Author's response to reviews

Title: Local Erythropoietin and Endothelial Progenitor Cells Improve Regional Cardiac Function in Acute Myocardial Infarction

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Author's response to reviews: see over
To
M. Norton
Editor in Chief
BMC Cardiovascular Disorders

Re.: Submission of revised manuscript 2979626173824946 for publication

Dear Sir,

Enclosed herewith, please find our revised manuscript entitled “Local Erythropoietin and Endothelial Progenitor Cells Improve Regional Cardiac Function in Acute Myocardial Infarction”. We would like to resubmit this manuscript for publication in the “BMC Cardiovascular Disorders”. Attached please find the specific comments to the reviewers.

All authors have seen and approved the paper. This work has not been and will not be published elsewhere.

Yours faithfully,

I. Ott MD, PhD
Comments to reviewer Peter van der Meer

1. Since MRI is a powerful imaging tool, more data should be presented. What were the effects on LV end diastolic diameter/volume and LV end systolic diameter/volume.

Ad 1. As suggested we added the endsystolic and enddiastolic diameter in the results section:

*Page 10 para 2 line 4: Moreover endsystolic and enddiastolic volumes were comparable in all groups (data not shown).*

2. No baseline cardiac function measurements (MRI or echo) were performed directly after myocardial infarction (and injection). This would allow the authors to really substantiate the claim that the effects shown are indeed related to the injected cells with EPO.

Ad 2. We agree with the reviewer that it would be interesting to have baseline MR examinations immediately after myocardial infarction. However prolonged anaesthesia, transportation to the MR and performing the MR would have been to stressful for the rats immediately after the operation.

3. The authors show nicely that capillary density increases with the combination of EPO and EPCs. Post myocardial infarction remodelling plays an important role. Did the intervention have an effect on the myocyte size? In addition the authors should also report the capillary to myocyte ratio in order to prove vasculogenesis. The authors did not include a sham group, so no information is available to which extent the cardiac parameters improved (including LV function and effects on vasculogenesis).

Ad 3. We reevaluated the slides to determine the ratio of myocytes and vessel density. Since the orientation of the myocytes on the slides was very variable we were not able to reliably determine their size.

4. The authors did not include a sham group, so no information is available to which extent the cardiac parameters improved (including LV function and effects on vasculogenesis).

Ad 4. In sham operated animals we found no effects on LV function or other MR parameters. We included this in the results section.

*Page 10 para 3 line 8: Values in sham operated rats were 92 ± 7 %. Page 11 para 1 line 7: In sham operated animals we did not see changes in regional wall movement as compared to control animals.*

5. Further information is needed on how many cells survived after 4 weeks. How many cells indeed incorporated into the capillaries, were there capillaries formed entirely from human C31+ cells? How reliable is double staining with TUNEL. Is it possible that cells undergoing apoptosis and labelled with TUNEL already lost the CD31 receptor which may lead to false negative findings. Please comment.
Ad 5. After 4 weeks we found no cells with positive human anti-CD31 staining. This suggests that the effects of the transplanted cells are only temporarily and paracrine effects may substantially contribute to the effect. Another explanation could be that the cells lost CD31 expression and therefore could not be detected.

Minor issues:
- As suggested we included the number of animals used in the abstract.
- Since we showed the results of each treatment group in one figure we find it difficult to describe them in 2 paragraphs. To separate the figures would mean to show the data of one group in 2 figures which would be even more confusing.

Comments to reviewer Nassos Manginas

1. The protocol is a little confusing and needs clarification, with regards to the number of experiments divided between EPO/eEPC/EPO+eEPC in each group of I/R or ligation. Are the experiments' number sufficient to demonstrate differences within the I/R group?

Ad 1: We clarified the number of experiments in the methods section. An expected increase in regional wall motion of 10% (alpha=0.05; power 80%) with additional Epo treatment would require 8 animals.

Minor Revisions:
1. We corrected labelling of figure 3
2. The authors should provide their insight on the discrepancy between improved regional contractility and unaltered global LVEF. Is this a result of relatively preserved EFs included? Could remote wall differences play a role?

Ad 2. We assume that measurement of the regional contractility is a more sensitive parameter than global EF. Therefore subtle changes can be only observed measuring regional contractility.

Comments to reviewer Ioakim Spyridopoulos

1. methodology is far too superficial. Here some examples:
a) PCR primers are not described (sequence, PCR condition, intron/exon localisation, bp product)

ad 1a: We described the PCR primers as follows:

page 6, para 3, line 4: TaqMan PCR was performed using Human Inflammatory Cytokines and Receptors PCR Array and Angiogenesis PCR Array (PAHS-011, PAHS-024, SABiosciences, Frederick, USA). Assays-on-Demand containing specific primers and probe for Bax (Hs00180269_m1) and GAPDH (Hs99999905_m1) were from Applied Biosystems.

b) CD34+ cell isolation. How was purity checked? Usually it requires 2 different antibodies raised to different epitopes in order to differentiate between the bead isolation and purity FACS
ad 1b: The method of CD34+ cell isolation is extensively described in a previous paper we referred to (Ott et al. FASEB 2005).

c) EPCs were not characterised. Not all CD34+ cells are EPCs

ad 1c: The method of characterisation of expanded CD34+ cells is extensively described in a previous paper we referred to (Ott et al. FASEB 2005).

d) RT-qPCR results are not confirmed on protein level

ad 1d: Due to small amount of material confirmation of PCR results was not possible on the protein level.

2. The main goal of the study is to demonstrate a beneficial effect of cell therapy on LV function. Nevertheless, global LVF is not different between groups, only regional wall motion

ad 2 We find that regional wall motion is a more sensitive parameter to measure LV-function than global EF. Yet it needs to be determined wheater our effects become clinically relevant in further studies.

3. Lack of mechanistic concept: There is no proof of mechanism or attempt other than random pick of regulated genes

ad 3: We find in our study that regional wall thickening was improved after treatment with eEPCs+EPO as compared to eEPCs, EPO alone or PBS (control). This was associated with an increase in inflammatory infiltrate and surviving eEPCs 3 days after myocardial infarction and with an increase in vasculogenesis after 4 weeks. Although we were not able to determine the underlying mechanisms in vivo, in vitro experiments showed that Epo inhibited apoptosis by a decrease in pro-apoptotic Bax. Moreover, the increase in local inflammation may be explained by an increased eEPC expression of Platelet factor 4 (chemokine (C-X-C motif) ligand 4), a strong chemoattractant for neutrophils and monocytes and chemokine receptor 8, involved in lymphocyte recruitment, after stimulation with Epo. Enhanced vasculogenesis in experimentel myocardion may be the result of secreted phosphoprotein 1, a cytokine involved in angiogenesis, that is released by eEPCs after stimulation with Epo.

Minor concerns:
We corrected the orthograpic mistakes