**Reviewer Assessment**

Catarina Hadamitzky, Hanes Perić*, Sebastian J. Theobald, Klaus Friedrich Gratz, Hendrik Spohr, Reinhard Pabst and Peter M. Vogt

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

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***Corresponding author: Hanes Perić,** myDent GmbH Tiergartenstrasse 114, 30559 Hannover, Germany, E-mail: hanesperic@aol.com

## Reviewers' Comments to Original Submission

### Reviewer 1: Thomas Kremer

Feb 04, 2018

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| **Reviewer Recommendation Term:** | Accept with Minor Revision |
| **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? | 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

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|---|---|
| **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 freezed 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 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.
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 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).
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.

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.

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 freezed 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 $cm^3$ 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 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.
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).

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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 |
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| 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, radiadion and vascularity is appropriately discussed in the resubmission.

## Reviewer 2: anonymous

Mar 10, 2018

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|---|---|
| **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 |
| How adequate is the data presentation? | 4 |
| Are units and terminology used correctly? | 4 |
| Is the number of cases adequate? | N/A |
| Are the experimental methods/clinical studies adequate? | 4 |
| Is the length appropriate in relation to the content? | 4 |
| Does the reader get new insights from the article? | 4 |
| Please rate the practical significance. | N/A |
| 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.