and adipose deposition, thus worsening lymphedema[21]. Other preclinical studies has linked the inflammatory cascade to obesity[22,23], which is supported by an absence of inflammation in our study. These findings may provide ground for future studies to enlighten the pathophysiology of lymphedema progression.

Preclinical research provides an indispensable platform for studying potential lymphedema treatments, before they are introduced to the clinic. However it is of importance to conduct such studies in animal models that reliably replicate the human disease. Inguinal and popliteal lymphosomes have been identified as primarily responsible for the lymphatic drainage of the lower limb[24], emphasizing the importance of disrupting both pathways when replicating the 2x10Gy animal model. Tissue compositions in humans and rodents are inherently different, as rodents heal primarily by contraction and lack subcutaneous fat. To promote wound healing by re-epithelization in rodents, a temporary skin gap is often needed to mimic the human wound healing process[25].
Previous studies that have inspected a lesser invasive approach for preclinical lymphedema, by not implementing a temporary skin gap have shown less edema[18,26]. In these studies, lymphedema was defined as a 5% relative increase in limb volume, measured between the ankle and hip joint, which was achieved in 80% of cases. While the studies were one of the early adapters of µCT for lymphedema assessment, the measured area is unreliable. Rodents are loose-skinned animals at their core and proximal extremities[27,28], making any cross-section cutoff in the inguinal at risk of including tissue associated with the abdominal and pelvic area. Due to inevitable soft tissue sliding within this region, measurements are further variable on the degree of rotation, abduction and flexion of the hip, contrasted to a more reproducible landmark such as the distal tibofibularia joint[10]. In our 2x10Gy model, over 20% excessive volume was achieved in 100% of cases and measured with reliable methods. Recent lymphedema mouse studies have opted for follow-up periods of 4 weeks or less[7,29–32], as preclinical lymphedema has been known to progressively fade over time. In this study, follow up time was 8 weeks and lymphedema had been stable over the course of several weeks without signs of withdrawal. While longer follow-ups are always encouraging, the advantage of further follow-up would not offset the ethical burden in animal research[33].

The greatest obstacles in preclinical and clinical lymphedema studies lie in the definition of lymphedema, and in the deficiency of standardized and objective measures. In humans, limb lymphedema can be symptomatic and diagnosable at just a 5% increase in circumference to the unaffected limb[1], however in a preclinical research setting; a greater relative difference is often needed to investigate the potencies of potential treatments. In preceding studies with sufficient follow up and absence of morbidity, lymphedema has been only transient or marginally detectable in the 5% range with biased volumetric and circumferential measurements[3,18,26]. In this study, titration of radiation showed that two fractions of 10Gy irradiation resulted in the model retaining
over 20% increased volume without concurrent adverse events. Reliable and reproducible outcome measurements further resulted in a low standard deviation and statistical significance across all domains in the 2x10Gy model using a limited number of animals. Hence, the 2x10Gy model is a Refinement of previous works, in that no serious morbidity was observed and allows for lymphedema to be consistently quantifiable with a Reduced number of animals[33]. While the described mouse model shares many of the key clinical lymphedema characteristics, the etiology of the condition is different to the long-term onset seen in humans[34]. As rodents primarily heal by contraction and humans by reepithelization[35], no lymphedema rodent model can resemble the human condition perfectly. We believe this deviation is somewhat insignificant, as the purpose of the model is to provide a platform for studying the course of lymphedema and potential treatments before they are trialed in the clinic. Injectable agents and tissue transplants are prominent lymphedema treatment modalities[36,37], and preclinical research is needed to unveil the underlying mechanisms of a potential therapeutic response.

**Conclusion**

In this study, we objectively evaluated several experimental lymphedema models over 8 weeks, and found that only the 2x10Gy irradiation model retained lymphedema without unacceptable soft tissue necrosis. In addition to the implementation of a standardized volume assessment, the paper further details objective methodologies for quantification of lymph flow and lymph vessel immunohistochemistry. These methodologies elucidate essential aspects of lymphedema, which can aid in the clinical translation of future studies. Similarly, the refined lymphedema mouse model can be used for future observational and interventional studies with clinical implication.
Acknowledgments

We are grateful for the assistance of Peter Bollen, head of section and the animal technicians at the Biomedical Laboratory for their care and overseeing of the animals in this experiment.

This study was funded by unrestricted grants from Karen A. Tholstrup’s Foundation, Timber Merchant Vilhelm Bangs Foundation, Dagmar Marshall’s Foundation, A.J. Andersen and wife Foundation and Vissing Foundation supporting independent research.

Conflicts of interests

None of the authors has a financial interest in any of the products, devices, or drugs mentioned in this manuscript.
Reference list


Figure legends

Fig. 1: Study design and overview of the experimental procedures and time points for outcome measures.

Fig. 2: Depiction of the surgical lymphatic tissue ablation. A) Right hind paw after patent blue injection. B) Popliteal lymph node with prenodal and postnodal lymphatic vessels. C) Tying of the two prenodal lymphatic vessels before their unification. D) Resection of the stained popliteal lymph node and surrounding fat.

Fig. 3: High-resolution 3D µCT scans for limb volume assessment. Presented is the lower extremity of one mouse in the 2x10Gy group, 8 weeks after lymphedema was induced. At this point, the lymphedemateous limb (red) is retaining more than 20% increased volume compared to the control limb (blue). The distal tibio-fibular joint was used as a proximal landmark, and the limb volumes distally for this boundary were calculated.

Fig. 4: Adverse soft tissue events and limb volumes from each week extracted from µCT scans. Graphed over time is the mean volume and standard deviation of the operated hindlimbs in each experimental group. For illustrative purposes, the Control group is comprised of all unoperated limbs. Photographs in the top left quadrant represent adverse soft tissue events in the 4 experimental groups with adverse tissue events. Photograph in the bottom right quadrant depicts a representative edematous hind limb in the 2x10 group after 8 weeks of follow up. n.s= no statistically significant volumetric difference to internal control limbs at any time point. ^= Statistically significant volumetric difference of p<0.05 from internal control limbs during the first two weeks.*= Statistically significant volumetric difference of p<0.001 from internal control limbs during the entire follow up.
Fig. 5: $^{99m}$Tc-HSA lymphoscintigraphy performed 4 weeks after surgical lymph ablation. SPECT/CT images were obtained 45min and 4hours after tracer injection. Tracer uptake can be seen in lymph nodes, bladder and intestine. Subcutaneous lymph stasis and backflow is extensively visible in the right hind limbs in the 2x10Gy group and to a lesser extend in the Silicone, 2x7.5Gy and 1x20Gy group. Diffuse lymph stasis in the right footpad is visible in the 2x15Gy, 2x20Gy and 1x30Gy group. No lymph backflow or diffuse stasis is visible in the Surgery Only group or in any of the unoperated left hind limbs, which served as internal controls with intact popliteal and inguinal lymph nodes. **Colored scale** bar represents tracer uptake intensity.

Fig. 6: Lymphatic clearance quantified with $^{99m}$Tc-HSA tracer injection at the 4th week follow up. After the tracer uptake reached steady state, clearance was calculated from 45min and 4 hour scans and compared between operated and unoperated limbs. ns= not significant. *= Significant difference of p<0.05 between operated and control limbs. **= Significant difference of p<0.001 between operated and control limbs.

Fig. 7: Depiction of the immunohistochemical analysis of lymph vessels 8 weeks after surgery. Representative images of LYVE-1 stained lymphatic vessels in a paraffin-embedded cross-section from the dorsal footpad. Lymph vessel dilation increased with higher irradiation dosages. Unoperated control limbs and Surgery Only, Silicone and 2x7.5Gy operated limbs cross-sections were predominanted with collapsed lymph vessels. **Black scale bar** represents 100µm.

Fig. 8: Immunohistochemically assessment of lymph vessels in the experimental groups without skin breakdown. Cross-sections were taken 10mm from the heel and lymph vessels were stained with LYVE-1 antibody. Lymph vessels were quantified in two dimensions by point counting in the dorsal footpad area, defined by the three middle metatarsals. A) Representation of the absolute area represented by lymphatic endothelia and vessel lumen for each experimental group. B) Depiction of
the total immunohistochemical area of interest and lymph vessel lumen ratio for each experimental group. ns= not significant. **=Significant difference of p<0.001 between operated and control limbs.

Fig. 9: Immunohistochemically assessment of lymph vessels in the experimental groups with skin breakdown. Cross-sections were taken 10mm from the heel and lymph vessels were stained with LYVE-1 antibody. Lymph vessels were quantified in two dimensions by point counting in the dorsal footpad area, defined by the three middle metatarsals.A) Representation of the absolute area represented by lymphatic endothelia and vessel lumen for each experimental group. B) Depiction of the total immunohistochemical area of interest and lymph vessel lumen ratio for each experimental group. ns= not significant. **=Significant difference of p<0.001 between operated and control limbs.