Author’s response to reviews

Title: A Novel Targeted Angiogenesis Technique Using VEGF Conjugated Magnetic Nanoparticles And In-Vitro Endothelial Barrier Crossing

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Reviewer reports:

Ali Yilmaz (Reviewer 1): In principle, the present manuscript is interesting and addresses the promising field of magnetic nanoparticles coupled with e.g. growth factors that can be used as potential drug carriers and also be navigated to the target issue/organ...

However, the present manuscript is just of descriptive nature (no quantitative analyses at all) and the presented methods and results - although being interesting - do not convincingly allow a potential clinical translation. Moreover, there are many issues that are either just mentioned without any comprehensive explanation or not addressed at all ...

Just a few examples:

As mentioned by the author, "the size of the nanoparticles is in the range of 200nm". Nanoparticles of such a size will be quickly eliminated by the reticulo-endothelial system after in vivo application and thus, will have a very short blood circulation time - preventing a meaningful in vivo application.

Response: Using Ni micromesh technique high gradients can be created using magnetic force of about 0.1 Tesla, and thereby the nanoparticles can be retained efficiently in intravascular compartment (Ref26).

The magnetic nanoparticles could also be injected extravascular to form collateral connections between left anterior descending artery and left internal mammary or intercostal vessels (percutaneous bypass); and thereafter these magnetic nano-particles can be externally controlled by the external magnetic field.

Future Potential Applications
These nanoparticles could be injected through percutaneous or sub-sternal/subcostal route, and an attempt to form collaterals from internal mammary to the coronaries i.e. in the form of a percutaneous bypass technique, as a drive for future research would be very interesting.

Reviewer: The author argues that "to control the magnetic nanoparticles the required magnetic field gradient strength is approximately 10T/M". Why that? There is no explanation at all ... and no information given on the magnetic properties of the particles used in this study.

Response: The magnetic nanoparticles were evaluated by interfacing a fluid suspension with magnetic nanoparticles between an electromagnetic field. The experiment was repeated at varying magnetic filed strengths and the displacement of the nanoparticles were studied in 4 different times. The osmolarity of the droplet suspension was 320mOsm/kg, which is near to serum osmolarity (280mOsm/kg).

With electromagnetic interfacing the magnetic nanoparticles were observed to polarize to one end of the drop (figure 5). This was consistently observed in all 4 different times. The tendency of polarization starts at 0.1 T. Access to higher strengths of magnetic field was not available. Hence, the highest limit evaluated was 0.7 T.

The magnetic particles show polarization in this study. This is the magnetic strength to overcome Vander Wall’s forces. The particles were evaluated in the past and the magnetic gradient required to control is 10T/M (Ref26).

The magnetic nanoparticles are small and they could be rapidly eliminated by monocytes. Aihua Fu et al., have generated high magnetic gradients using Ni micro-mesh, and through these remarkable high gradients, the magnetic nanoparticles of even smaller sizes (<100nm) could be controlled to retain them in the intravascular compartment (Ref26). The particles accumulated in the target areas using the Ni micro-mesh technique. The Ni mesh used in the study was 76 µm pitch, 12 µm wire width and 5 µm wire width and using this large gradients of 10e5 to 10e10 gradient were developed within 10 µm of Nitinol edges.

Reviewer: In order to simulate the endothelial barrier, the author used a "setup in a microfluidic chip with a monolayer of HUVEC cells, and the nanoparticles were placed above in the nutrient layer". I'm afraid, but this setup does not allow to simulate the in vivo endothelial barrier...

Response: The experiment was set up as a vertical sandwich structure inside a micro-fluidic chip. Lower layer of collagen hydrogel was placed. A monolayer of HUVEC cells was built on top of
the hydrogel layer. The culture medium with nanoparticles was placed in the upper layer. The experiment was performed at 37 degree C and 5% Carbon-di-oxide. The ability and the extent of the nanoparticles to cross the HUVEC layer to reach the lower hydrogel layer were studied using Z-stack confocal microscopy. The confocal pictures were taken at 10 min and at 48 hours to visualize the extent of crossing by the nanoparticles. The confocal observations were also made in serial intervals of time.

Endothelial Barrier Crossing

It is difficult to simulate in-vitro the phenomenon of endothelial barrier crossing. A better method to demonstrate could have been usage of growth factor reduced matrigel, which could mimic tight junctions (Corning TM) instead of hydrogel used in this study (Ref24, 25). Matrix Matrigel is a recombinant basement membrane extract. Though absolute quantification of crossing was not studied this is the first evaluation of such a simulation. Moreover, this endothelial penetration property of the particles could be increased with external magnetic force.

Savneet Kaur (Reviewer 2): The study by Arokiaraj et al. describes the conjugation of magnetic nanoparticles (MNs) with vascular endothelial growth factor (VEGF) and demonstrates the capability of the MNs-VEGF to cross the endothelial barrier and in vitro angiogenesis. The pilot study presents an important method of targeted angiogenic therapy in ischemic areas using MNs coupled with VEGF.

However, I have the following concerns and suggestions:

1. The results are interesting but have been poorly described. They lack clarity of expression.
   Response: More information with description, and grammar corrections have been included in the revised manuscript.

2. In the second experiment, the authors describe the movement of the HUVECs towards MNs conjugated with VEGF, however there is no quantification technique to assess how many cells actually migrate. This should be quantitatively assessed.
   Response: Quantification of the HUVECs displacement was not performed, however, by visual assessment a significant number of HUVECs cross the demarcation line. In the presence of external magnetic force a significant displacement of MN+ VEGF towards HUVECs is possible, which will facilitate angiogenesis.
3. Why were the HUVECs modified to HUVEC spheroids for the experiments? This should be explained somewhere in the discussion or the methods.

Response: HUVEC endothelial cells were modified to form clusters of HUVEC spheroids as the spheroids are better known to mimic natural cell responses and interactions (Ref18,19). The extracellular matrix exerts its interaction with the cells, which again is influenced by the cellular architecture, and thereby determining the genetic and nuclear expression of the cells, which is a good response with the spheroids (Ref20, 21).

4. In each experiment, the inclusion of controls consisting of only 'VEGF' and only 'MNs' (without VEGF) should be considered.

Response: Only MN as control was not evaluated in tissue culture. However, we would perform this in future studies. Only MNs were studied for response to magnetic field, which showed polarization to one side of the droplet.

5. In the conclusion, the authors mention that the MNs could be controlled by magnetic force. However, I did not find any such demonstration in the results section. It would be worthwhile to see the results with varying magnetic force.

Response: Magnetic polarization

With electromagnetic interfacing the magnetic nanoparticles were observed to polarize to one end of the drop (figure 5). This was consistently observed in all 4 different times on a glass slide. The tendency of polarization starts at 0.1 T. Access to higher strengths of magnetic field was not available. Hence, the highest limit evaluated was 0.7 T.

In another experiment, the droplet with magnetic nanoparticles over a bar magnet retains its shape in all directions (supplemental figure 2); whereas a control droplet without magnetic nanoparticles loses its shape easily.

Reference 26 describes the magnetic properties and the controllability of these nanoparticles in the presence of mild magnetic fields. (0.1 T)

6. The manuscript needs language editing at some places. Eg. At some places, it is written as HUVEC cells, this should be corrected to only 'HUVECs' or 'HUVEC spheroids'.
Response: HUVEC cells have been modified to HUVEC spheroids or HUVECs. In endothelial barrier crossing experiment HUVEC cell’s monolayer was used.