Author's response to reviews

Title: Intraoperative use of enriched collagen elastin matrices with freshly isolated adipose-derived stem/stromal cells: A possible clinical approach for soft tissue reconstruction

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Author's response to reviews: see over
Reviewer #1

Major:

Why have they chosen an in-vitro/ex-vivo approach?
Was it to determine the best interval, when considering an adipose tissue transfer with a timely delay (eg. patient -> lab -> patient)? However, when choosing delay involving lab facilities, why did the authors not attempt to process the fat cells, as this might be the only advantage vs. a direct fat transfer in the same operating session?

We should notice here that we worked on a method of a direct transfer of adipose-derived stem/stromal cells on available commercial matrices during surgery without using a lab necessary GMP facilities or time-consuming cell culture steps.

Is this experiment really clinically relevant, and if so under which circumstances?
Why submit a patient to two operations? Autologous fat cell transfer in one simultaneous operation has a significantly higher survival in an "autologous in-vivo bed" as compared to an in-vitro environment

This experiment should prove that under clinical condition, seeding of freshly isolated ASCs onto collagen matrices is possible during one operation without leaving the operation theater.

The intention of different time intervals was to determine whether 3 hours are needed for cellular sedimentation on scaffolds or even one hour sedimentation time is enough for cells to attach and migrate onto Matriderm™ before any possible transplantation of the enriched construct in the operating theatre. Thus; we did not intend to process cells in order to keep the clinical simulation of the experiment. In this way, the patient would have one operation with a possible enrichment of a selected collagen based matrix with, for example, split thickness skin transfer for soft tissue reconstruction. We admit that it was unclear to express the methodology but in this context, we implemented all above mentioned points again in the text for better understanding.

The authors discussed other publications relating to this subject (eg. Ref 7), but failed to introduce them at the beginning, when relevant for formulating the research question. It is not clear, what this work distinguishes from previous publications? I suggest to focus on the methodology in the introduction section, instead of discussing it as performed in the discussion only. What makes the chosen methodology stand out as compared to previous approaches?
Publications which are related to the same subject are now introduced in the Introduction section before formulating the research question. The main difference to other studies:

1. A direct use of isolated cells from adipose tissue has been performed with short enrichment time on Matriderm™ (e.g. 1 or 3 hours vs. 24 hours) in order to investigate the feasibility of this approach in the clinical setting without the need of long enrichment time in the lab. All studies include cell culture prior cell seeding or cell transplantation, respectively. Furthermore, most studies incubate ASCs on matrices for at least 16-24h and may be up to days in the cell culture incubator.

2. In this regard, the more focus on methodology is now in the Introduction section. Accordingly, we concentrated on early time points of adhesion and demonstrated that a number of ASCs could be visualized on top of the Matriderm sheet also in histological evaluation after 1h after cellular seeding which was also supported by 2-photon microscopy.

The authors need to provide a statistical analysis of their experimental results: eg. 1 hrs vs. 3 hrs vs. 1 day. No statistical analysis was presented in the manuscript. 
Sorry, for this mistake. Surely, we performed statistical analyses of our results. Unfortunately, we uploaded an older version of figures. After consulting a statistician, we give now a better ANOVA-based statistical analysis and better visualization using box plots, which shows min.max values median and Quartiles 25-75%.

Minor:

References:

References are now ordered and formed according to the journal style

We thank the reviewer for the discussion and corrections, and we hope that we could address all comments.
Reviewer #2

Major:

The same work group published a study called Conventional vs. micro-fat harvesting: How fat harvesting technique affects tissue-engineering approaches using adipose tissue-derived stem/stromal cells. Z Alharbi et al. Journal of Plastic, Reconstructive & Aesthetic surgery 56, 1271-1278 2013 which describes the same ASC samples (as judged by the number of the samples and the ages of the patients) inserted in Matriderm. As the focus of this study was different that might be no problem but the authors should remove all parts of the manuscript that overlap and they should refer to the other study in their introduction and discussion. This means the 24 hrs time points should be carefully checked as they are included in the other study.

As already explained by the reviewer, we worked on a method of a direct transfer of adipose-derived stem/stromal cells on available commercial matrices like the collagen elastin matrices (Matriderm™) to support transplantation. A clinical situation was oriented for the direct use of isolated cells rather than cultivation steps in the lab and, thus to avoid the need of GMP facilities. Therefore, this approach would go with only one step operation to avoid delay.

Overlap parts have been carefully removed and the previous study has been clearly referred in the introduction part as well as the discussion part. The intention of different time intervals was to determine whether 3 hours are needed for cellular sedimentation on scaffolds or even one hour sedimentation time is enough for cells to attach and migrate onto Matriderm™ before any possible transplantation of the enriched construct in the operating theatre. Furthermore, we performed same tests after 24 hours cellular cultivation time on Matriderm™ to compare it with shorter cultivation time, although our previous study concentrated on this time point despite different methods of liposuction procedures.

All above mentioned points have been implemented in the text in their best positions.

Figures 1 and 2 contain no real information needed to understand the data. Figure 2 is misleading as it suggests that clinical application has been done.

Figures 1 and 2 have been changed as only one figure which explains the methodology part of the study concentrating on early and direct transfer of adipose-derived stem/stromal cells on Matriderm™.
How many times were the measurements of metabolic activity repeated and in how many replicates? Why the data was not evaluated statistically and presented as means with standard deviation. Were negative and positive controls included? And were the supernatants measured or the cell-seeded matrices? Can it be excluded that spatial distribution and the matrix itself have an influence on this fluorescence based technique? Based on your description in your former manuscript, which is much more detailed, I would suppose that you took the supernatants which should avoid these problems but it should be explained in the methods section.

Medium/alarBlue™ mix was repeated and carefully removed from the well then measured at room temperature using a fluorescence spectrometer (Fluostar Optima; BMG Labtech, Offenburg, Germany; excitation wavelength 540 nm, emission wavelength 590 nm). We intended not to measure matrix itself to avoid the influence of matrix on fluorescence. As negative control, AB was added to matriderm™ sheets without cells. All detailed methods are now formatted and explained in the method section. Furthermore, we strongly regret that we did not provide the very important information regarding statistical presentation. Sorry, for this mistake. Surely, we performed statistical analyses of our results. Unfortunately, we uploaded an older version of figures. After consulting a statistician, we give a better ANOVA-based statistical analysis and better visualization using box plots, which shows min.max values median and Quartiles 25-75%. Such facts have now been implemented in the diagram.

Maybe the authors can provide higher magnifications of their images 4-6. Although I can see a number of cells on top of the Matriderm after 1 and 24 hours, I can only detect small amount of cytoplamic fragments in the middle of the Matriderm of the 3 and 24 hours samples. Can you indicate the vital cells that you observed?

Cells are seen on the upper layer of Matriderm just after one hour seeding time of cells on scaffolds. With H&E staining it is not possible to discriminate between dead or vitals cells. However, only cells which were adhered tightly on the matrix can be detected indicating that after 1 h, enough cells were adhered despite washing steps. By using 2-photon microscopy and vital staining, we could demonstrate that living cells were adhered on the surface of the matrices. But this method is limited to a depth of 100-200µm, thus; a migration of cells deeper into the matrices can only be seen by classical histology and H&E staining as we performed.
Detection of cell surface markers might be interesting as your study is in contrast to other studies incorporating ASCs in Matriderm using passaged cells. Your cells could be characterized directly after isolation and at the given time points after seeding. This point is very important and should be taken into consideration. It was, for sure, very helpful to identify cellular population directly after isolation, although several studies focused on that issue, and to concentrate on cellular population after designed time points after transplantation on matrices. The later point could be very interesting to address, which, however, is going to be a cornerstone in our ongoing study. Nevertheless, we believed at the beginning, as we designed the protocol of the study, that we concentrate on the clinical feasibility of cellular transplantation on scaffolds rather using selected populations for a specific regenerative medicine approaches; for example in bone, cartilage or nerve regeneration. All these facts have been carefully discussed in the discussion part.

It is explained at which time points the samples were analyzed under the 2-photon microscope. The number of analyses n=3 suggests that only one time point was analyzed. If 24 hours was chosen there is another overlap with the former study although the images look quite different although the same staining protocol was used, please explain. Also a more dimensional view like in your former study might help to judge the spatial distribution of the cells. Maybe 1 hour and 3 hour samples can be compared to visualize the migration. Yes, same staining protocol was used under 2-Photon Microscopy. We have, therefore, contacted Dr. Vogt, our expert for multi photon Imaging to provide us with images at early time points with different dimensional view to identify cellular distribution inside the Matriderm™ as advised by the reviewer. We deleted the one taken after 24 hours to avoid duplication of information. We should notice here that our imaging is getting better because we are learning with time especially in terms of the wavelength use, second harmonic generation and handling process of Matriderm itself under microscopy.

I do not understand the results of Figure 8. Why a single rounded cell is seen in a completely dark surrounding. Is there any additional information compared to figure 7? The intention of that figure was to provide more close view but we have deleted it now due to its irrelevancy for the paper especially if more images in different time points will be provided with more dimensional views as stated earlier.
Discussion: The first paragraph is redundant. Paragraph 5 has no relation to your data. The discussion in general is not clearly focused on the results and the intention of your study. Do you really suggest that Alamar Blue assays should be performed before transplanting ASCs?

First paragraph is now deleted and completely reformed. Paragraph 5 is also deleted and relevant information to data are now included. More focus on the result of the study is now included in the discussion part as advised by the reviewer. Therefore, testing procedures and possibilities are either deleted or shifted to the introduction part if possible. We performed AlmaraBlue™ to justify the results regarding metabolic activity but we believe that a specific number of isolated cells from adipose stromal vascular fraction should be identified before transplantation to any selected matrices. However, this assay should be faster than the Alamarblue assay. These points were taken into consideration in the discussion part.

Minor:

A lot of typos and grammatical errors, for instance “fresh isolated” instead of “freshly isolated” in the title, need careful editing of the whole manuscript.

Intensive editing has been made for the whole manuscript including grammatical errors.

Figure 4-6: no scale bars

Figures were united in on figure. Scale bars are now provided

The figure legends are not sufficient as they do not give the necessary details to understand the figures, e.g. no explanation of the acronym AU (figure 3)

Figure legends have been reformed to be more understood. AU is now refereed to the term (arbitrary unit; AU)
**Discretionary Revisions:**

You may comment on the stability of cells seeded on the Matriderm after short time periods, as it would happen when your suggested protocol is used in clinical settings. And do you think nutrition by diffusion will suffice for cell maintenance after transplantation? Thank you very much for this important point. The discussion part contains now information related to that point. We think that nutrition by diffusion alone is not sufficient for all cells but we do take into consideration that several growth factors play important role for the survival process such as the vascular endothelial growth factors. Cellular signaling and the possible effect of collagen may also play a role in this regard. All of these points are now carefully implemented in the discussion part.

We would like to thank the reviewer for the talented discussion. We hope that we could implement all comments and answered all questions.

Yours,

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