Effects of amniotic membrane extract and deferoxamine on angiogenesis in wound healing: an in vivo model

Objective: Angiogenesis, formation of new vessels from pre-existing vessels, is an essential part of wound healing. We aimed to compare amniotic membrane extract with deferoxamine in angiogenesis and to assess any synergistic effect.

Method: We examined four groups of rats (five per group): control, deferoxamine, amniotic membrane extract, and deferoxamine and amniotic membrane extract in combination. A distal-based skin flap was created. Deferoxamine (100mg/kg), amniotic membrane extract (0.1mg/ml), and the combination of both were injected subcutaneously every other day in 10 separate points (0.1 ml at each point) in the skin flap. On day 11, the animals were euthanised for histopathological evaluation.

Results: Results indicated that the amniotic membrane extract raised angiogenesis or neovascularisation, the development of new blood vessels, can facilitate oxygen or nutrients distribution in the ischaemic wound. Therefore, enhancing angiogenesis could be a way to prevent insufficient oxygen and nutrients, following the blockage of vessels by accumulation of either free radicals and cytokines or thrombosis in the skin flap, which is used for testing the potency of agents in covering the damaged tissue. Induction of this pathway, could be the key point for the treatment of disorders such as cardiovascular disease, chronic inflammatory disease and wound healing, due to its crucial role in both proliferation and reconstruction of damaged tissue; on the other hand suppression of angiogenesis in conditions like cancer could be beneficial. In light of previous studies, some therapies via angiogenic properties like growth factors, amniotic membrane extract, deferoxamine, and other agents by increment of perfusion rate, such as vasodilators, calcium channel blockers, prostaglandin suppressant, anticoagulant, glucocorticoids, and hyperbaric oxygen, were analysed for wound healing.

Conclusion: Amniotic membrane extract or deferoxamine could be used interchangeably in angiogenesis within wound healing due to their high safety and availability.

Declaration of interest: The authors have no conflicts of interest to declare.

amniotic membrane extract ● angiogenesis ● deferoxamine ● wound healing

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deferroxamine in angiogenesis. Moreover, by removing radicals and binding with iron responsible for hydroxyl radical production, deferoxamine is able to prevent necrosis as a consequence of free radicals activity in skin graft. This study aimed to investigate the efficacy of amniotic membrane extract compared with deferoxamine in sprouting vessels by assessment of particular angiogenic markers including CD31+, CD34+, Von Willebrand Factor (vWF), and new vessels numbers. In addition, because of different mechanisms in angiogenesis following each agent, we assessed the synergistic effect of their combination in wound healing.

Materials and methods

Materials

Amniotic membrane extract 0.1mg/ml of total protein (Roya Institute, Tehran, Iran), Deferoxamine Mesylate (Ronak Daru, Tehran, Iran), CD31 (antibody, clone JC/70A, mouse anti-human, Dako, Denmark), CD34 (antibody, clone, QBEnd-10, mouse anti-human, Dako, Denmark), vWF (antibody, rabbit anti-human, Dako, Denmark), digital camera (Olympus CH30, Tokyo, optical, Japan) were used in this study.

Animals

Healthy male Wistar rats weighing 350–400g were purchased from Razi Institute. They were housed in separate cages under standard circumstances including 12/12 hour light/dark cycle, temperature of 26±1°C, and free access to standard pellet diet and water ad libitum. This study was conducted according to the guidelines set out by the Helsinki Declaration and Ethical Committee of Imam Khomeini Hospital.

Flap technique

Rats were anaesthetised by 90mg/kg of ketamine 10%, and 9mg/kg of xylazine 2%, intramuscularly. Following this, the dorsal part of each rat was shaved and disinfected by betadine and alcohol. The animals then received 60mg/kg of cefazolin intramuscularly for prophylaxis. Animal unconsciousness was examined by pinch flexion/withdrawal test. Distal-based skin flap (3×6cm) with the apex of pelvic joint on the dorsum of the animals was determined based on modified McFarline method. The skin flap was elevated up to the underneath of the panisicus carnosus and the cauterisation of axial or perforating vessels was performed. All surgical procedures were carried out under sterile condition.

Amniotic membrane extract preparation

This extract was derived from healthy and live donors, and contained stem cells with no tissue compatible genes resulting in type 2 immune response. The placenta, including amnion membrane, was rinsed by buffer solution of phosphate-buffered saline (PBS, pH=7.2) containing penicillin (1000U/ml) and streptomycin (50μg/ml) in order to remove blood residues. The amnion layer was then separated from the rest of the placenta, rinsed by PBS and divided into smaller pieces. The small sections were turned into powder form by liquid nitrogen. Following this, 2ml of injectable sterile water was added to 1g of the powder. The final solution was sonicated to disrupt the cell membrane and release cellular contents such as proteins. The tubs were put in ice during sonication to prevent protein denaturation. The uniform solution was centrifuged (4000rpm) and the supernatant was then centrifuged (1500rpm) again. The solution containing proteins was sterilised with 0.2μm filter and stored at –70°C. Finally, the total protein of the extract was calculated using Bradford Assay test.

Experimental procedure

A sample of 20 rats were randomly divided into four groups, with five rats in each group:

- control
- defereroxamine
- amniotic membrane extract
- defereroxamine and amniotic membrane extract in combination.

Following the skin flap formation, in rats in the control group, 1ml of normal saline was injected subcutaneously into the skin flap into 10 predetermined points, equidistant from each other (0.1ml in each point). In rats in the amniotic membrane extract group, 0.1ml of prepared amniotic membrane extract was injected in each point, and in the defereroxamine group, 100mg/kg of this solution was injected in each point, subcutaneously. In the combination group, defereroxamine (100mg/kg) was injected subcutaneously first, immediately followed by a subcutaneous injection of amniotic membrane extract (0.1ml).

The overall trial duration was 10 days and the injections were given every other day (totalling five sets of injections) in all four groups. On day 11, the animals were euthanised by CO2 gas and the skin flaps were detached and kept in formalin 10% before performing histopathological tests.

Immunohistochemistry (IHC) process

Tissues samples of 1–2μm thickness were incised from each rat and attached to lamella for 24 hours at 37°C. The lamellas were then sunk in xilene for seven minutes, alcohol (70–100%) for five minutes, and PBS or tris-buffered saline (TBS) for five minutes. For release of related antigens and breaking the formalin-protein bonds, the lamellas were soaked in citrate buffer (pH6), heated in a 900-watt microwave (which reached about 120°C temperature) for 5–7 minutes, retrieved for 35–45 minutes, and finally rinsed with sterile water. After blocking with H2O2, the samples were dipped in H2O2 (3/5–5%) for 7–10 minutes to inhibit the intracellular H2O2 and then rinsed with sterile water. The lamellas were immersed in PBS to reach a pH of 7.2–7.4. The specific primary antibodies for CD31+ (1:20 dilution), CD34+ (1:30 dilution) and vWF (1:30 dilution) were used in this study.

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with 40× magnification. Finally, the mean of total blood vessels density was reported. It is important to note that the cluster form of endothelial cells was considered as a blood vessel.\(^\text{19}\)

**Statistical analysis**

As the study parameter values were not normally distributed (Shapiro-Wilk test and Q-Q plot), they were analysed with nonparametric statistical methods. Kruskal Wallis tests with post-hoc multiple comparisons by Mann-Whitney U test with Bonferroni correction. Data were expressed as mean (SD) and median (interquartile range). The level of significance was set at \(p=0.05\) and \(p=0.008\) respectively. Data were analysed using IBM SPSS statistics version 22.

**Results**

The angiogenic markers level was expressed as the mean (SD) and median (interquartile range) values of each group and is shown in Table 1.

### New vessels counting

The areas of the IHC-stained slides with possible new vessels were observed using an optical microscope at 10× magnification. We examined 10 fields containing the most vessel density (hot spots) and then counted the blood vessel number per mm\(^2\) of each field with 40× magnification. Finally, the mean of total blood vessels density was reported. It is important to note that the cluster form of endothelial cells was considered as a blood vessel.\(^\text{19}\)

### New vessels number

The difference in new vessels number between groups was statistically significant (\(p=0.007\), Kruskal-Wallis test). The treatment groups had a significantly higher new vessels count than the control group (\(p<0.008\),

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<table>
<thead>
<tr>
<th>Groups of study*</th>
<th>Mean (SD)</th>
<th>Median (IQR)</th>
<th>p-value†</th>
<th>Statistically significant pairs differences‡</th>
</tr>
</thead>
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<tr>
<td>New vessels number</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>25.4 (4.1)</td>
<td>25 (22–29)</td>
<td>0.007</td>
<td>G1G2</td>
</tr>
<tr>
<td>G2</td>
<td>47 (4.95)</td>
<td>45 (43–51)</td>
<td></td>
<td>G1G3</td>
</tr>
<tr>
<td>G3</td>
<td>54.8 (12.32)</td>
<td>48 (46–66.5)</td>
<td>G1G4</td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>51.2 (6.42)</td>
<td>52 (45–57)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD31+ level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>1 (0)</td>
<td>1 (1)</td>
<td>0.036</td>
<td>G1G3</td>
</tr>
<tr>
<td>G2</td>
<td>1.2 (0.45)</td>
<td>1 (1–1.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>2 (0.71)</td>
<td>2 (1.5–2.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>1.2 (0.45)</td>
<td>1 (1–1.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34+ level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>1.6 (0.55)</td>
<td>2 (1–2)</td>
<td>0.24</td>
<td>G1G2</td>
</tr>
<tr>
<td>G2</td>
<td>1.6 (0.55)</td>
<td>2 (1–2)</td>
<td></td>
<td></td>
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<tr>
<td>G3</td>
<td>2.2 (0.45)</td>
<td>2 (2–2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>1.8 (0.45)</td>
<td>2 (1.5–2)</td>
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<tr>
<td>vWF level</td>
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<td></td>
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<tr>
<td>G1</td>
<td>1 (0)</td>
<td>1 (1–1)</td>
<td>0.011</td>
<td>G1G2</td>
</tr>
<tr>
<td>G2</td>
<td>1.8 (0.45)</td>
<td>2 (1.5–2)</td>
<td></td>
<td>G1G4</td>
</tr>
<tr>
<td>G3</td>
<td>1.6 (0.55)</td>
<td>2 (1–2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G4</td>
<td>2 (0)</td>
<td>2 (2–2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*G1—Control, G2—Deferoxamine, G3—Extract of Amniotic Membrane, and G4—Extract of amniotic membrane and Deferoxamine; SD—standard deviation; IQR—interquartile range; †Kruskal Wallis test; ‡post-hoc multiple comparisons by Mann-Whitney U test with Bonferroni correction.
There was no significant difference between other pairs of treatment groups (p>0.008, Mann-Whitney U test with Bonferroni correction) (Fig 1). CD31+ and CD34+ level

The difference in CD31+ between groups was statistically significant (p=0.036, Kruskal-Wallis test). The CD31+ in Amniotic membrane extract group was significantly greater than control group (p=0.003, Mann-Whitney U test with Bonferroni correction). There was no significant difference between other pairs of groups (p>0.008, Mann-Whitney U test with Bonferroni correction). The difference in CD34+ between groups was not statistically significant (p=0.24, Kruskal-Wallis test) (Fig 2 and 3).

vWF level

The difference in vWF between groups was statistically significant (p=0.011, Kruskal-Wallis test). The Deferoxamine and combination groups had a significantly greater vWF level than the control group (p<0.008, Mann-Whitney U test with Bonferroni correction). There was no significant difference between other pairs of groups (p>0.008, Mann-Whitney U test with Bonferroni correction) (Fig 4).

Discussion

Angiogenesis as a development of new vessels has drawn considerable attention in the wound healing field, particularly with regard to some ischaemic wounds such as diabetes, peripheral vascular disease, and post-radiotherapy. Therefore, various agents with angiogenic properties have been used in the acceleration of wound healing. With regards to the established angiogenic effect of amniotic membrane extract and deferoxamine, we compared these agents and their combination by measuring angiogenic markers as specific and accessible indicators besides new vessels numbers.

Our study showed that the amniotic membrane extract increased angiogenesis by significantly elevating angiogenic indicators, particularly new vessels number and CD 31+, compared with the control group. Choi et al. in 2009 evaluated the effectiveness of amniotic membrane extract in cultured human corneal epithelial cells and asserted that this suspension contains growth factors that motivated the epithelium migration and proliferation in order to accelerate the wound restoration. Mirabella et al. in 2011 designed a study in which they proved that the amniotic-derived stem cells amplified the angiogenesis by either their own angiogenic factors like monocyte chemoattractant protein-1 (MCP-1) and VEGF, or recruitment of host reparative cells in the murine model. A broad spectrum of pathways for angiogenic effects of amniotic membrane extract have been postulated within recent investigations. For example, amniotic membrane extract, containing chemokines such as...
interleukin-8 (IL8), stromal cell-derived factor-1α (SDF-1) and MCP-1, plays an important role in proliferation and differentiation of endothelial cells. The angiogenic effect of the extract is also attributed to numerable growth factors such as basic fibroblast growth factor (b-FGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF), keratocyte growth factor (KGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), and PDGF that cause microvascularisation. It stimulates the most potent angiogenic factor, VEGF, via both VEGF receptor-1 (VEGFR-1) and VEGFR-2 resulting in downstream cascade for endothelialisation. It is noteworthy to mention that there are several interplays between these mentioned angiogenic elements to reinforce the neovascularisation effects of amniotic membrane extract including upregulation of VEGF by TGF-β leading to MCP-1, growth factors, and matrix metalloproteinase (MMP) overgeneration. MMP facilitates the vascular invasion in the tissue besides great formation of extracellular FGF and VEGF. Last but not least, it was well-established that VEGF has favourable impacts on b-FGF, which plays a part in increase of HGF.

The second agent in our study, deferoxamine, illustrated a great capacity in revascularisation, specifically with regard to vWF and new vessels number compared to the control. Mericli et al in 2015 examined the probable effects of topical deferoxamine on vascularisation in rats with transverse rectus abdominis muscle (TRAM) flap, and discovered that deferoxamine strengthened the vascularity and elasticity of the tissue by affecting the HIF-α which is responsible for angiogenesis. In 2014, Wang et al. assessed the efficacy of topical deferoxamine on a random flap in diabetic rats. The further activity of HIF-α and VEGF in addition to endothelial progenitor cell migration underlined the vital role of deferoxamine in ischaemic diabetic flap as an angiogenic stimulus.

Deferoxamine has shown that it could increase the blood perfusion in the ischaemic site by phosphorylation of endothelial nitric oxide synthase (eNOS) through PI3K-Akt pathway. Moreover, deferoxamine stabilises the HIF-α, a key transcription factor for angiogenic genes expression. Therefore, HIF-α attaches to hypoxia response elements and transcribes the angiogenic genes such as VEGF in addition to endothelial progenitor cell (EPC) production. Some documents claimed that deferoxamine elevates the cyclooxygenase-2 (COX2) amount which results in positive feedback on VEGF. It is assumed that deferoxamine could inhibit the endothelial cells apoptosis by neutralising reactive oxygen.

Regarding the comparison of the two agents, amniotic membrane extract and deferoxamine, our results did not indicate any significant difference in the amount of angiogenic markers. A liable synergistic interaction between amniotic membrane extract and deferoxamine might be proposed. Deferoxamine interacts with VEGF by either HIF-1α stabilisation or COX2 overgeneration and, in turn, amniotic membrane extract is a vigorous stimulus of VEGF resulting in later events in order to endothelialisation, so there is a cycle in which the VEGF, as a main recognised element in angiogenesis, is boosted positively. The combination group did not become predominant compared to single compound groups, in spite of the different mechanisms of each agent in addition to the synergistic interaction, as described above. However, the combination was more capable of increasing vWF factor compared with single compounds without any significance. We assumed that insignificant differences might be as a consequence of the small sample size, and the use of a larger number of rats should be regarded within prospective animal studies. Furthermore, other markers such as VEGF, angiopoietin proteins Ang 1 and Ang 2, FLT-1, Tie-1, and angiotenin can be also measured and further studies in vivo are needed to certify their effect.

**Conclusion**

In conclusion, amniotic membrane extract and deferoxamine are equally as effective in angiogenesis during the wound healing process. The combination did not surpass the single groups. Therefore, regarding the pronounced angiogenic impact of these agents and the importance of angiogenesis in the wound healing process, especially ischaemic wounds, we suggest a clinical study should be performed.

**Acknowledgement:** We would like to express our gratitude to the Pathology Department staff of Imam Khomeini Hospital Complex.

**References**

2. Davis RE, Wachholz JH, Jassir D et al. Comparison of topical anti-ischemic agents in the salvage of failing random-pattern skin flaps in...
What is the role of deferoxamine in angiogenesis?

Is there any synergistic, or at least additive effect, following the combination of deferoxamine and amniotic membrane extract in angiogenesis?


