

Original Article

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Effect of cryopreservation on lymph node fragment regeneration after autologous transplantation in the minipig model

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Abstract

Introduction: Lymphoedema is a worldwide pandemic causing swelling of tissues due to dysfunctional transport of lymph fluid. Present management concepts are based in conservative palliation of symptoms through manual lymphatic drainage, use of compression garments, manual lymph drainage, exercise, and skin care. Nevertheless, some curative options as autologous lymph node transplantation were shown to reduce lymphoedema in selected cases. Lately, some concern has arisen due to reports of donor site morbidity. A possible solution could be the development of artificial lymph node scaffolds as niches of lymphatic regeneration. Engineering these scaffolds has included cryopreservation of lymph node stroma. However, the effects of cryopreservation on the regeneration capacities of these organs were unknown.

Materials and methods: Here, we used the minipig animal model to assess lymphatic regeneration processes after

cryopreservation of autologous lymph nodes. Superficial inguinal lymph nodes were excised and conserved at -80°C for 1 month. Thereafter, lymph node fragments were transplanted in the subcutaneous tissue.

Results: Regeneration of the lymph nodes was assessed five months after transplantation. We show that lymph node fragment regeneration takes place in spite of former cryopreservation. Transplanted fragments presented typical histological appearance. Their draining capacity was documented by macroscopic transport of Berlin Blue dye as well as through SPECT-CT hybrid imaging.

Discussion: In conclusion, our results suggest that processes of cryopreservation can be used in the creation of artificial lymph node scaffolds without major impairment of lymph node fragments regeneration.

Keywords: artificial lymph node; cryopreservation; lymph node transplant; lymphoedema.

Introduction

Lymphatic fluid, products of cell catabolism, antigens and debris are transported from the tissues and organs through a fine network of transparent valved vessels called lymphatics [1, 2]. These drain into lymph node chains, where lymphatic fluid is progressively thickened through selective fluid filtration into the blood capillaries of these organs [3]. This reabsorption not only promotes homeostasis of drained tissues but also allows exposure of nodal dendritic cells and macrophages to potential pathogens, promoting antigen phagocytosis. Dendritic cell-driven presentation of antigen surface molecules to lymphocytes eventually activates tissue-specific immunity [4].

Impairment of lymph node function or lymphatic transport can lead to tissue swelling called lymphoedema. This disease has multiple causes. Primary lymphoedema has a reduced overall incidence. In developing countries, secondary lymphoedema mainly occurs due to filarial worm

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infection. According to World Health Organization data, about 40 million patients have lymphoedema due to these parasites [5, 6]. In developed countries, secondary lymphoedema is often related to oncologic causes or trauma [7].

In malignant diseases such as breast cancer, melanoma or prostate cancer, draining lymph nodes participate in the recognition of tumour cells and have become important markers for metastatic disease and overall prognosis [8]. Surgical removal of lymph nodes is often necessary. About 20%–30% of the patients develop limb lymphoedema after axillary or inguinal lymph node removal [9, 10]. Lymphoedema is a lifelong condition that reduces the quality of life and productivity of affected patients [10]. Its continuous conservative management has high costs for the health system [11].

As the immune system and its organs show great plasticity, concepts of autologous lymph node transplantation were developed for the treatment of secondary lymphoedema [12–14]. The aim of these surgical procedures is to substitute damaged lymph nodes through functioning nodes contained in autologous tissue flaps. Clinical studies seem to show some benefit, with reported cure in 32%–40% of the patients, and improvement in the great majority [12–16]. However, donor site morbidity in the form of lymphoedema of the donor area was also described [17–19]. It might therefore be important to develop alternative donor solutions to prevent this major complication.

Concepts of artificial lymph node engineering originally arose to face the challenge of patients with congenital paucity of lymph nodes without autologous harvesting possibilities (primary lymphoedema). Nevertheless, present evidence of possible donor site morbidity might extend the potential utility of artificial lymph node constructions to the treatment of secondary lymphoedema.

Although still in development, artificial lymph node constructs seem to induce efficient regeneration when biocompatible scaffolds and stromal cells are used [20]. This induces migration of immune cells into the artificial constructs, resulting in the presence of dendritic cells as well as B- and T-lymphocyte clusters with typical cortical and paracortical distribution [21]. Some processes of tissue engineering associated with artificial lymph node constructs apply methods of cryopreservation to allow laboratory manipulation [21]. Nevertheless, it was unknown if such cryopreservation could permanently damage the regenerative capacity of nodal tissues [22].

The purpose of this study was to analyse lymph node fragment regeneration after cryopreservation in the minipig model. The viability of cryopreserved lymph node fragments was assessed after avascular transplantation. Here, physiological drainage capacities were analysed through SPECT-CT hybrid imaging and subsequent histologic analysis provided insights on regeneration processes.

Methods

Minipig model and experimental groups

A total of eight wild-type Göttinger minipigs weighing 6–11 kg were obtained from the animal facility of the University of Göttingen (Relliehausen, Germany). They were housed in our animal facility, according to German legal specifications, and fed mixed food and water *ad libitum*. Animal procedures were approved by the government of Lower Saxony (Ref. No. 509.6-42502-07/1402).

Animals were divided in two groups (Figure 1). The number of animals allocated to each group was different, as group A was prioritized on being given the possibility of intra-individual limb volume control (negative control in the non-operated left limb). Group A

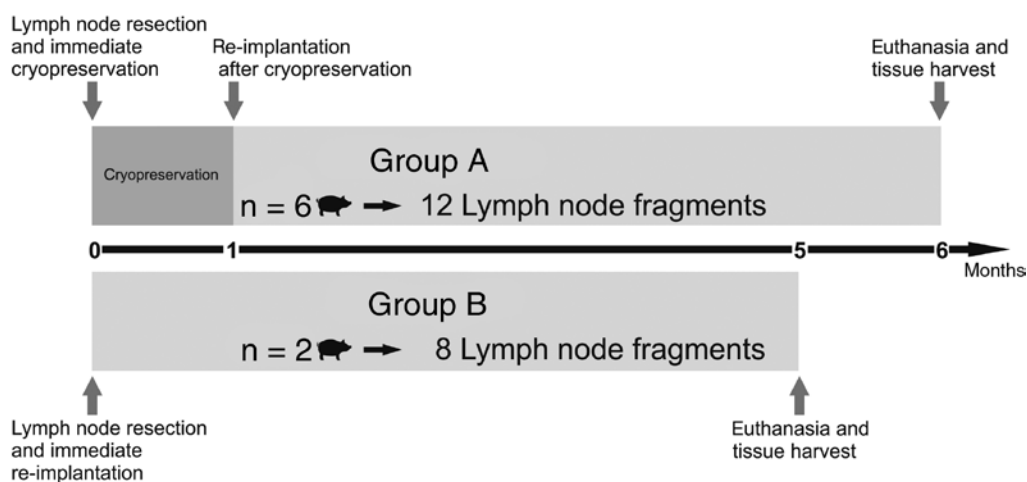


Figure 1: Schematic overview of the study outline during the 6-month follow-up.

(n=6) was therefore submitted to resection of the right lymph node under general anaesthesia, as previously described [23], with the left side of the animal being left intact. The single superficial groin lymph node present in this species was operatively excised on the right side. Afferent and efferent lymphatics were carefully sharply resected in the immediate vicinity of the organ capsule. The blood vessels of the hilum were also resected. No ligatures were applied to the organ. The excised lymph node was then cut transversally in two fragments of about 1-cm³ size and introduced in sterile recipients. After hermetic closure, recipients were immediately submersed in a bath of nitric oxide. Soft plastic recipients were used to maximise the surface of thermal contact. No chemical cryoprotection was performed, as the maximal distance for cold penetration was about 5 mm. Therefore, the risk of water crystals formation was considered negligible, rendering additional exposure of the lymph node fragments to alcohols or sulfoxides avoidable. Fragments were transported to final storage at -80 °C immediately after the operation and were conserved in these conditions for 1 month. Thereafter, animals were re-operated under general anaesthesia, and the corresponding two fragments (autologous transplantation) were re-implanted in the subcutaneous fatty tissue of the groin and left to unfreeze *in vivo*. The left groin was used as negative control for limb swelling.

In both animals of group B (positive control, n=2, total of four lymph nodes), both groin lymph nodes were excised and transversally fragmented. Immediate avascular re-implantation occurred in the same operative procedure.

In group A, a degeneration of cryopreserved, avascular lymph node transplants would be plausible and could become the origin of necrosis and inflammation at the operative site with subsequent lymphoedema. Therefore, transplants were only performed unilaterally, allowing comparison of the limbs.

In control group B (positive control), based on our previous unpublished observations, we expected no lymphoedema development and therefore proceeded to bilateral transplants.

All transplantation areas were closed by atraumatic sutures and the skin was sutured. Wound healing was regularly checked. Pre-medication, anaesthesia and wound prophylaxis were performed, as previously described [23].

Lymph node fragment regeneration assessment through SPECT-CT hybrid imaging and histology

Five months after transplantation, animals in both groups were injected a mixture of 10 MBq ^{99m}Tc-nanocolloid in combination with Berlin blue in the hind paws, as previously described [23]. Animals were then left alive 1 h under anaesthesia to allow lymphatic drainage of the extremities. After *exitus letalis*, SPECT-CT hybrid imaging was performed, as previously described [23]. Shortly after, histologic probes of regenerated lymph node fragments were taken based on gamma probe signalling and macroscopic blue dye caption. A photographic register of the skin where positive samples were marked *in situ* allowed direct correspondence between SPECT-CT hybrid imaging and later histologic results. Lymph node samples were frozen in liquid nitrogen immediately after sampling and left to lose their radioactivity for 3 days. To avoid excessive staining due to the *pre mortem* administration of Berlin blue in the paws, samples were sectioned and simply stained with haematoxylin-eosin. Full slices of macroscopic regions of interest were analysed initially at $\times 10$ magnification under the optical

microscope. Samples presenting lymph node fragments were selected for further characterisation. Lymph node fragments were considered vital when they presented typical B- and T-lymphocyte distribution in the cortex and paracortex, respectively, showed uptake of Berlin blue in the sinuses and had lipomatosis and fibrotic degeneration features in less than 50% of the fragment surface on at least three sections. Histologic analysis was performed blind by one observer. Later, histologic probe numbers were associated with each SPECT-CT hybrid imaging to allow final assessment of results.

Data analysis

The experimental data generated were reported as fragment regeneration rate and expressed as proportion to the total number of transplanted fragments. The restricted number of observations in the reported big animal model allowed direct interpretation of the results without additional statistical analysis. Histologic pictures were taken, with the Panoramic Viewer (3dHistech, Budapest, Hungary) as viewing software.

Results

In this study, a total of 8 minipigs and 10 groin lymph nodes divided into 20 fragments were analysed (two animals having had a bilateral procedure). Operative procedures were well tolerated in all cases. Animals were controlled weekly for any major complications such as wound healing problems, seromata or macroscopic lymphoedema (defined for this purpose as pitting of the subcutaneous tissue after deep thumb pressure for 1 min). No complications were registered during follow-up.

Avascular lymph node fragments can regenerate in the subcutaneous tissue

Regardless of the groups, 14 avascular lymph node fragments were able to regenerate and connect to the surrounding blood and lymphatic vessels. Six fragments were histologically necrotic and/or showed no locoregional draining activity, as they did not capture ^{99m}Tc-nanocolloid or Berlin blue injected in the hind paws. Therefore, avascular lymph node fragment survival in healthy subcutaneous tissues was observed in ca. 70% of the cases.

Cryopreserved fragments have similar regeneration rates than fresh fragments

To assess the regeneration rates of transplanted lymph node fragments, blind analysis of histologic probes

was performed, and results were later combined with obtained SPECT-CT hybrid imaging. A 100% correlation of both methods was observed. Regenerated fragments were clearly identifiable in SPECT-CT hybrid imaging as functional and draining the lymphatic fluid of the injected hind paws (Figure 2). Later, histologic probes were sampled based on macroscopic caption of injected Berlin blue dye, but were also located *post mortem* with a gamma probe. Histologic sampling occurred in the whole region of interest to account for eventual necrotic fragments not visible in the process of peripheral injection. Regional probes containing only fatty tissue or necrotic lymph nodes were considered as non-regenerated transplants (Figure 3B and C). In all cases, they corresponded to regions failing to cause “hot spots” in SPECT-CT hybrid imaging. Histologic analysis of fragments capturing Berlin blue/ ^{99m}Tc -nanocolloid revealed a functional organisation of the lymph node architecture, presence of a capsule and peripheral sinuses engorged with blue dye (Figure 3A and D–F). Additionally, indirect signs of immune function were given by the presence of germinal follicles (Figure 3F). According to our criteria, 8 of 12 fragments were able to regenerate for the cryopreserved lymph nodes in group A.

In group B, 6 of 8 immediately transplanted fragments were regenerated after the 5-month follow-up. Therefore, the regeneration rate in group A (67%) was comparable to the regeneration in group B (75%), despite the relatively low number of total observations (Figure 4). These findings suggest no major impact of cryopreservation in the capacity of lymph node fragments to regenerate in healthy subcutaneous fatty tissue.

Discussion

Lymphoedema is one of the pandemics that reached the twenty-first century without an established cure [7]. Meeting the challenges of lymphoedema treatment implies fine tuning of several management options [24], as lymphoedema has several causes. Because there is no universal cure for lymphoedema, it is paramount to thoroughly understand lymphatic regeneration and explore possible solutions to attain successful management options. The possibility of generating artificial lymph nodes might be therapeutic in specific cases when patients lack functioning donor nodes [25].

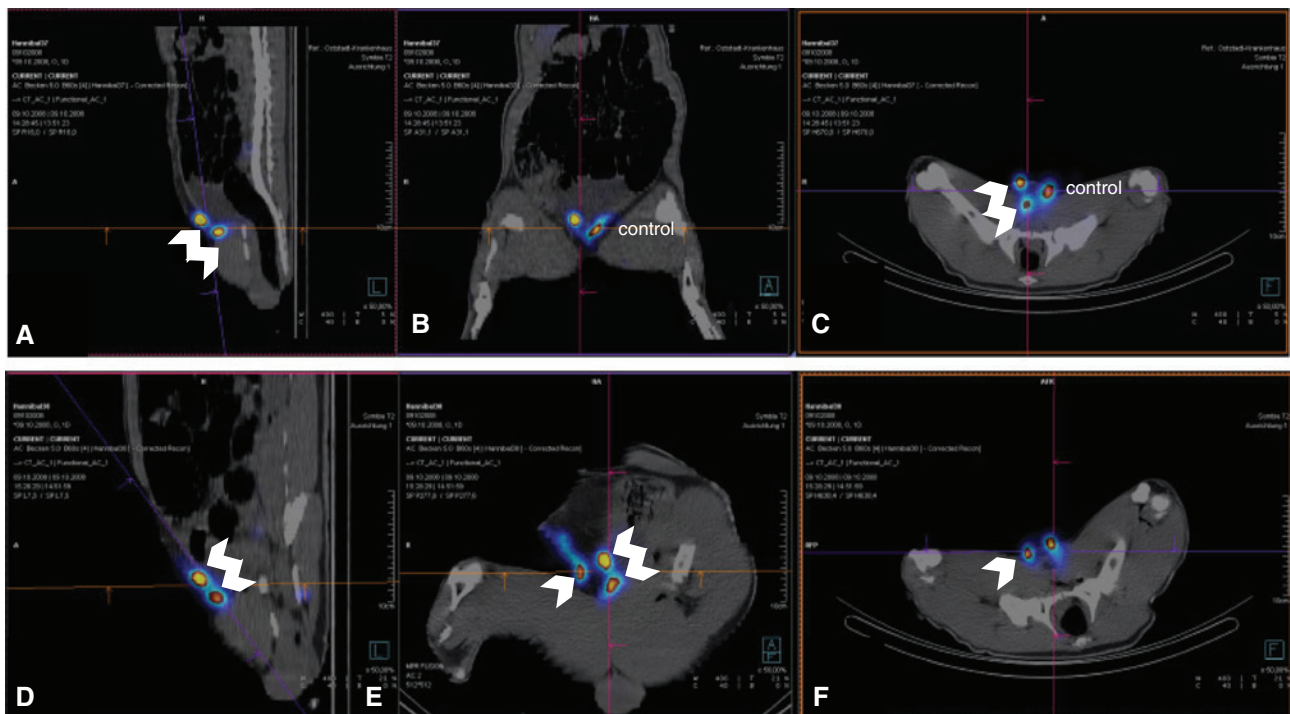


Figure 2: Lymphatic imaging for functional analysis of transplanted lymph node fragments.

SPECT-CT hybrid imaging of a minipig in group A (A–C) with two avascular cryotransplants in the right groin (double arrow). The left groin was used as control. All images were taken after injection of ^{99m}Tc -nanocolloid bilaterally in the hind paws. (D–F) SPECT-CT hybrid imaging of a minipig in group B (bilateral transplantation). In the right groin, only one of the two transplanted fragments is vital (single arrow); in the left groin, both transplanted fragments are vital and draining the periphery (double arrow). (A, D) Sagittal plane. (B, E) Coronal plane. (C, F) Transverse plane.

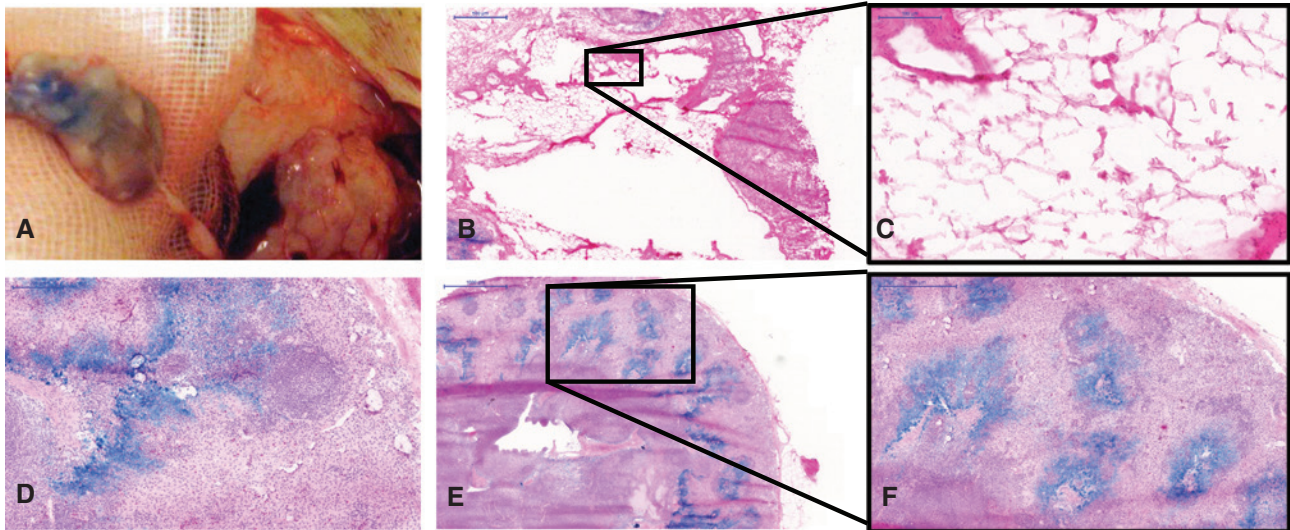


Figure 3: Macroscopic and microscopic aspects of transplanted lymph node fragments (haematoxylin-eosin). (A) Intraoperative macroscopic view. (B, C) Necrotic fragments presented fatty degeneration features observable within the lymph node capsule without major presence of lymphocytes. Vital fragments, in contrast, showed typical lymph node architecture (D–F) and sometimes germinal follicles (F). Here, blue-stained sinuses result from the peripheral injection of Berlin blue stain in the hind paws *ante mortem*. Scales: (B and F) 500 μm , (C) 100 μm , (D) 200 μm , and (E) 1000 μm .

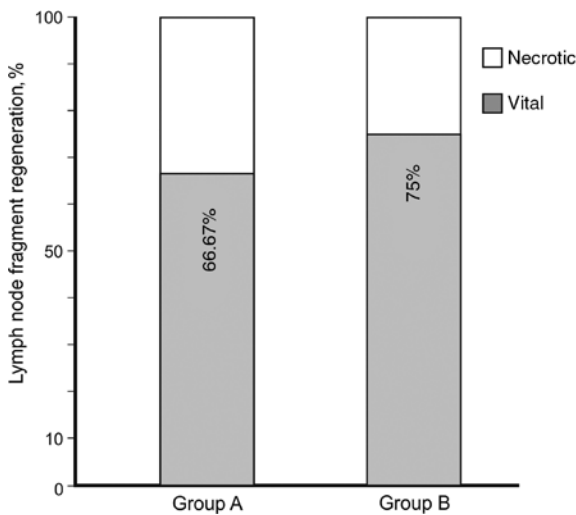


Figure 4: Regeneration rates (in percent) for each group.

Animal model

This study aimed at a proof of concept. The animal model used is not a paradigm for lymphoedema, as none of the animals in this study developed this condition. It is still extraordinary that even bilateral resection of both superficial groin lymph nodes in group B did not generate any kind of macroscopic swelling of the hind paws. These findings suggest an intact regeneration capacity of lymphatics in these relatively young animals, a characteristic that might be lost in patients with secondary lymphoedema,

e.g. after oncologic therapy including operative scars and radiotherapy of the surrounding tissues [8]. Since avascular organ transplants are initially completely dependent of the local diffusion capacities of the tissues, modelling the subcutaneous conditions in secondary lymphoedema patients is paramount for the predictive value of our findings. Although our models presented vast subcutaneous scars from radical lymphadenectomy of the groin, they were nevertheless free of tissue radiation, and therefore, the scar quality and tissue regeneration improved with time. This does not reproduce the subcutaneous evolution of scars in most irradiated oncologic patients [1, 3]. In addition, this animal model is less prone to lymphatic swelling due to the shortness of its limbs in comparison with the length of his body. The hind limb/body length ratio in an adult minipig is about 1/2, whereas this proportion in adult humans is roughly 1/1 for the arms and 5/6 for the legs [26]. Human extremities therefore possess an overproportioned terminal vessel network. Additionally, this big animal model also presents a physiological advantage against swelling by having the groin at the same level of the heart [27], a feature mostly observed in humans only during sleep. Notwithstanding all limitations, this big animal model provides a similar proportion of subcutaneous fat, mimicking the tissue environment of avascular lymph node stroma transplantations in healthy humans.

Assessment of lymph node fragment regeneration was very consistent between SPECT-CT hybrid imaging

evaluation and histologic analysis. This confirms the validity of SPECT-CT hybrid imaging for functional assessment of the lymphatic system. Because the radionuclide is injected in the paws, only lymph node fragments draining the periphery could capture the signal. Additional histologic information was added to this functional characteristic by using a combination of ^{99m}Tc -nanocolloid and Berlin blue dye. It allowed macroscopic visualisation of functional fragments whilst retrieving the probes and provided histologic staining of the lymph node sinuses. The observation of lymph node sinuses and a functional lymph node capsule is paramount, as it allows the differentiation between a regenerated organ and the occurrence of tertiary lymphoid tissue [28]. Nevertheless, this strong correlation between lymph node fragment function proven by SPECT-CT hybrid imaging and histologic confirmation of the viability of radionuclide-positive, blue-stained nodes did not exclude false negatives. This possible weakness was countered by retrieving histologic probes of the subcutaneous regions of former lymph node fragment transplantation areas, even when no radionuclide signalling was visible with the gamma camera and no blue dye was present. Nevertheless, in most of these, only fatty tissue was detectable, so that a formal verification of lymph node fragment necrosis was not possible in most cases. Fatty degeneration of unviable tissues within the lymphatic tissue is a common observation within primary lymphatic organs as the thymus [29] or ageing human lymph nodes [30]. This might explain why the histologic location of unviable fragments was not feasible in most cases, and only fatty tissue could be observed in non-signalling regions of transplantation. Nevertheless, even if histologic false negatives occurred, and regenerated fragments were missed, these would have presented a local vascular connection only and therefore would have not been relevant for limb drainage or else they would have captured peripheral radionuclide.

The observed microarchitecture of regenerated lymph nodes in the histologic analysis suggests a participation in immune processes [31], although the animals were kept in a clean facility. The presence of an organ capsule, sinuses, and germinal follicles in the paracortex stressing a compartmentation of immune cells are all indirect signs of immune competence of regenerated lymph node fragments. Clear confirmation of these findings would nevertheless require immune challenge analysis as well as immunohistochemistry; thus, further studies are needed [31].

The interpretation of our regeneration rates was limited by the low number of samples. This is a common feature with most studies using big animal models. In the

current study, the cryopreservation group A was bigger than the positive control group B (12 lymph node fragments in group A vs. 8 in control group B; therefore, group B was only two thirds of the size of group A). This created restrictions in the comparison of both groups, as a fragment in group A accounted for 8.3% of the results within the group, whereas in group B, each fragment represented 12.5% of the group results. Therefore, registered differences in the regeneration rates of both groups ([regeneration rate in group A] – [regeneration rate in group B] = 9%) do not account for significant differences, as a single observation of fragment regeneration or necrosis within a group could be mathematically sufficient to close this gap.

A comparative analysis of the regeneration rates of vascularised lymph node flaps and avascular lymph node fragments is laborious. Vascularised flaps containing lymph nodes were mostly investigated in humans [12–16], and therefore, survival of the flaps was analysed indirectly by measuring the ability to improve lymphoedema. Radioisotope controls of post-operative flap draining abilities were not performed systematically [12–16]. Additionally, a thorough anatomical characterisation of the flaps is failing, so that the number of lymph nodes contained in each flap is a mere estimation. In humans, lymph node flaps have about three to five lymph nodes. The capacity to dramatically improve lymphoedema after transplantation procedures is established to be about 30%–40% of the cases [12–16], depending on lymphoedema stage, but some degree of improvement could be observed in most patients [12]. It is arguable if these partial improvement cases correspond to loss of draining function of transplanted nodes despite blood vessel micro-anastomosis. Nevertheless, it seems safe to admit that vascularised lymph node flaps present a non-negligible quantitative advantage compared with avascular lymph node fragments, as the first contain several lymph nodes and the latter only have two regeneration foci per harvested lymph node. The regeneration of autotransplanted lymph node fragments in rats can be enhanced by injection of the growth factor vascular endothelial growth factor C in the draining area [32, 33].

Meanwhile, the risk for the donor area was described for the microsurgical lymph node flaps [17–19].

Additionally, the comparison of our regeneration rates with those of artificial lymph nodes needs further studies, as current publications on artificial nodes are focussed on single cases and the success rates of the attempts were not measured or were left unmentioned [20–22].

Processes responsible for lymph node regeneration seem to be relatively independent of nutrient diffusion through the tissue. The average amount of tissue

estimated to survive under diffusion conditions is about 1 cm [22], but this distance is increased in some tissues, e.g. cartilage, and decreased for highly metabolic tissues, e.g. heart muscle. Hypothetically, fragmentation, whilst multiplying regeneration foci, would improve initial lymphangiogenesis induced by the transplants when reconnecting to the surrounding tissues. Nevertheless, it could probably only partially guarantee the survival of nodal cell populations through diffusion [22].

Stroma cells seem to be the key players of lymph node regeneration [34]. Lymphocyte populations were shown to later recolonise viable transplants and recover typical architecture and immune function [33, 34]. Lymph node tissue regeneration is clinically relevant in the described lymph node transplant procedure [12–16]. Nevertheless, some patients present with lymphoedema combined with a paucity of potential donor lymph nodes, as in some primary conditions [25]. In such cases, creation of artificial lymph nodes based on human material could be a possible solution for inducing lymphangiogenesis and improving local immune responses [20]. Successful creation of artificial lymph nodes can use lymph node stroma cells as inducers of the whole regeneration cascade. Although migration of peripheral dendritic cells into the nodes is possible [35], this only takes place if nodal stroma cells are present for signalling the existence of this organ when it contacts its environment [34]. It was not known until now if cryopreservation processes could affect the capacity of stroma cells in inducing regeneration [22].

The present study in a large animal model shows us that cryopreservation methods do not seem to affect the regeneration of lymph node fragment function significantly. This feature might allow future laboratory use of lymph node stroma to make generation of artificial lymph nodes possible.

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Author Statement

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is not applicable. **Ethical approval:** The research related to the animal use complies with all the relevant national regulations and institutional policies. It was performed in accordance to the criteria approved by the institutional review board of the region of Lower Saxony.

Author Contributions

Catarina Hadamitzky: overall data analysis and writing of the manuscript; Hanes Perić: overall data analysis and writing of the manuscript; Sebastian Theobald: histologic data analysis; Klaus Friedrich Gratz: data analysis on SPECT-CT hybrid imaging; Hendrik Spohr: design of the study; Reinhard Pabst: design of the study and approval of the manuscript; Peter Maria Vogt: approval of the manuscript.

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Supplemental Material: The article (<https://doi.org/10.1515/iss-2018-0003>) offers reviewer assessments as supplementary material.



Reviewer Assessment

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Reviewers' Comments to Original Submission

Reviewer 1: Thomas Kremer

Feb 04, 2018

Reviewer Recommendation Term: Accept with Minor Revision
Overall Reviewer Manuscript Rating: 95

Custom Review Questions

Custom Review Questions	Response
Is the subject area appropriate for you?	5 - High/Yes
Does the title clearly reflect the paper's content?	5 - High/Yes
Does the abstract clearly reflect the paper's content?	5 - High/Yes
Do the keywords clearly reflect the paper's content?	5 - High/Yes
Does the introduction present the problem clearly?	3
Are the results/conclusions justified?	5 - High/Yes
How comprehensive and up-to-date is the subject matter presented?	5 - High/Yes
How adequate is the data presentation?	5 - High/Yes
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	3
Are the experimental methods/clinical studies adequate?	5 - High/Yes
Is the length appropriate in relation to the content?	3
Does the reader get new insights from the article?	5 - High/Yes
Please rate the practical significance.	5 - High/Yes
Please rate the accuracy of methods.	5 - High/Yes
Please rate the statistical evaluation and quality control.	N/A
Please rate the appropriateness of the figures and tables.	4
Please rate the appropriateness of the references.	5 - High/Yes
Please evaluate the writing style and use of language.	4
Please judge the overall scientific quality of the manuscript.	5 - High/Yes
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

The authors present a manuscript reporting on the effects of cryoconservation of lymphnodes Prior to avascular re-Implantation. Interestingly, two thirds of the replantet fragments survive and show lyphatic Drainage capacity.

Abstract: conclusive, well written

Introduction: The introduction accurately describes the scientific Background. However, the section on lymphedema, causes (Primary - secondary), and Treatment Options is not the focus of the manuscript, since the main focus is on the experimental subject of cryoconservation. I would recommend to shorten the introduction and to focus on the main Topic.

Materials and Methods: the experimental protocol is well described and clearly documented. Please describe why the different Group sizes and experimetal protocolls were chosen fpor Groups A and B (12 Lymphnodes and unilateral procedure in Group A, and 8 Lymphnodes and bilateral procedure in Group B).

Results: no changes required.

Disussion: The animal model as well as the experimental protocol and the results are well discussed. The differences between minipigs and humans regarding positioning and extremity length are discussed. However, I miss a small section on the differences regarding the healing - Diffusion capacity of the tissues - These are normal in this model and might be very different in the clinical Setting due to scarring or postradiation alterations.

Reviewer 2: anonymous

Feb 14, 2018

Reviewer Recommendation Term: Revise with Major Modification
Overall Reviewer Manuscript Rating: 50

Custom Review Questions	Response
Is the subject area appropriate for you?	5 - High/Yes
Does the title clearly reflect the paper's content?	4
Does the abstract clearly reflect the paper's content?	4
Do the keywords clearly reflect the paper's content?	4
Does the introduction present the problem clearly?	4
Are the results/conclusions justified?	2
How comprehensive and up-to-date is the subject matter presented?	3
How adequate is the data presentation?	3
Are units and terminology used correctly?	4
Is the number of cases adequate?	2
Are the experimental methods/clinical studies adequate?	2
Is the length appropriate in relation to the content?	3
Does the reader get new insights from the article?	2
Please rate the practical significance.	4
Please rate the accuracy of methods.	2
Please rate the statistical evaluation and quality control.	3
Please rate the appropriateness of the figures and tables.	3
Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	3
Please judge the overall scientific quality of the manuscript.	2
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

Nice approach. There are some problems with the methods.

1. Cryopreservation follows different rules. Nowadays special protocols to avoid freeze-thaw damage are used. I understand, that they just froze the lymph nodes without cryoprotection.
2. I would think that the rate of vessels within the specimen are relevant for later revascularisation! Any data on this?
3. If one wants to asses the take rate I suggest to look at the lymph nodes after 5-7 days. If I want to asses their lymphatic capacity the 5 months may work.

So the conclusions are not really justified. Some details remain unclear and need specification: e.g. "In the current study, the cryopreservation group A had an additional 1/3 of observations in comparison with the positive control group B"

Authors' Response to Reviewer Comments

Feb 22, 2018

Dear Prof. Dr. Jaehne,

We are very grateful for the possibility of revising and improving our manuscript according to the provided comments. We hereby send the point-by-point analysis of the comments, as well as our manuscript now entitled "Effect of cryopreservation on lymph node fragment regeneration after autologous transplantation in the minipig model" (ISS-2018-0003), as you suggested. All changes were inserted with the "track changes" option.

POINT-BY-POINT RESPONSE TO THE REVIEWERS' COMMENTS:

Reviewer #1:

The authors present a manuscript reporting on the effects of cryoconservation of lymph nodes prior to avascular re-implantation. Interestingly, two thirds of the replant fragments survive and show lymphatic drainage capacity.

Abstract: conclusive, well written

Introduction: The introduction accurately describes the scientific background. However, the section on lymphedema, causes (Primary - secondary), and Treatment Options is not the focus of the manuscript, since the main focus is on the experimental subject of cryoconservation. I would recommend to shorten the introduction and to focus on the main Topic.

When re-reading the manuscript with this comment in mind, we also noticed that our fascination for lymphoedema eventually led to a disbalance in the quantity of information given about this disease. We therefore shortened the introduction of a total of 553 characters in the suggested parts that were not focussed on the main topic (corresponds to about 25% shortening) (please see lines 66-92 of the Introduction section in the manuscript).

Materials and Methods: the experimental protocol is well described and clearly documented. Please describe why the different Group sizes and experimental protocols were chosen for Groups A and B (12 Lymph nodes and unilateral procedure in Group A, and 8 Lymph nodes and bilateral procedure in Group B).

Because of the costs associated with the maintenance of big animal models during a five month follow up, the budget allocated to this proof of concept study allowed only a total of eight animals. In other experiments (not yet published) we had observed no lymphoedema after radical lymphadenectomy and immediate lymph node fragment transplantation. Therefore, we proceeded to a bilateral procedure in the positive control animals where a low probability of swelling was expected.

In contrast, in the cryotransplanted models we did not know what to expect. One plausible hypothesis was that all transplanted fragments would degenerate, and the animals could get lymphoedema through necrosis and inflammation in the operative site. Therefore, these animals were operated unilaterally as to possess an intraindividual limb control in case of macroscopic swelling. This ended up being redundant, as a certain degree of regeneration took place after cryotransplantation, and lymphoedema did not occur.

We explained the above-mentioned circumstances in the methods section in the following sentences:

"The number of animals allocated to each group was different, as priority was given to the possibility of intra-individual limb volume control in group A (negative control in the non-operated left limb)." (please see lines 124 – 126 of the Methods section)

and:

"In group A, a degeneration of cryopreserved, avascular lymph node transplants would be plausible, and could come to originate necrosis and inflammation at the operative site with subsequent lymphoedema. Therefore, transplants were only performed unilaterally, allowing comparison of the limbs.

In control group B (positive control), due to prior unpublished observations of our group, we expected no lymphoedema development and therefore proceeded to bilateral transplants." (please see lines 149 – 155 of the Methods section).

Results: no changes required.

Discussion: The animal model as well as the experimental protocol and the results are well discussed. The differences between minipigs and humans regarding positioning and extremity length are discussed. However, I miss a small section on the differences regarding the

healing - Diffusion capacity of the tissues - These are normal in this model and might be very different in the clinical Setting due to scarring or postradiation alterations.

These important ideas were added to the discussion in the revised version:

“Since avascular organ transplants are initially completely dependent of the local diffusion capacities of the tissues, modelling the subcutaneous conditions in secondary lymphoedema patients is paramount for the predictive value of our findings. Although our models presented vast subcutaneous scars from radical lymphadenectomy of the groin, they were nevertheless free of tissue radiation, and therefore the scar quality and tissue regeneration improved with time. This does not reproduce the subcutaneous evolution of scars in most irradiated oncologic patients [1,3].” (please see lines 260-267 of the Discussion section of the manuscript).

Reviewer #2:

Nice approach.

There are some problems with the methods.

1. Cryopreservation follows different rules. Nowadays special protocols to avoid freeze-thaw damage are used. I understand, that they just froze the lymph nodes without cryoprotection.

Although cryoprotection has been used since decades, several protocols are common including a vast range of chemical substances. No single cryoprotection method can be described as standard for lymph nodes. Fortunately, lymph node fragments were about 1 cm³ size. Because soft sterile containers were used that allowed thermal contact all around the organ fragments, the maximum distance for cold penetration in liquid nitrogen was about 5 mm in our case. Therefore, fast freezing could take place with negligible risk of water crystal formation. Also, the transformation rate of vitreous ice to expanded crystal ice during the relatively short freezing phase of one month was estimated to be low. For this reason, we abstained from chemical cryoprotection altogether.

Because the manuscript should support the share of good scientific practice, and the process of cryopreservation was not specified, we added the following sentence to the text:

“[...] Soft plastic recipients were used to maximise the surface of thermal contact. No chemical cryoprotection was performed, as the maximal distance for cold penetration was about 5 mm. Therefore, the risk of water crystals formation was considered negligible, rendering additional exposure of the lymph node fragments to alcohols or sulfoxides evitable. Fragments were transported to final storage at -80°C immediately after the operation and were conserved in these conditions for one month.” (Please see lines 135 – 141 of the Methods section of the manuscript)

2. I would think that the rate of vessels within the specimen are relevant for later revascularisation! Any data on this?

The inguinal donor nodes were carefully resected with sharp separation of all afferent and efferent lymphatics in immediate vicinity of the organ capsule, as well as the organ's hilum. Therefore, revascularisation of the fragments occurred as neovascularisation, as no blood vessels or lymphatic channel stumps were left in place.

Because this information was missing, we completed the data with the following sentence:

“Group A (n=6) was therefore submitted to resection of the right lymph node under general anaesthesia as previously described [23], the left side of the animal being left intact. The single superficial groin lymph node present in this species was operatively excised on the right side. Afferent and efferent lymphatics were carefully sharply resected in the immediate vicinity of the organ capsule. The blood vessels of the hilum were also resected. No ligatures were applied to the organ.” (please see lines 126-132 of the Methods section of the manuscript)

3. If one wants to assess the take rate I suggest to look at the lymph nodes after 5-7 days. If I want to assess their lymphatic capacity the 5 months may work. So the conclusions are not really justified.

We agree with the reviewer, and therefore have adapted the conclusions to considerations on lymphatic function only, and deleted assumptions on the early processes of organ take after initial osmotic diffusion:

“[...] here deleted sentence on take...] Hypothetically, fragmentation, whilst multiplying regeneration foci, would improve initial lymphangiogenesis induced by the transplants when reconnecting to the surrounding tissues. Nevertheless, it could therefore probably only partially guarantee the survival of nodal cell populations through diffusion [22].” (please see lines 353 – 358 of the Discussion section).

and later:

“The present study in a large animal model shows us that cryopreservation methods do not seem to affect the regeneration of lymph node fragment regeneration function significantly.” (please see lines 373 – 375 of the Discussion section of the manuscript).

Some details remain unclear and need specification: e.g. “In the current study, the cryopreservation group A had an additional 1/3 of observations in comparison with the positive control group B”

This comment was already mentioned by the first reviewer, so the necessity of improvement is confirmed. Additionally to the above specified clarifications based on the second comment of the first reviewer, we also changed this cited sentence from:

“In the current study, the cryopreservation group A had an additional 1/3 of observations in comparison with the positive control group B.” to:

“In the current study, the cryopreservation group A was bigger than the positive control group B (Group A: twelve lymph node fragments vs. eight in control group B, therefore group B corresponded only to 2/3 of the size of group A). This created restrictions in the comparison of both groups,[...]”. (please see lines 313-318 of the Discussion).

With the above-mentioned corrections, we hope to have met your criteria of quality. We would also wish to thank the reviewers for the invested time and the added value of their comments. With this original article, we hope to raise a much-needed awareness on lymphoedema and motivate further research on lymph nodes.

Very truly yours,

Reviewers' Comments to Revision

Reviewer 1: Thomas Kremer

Mar 06, 2018

Reviewer Recommendation Term:	Accept
Overall Reviewer Manuscript Rating:	95
Custom Review Questions	Response
Is the subject area appropriate for you?	5 - High/Yes
Does the title clearly reflect the paper's content?	5 - High/Yes
Does the abstract clearly reflect the paper's content?	5 - High/Yes
Do the keywords clearly reflect the paper's content?	5 - High/Yes
Does the introduction present the problem clearly?	3
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	5 - High/Yes
How adequate is the data presentation?	5 - High/Yes
Are units and terminology used correctly?	5 - High/Yes
Is the number of cases adequate?	4
Are the experimental methods/clinical studies adequate?	5 - High/Yes
Is the length appropriate in relation to the content?	5 - High/Yes
Does the reader get new insights from the article?	4
Please rate the practical significance.	5 - High/Yes
Please rate the accuracy of methods.	4
Please rate the statistical evaluation and quality control.	5 - High/Yes
Please rate the appropriateness of the figures and tables.	5 - High/Yes
Please rate the appropriateness of the references.	5 - High/Yes
Please evaluate the writing style and use of language.	5 - High/Yes
Please judge the overall scientific quality of the manuscript.	5 - High/Yes
Are you willing to review the revision of this manuscript?	Yes

Comments to Authors:

I appreciate the authors reply. My concerns regarding the length and focus of the introduction, the explanation for different group sizes are well addressed. My comment regarding differences of the animal model and lymphedema patients in Terms of scarring, radiation and vascularity is appropriately discussed in the resubmission.

Reviewer 2: anonymous

Mar 10, 2018

Reviewer Recommendation Term:	Accept
Overall Reviewer Manuscript Rating:	N/A
Custom Review Questions	Response
Is the subject area appropriate for you?	5 - High/Yes
Does the title clearly reflect the paper's content?	4
Does the abstract clearly reflect the paper's content?	4
Do the keywords clearly reflect the paper's content?	4
Does the introduction present the problem clearly?	4
Are the results/conclusions justified?	4
How comprehensive and up-to-date is the subject matter presented?	4
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Please rate the accuracy of methods.	4
Please rate the statistical evaluation and quality control.	N/A
Please rate the appropriateness of the figures and tables.	4
Please rate the appropriateness of the references.	4
Please evaluate the writing style and use of language.	4
Please judge the overall scientific quality of the manuscript.	3
Are you willing to review the revision of this manuscript?	No: already revision

Comments to Authors:

The authors have addressed the significant points in the revision. However further studies require a better experimental setting.
