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Topical application of lyophilized and powdered human amniotic membrane promotes diabetic ulcer healing

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ABSTRACT

Background: Amniotic membrane has been widely used in the treatment of several acute and chronic diseases, and surgical reconstructions. Here we report the successful application of a commercial lyophilized, powdered, amniotic membrane (AMX) to a patient with a treatment-refractory diabetic ulcer, with further investigations on AMX angiogenic and anti-inflammatory properties.
Objective: To investigate the topical, continuous, treatment with AMX on a chronic diabetic foot ulcer, along with its angiogenic *in vitro* properties and proteomic profile.
Methods: A 77-year old woman was treated with AMX 2–3 drops 4–6 times a day for 2 months. Amniotic fluid stem cells were tested for proliferation and angiogenesis potential with or without AMX. ELISA quantification was conducted on NGF, TGF-alpha, NT-3, and IL1-ra. Proteomic analysis was also performed on AMX.
Results: At 2-month follow-up, the ulcer was reduced by 60% and it remained steady after 3-months. At 4-month follow-up, the lesion showed signs of re-epithelization. Cells, with or without AMX, were prone to form endothelial tubules with similar characteristics. Proteomic analysis confirmed that AMX retains proteins involved in anti-inflammatory and angiogenesis pathways.
Conclusions: AMX ameliorated pain and extent of a chronic diabetic ulcer. The effect could be ascribed to angiogenesis and epithelium stimulation that was confirmed in *in vitro*. Multiple wound healing-involved proteins were detected but further studies are needed to assess their specific role.

1. Introduction

Amniotic membrane (AM) is the innermost layer of placenta that lies in direct contact with the amniotic fluid, providing a metabolically active filter and a source of cytokines and growth factors to the fetus. It has been widely used in the treatment of several acute and chronic diseases, surgical reconstructions, and growing applications are emerging thank to the improved shelf life eased by new conservation methods like cryopreservation and freeze-drying. AM is extensively applied in ophthalmology for the treatment of persistent corneal or conjunctival ulcers, neurotrophic ulcers, perforations, ocular cicatricial pemphigoid, Stevens-Johnson syndrome, pterygium, tumor removal, chemical and thermal burns, symptomatic bullous keratopathy, and other diseases leading to limbal stem cell deficiency [1]. AM application showed an improvement in visual acuity after stem cell transplant in the treatment of symblepharon, after thermal and chemical burns, pterygium, and tumor removal [2] with an improvement of the anatomy of the ocular surface and a better cosmetic appearance with discomfort reduction. AM transplant determine a faster healing rate of the epithelial defect if compared to traditional therapy in the treatment of acute chemical or thermal burns [3]. AM transplant also gives important pain relief and helps healing corneal defects in the treatment of bullous keratopathy of different etiologies [4]. Applications in other body districts comprises massive post-traumatic skin wounds, where

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AM is able to promote re-epithelization [5], and oral and maxillofacial applications [6]. Diabetic lower limbs infections are associated with substantial morbidity and mortality [7]. AM treatment can avoid the amputation in full thickness of foot and or leg wound of different etiologies [8]. It permits faster healing of more than 4-weeks-old diabetic leg ulcers [9] and dramatically shortens duration of graft take in burns wound [10].

The mechanisms underlying AM efficacy are not yet fully understood but it seems likely that a combination of mechanical support, growth factors, and extracellular matrix interactions, would re-create a suitable environment for controlled wound closing and healing [11].

AM is generally employed as fresh graft, however this approach is not practical for routine or mid/long term clinical use. For this reason, AM products undergo a range of processing and preservation techniques, including dehydration and cryopreservation, which are commercially available. One of the safer techniques for clinical use is lyophilization, a process in which the material is frozen and then the surrounding pressure is reduced to allow sublimation of the frozen water that thus passes directly from the solid phase to the gas phase. The main advantages of lyophilization are gathered on the preservation of the ultrastructure, the easiness and cost effective storage, and transportation that permit to extend the uses of AM in different body locations and for longer periods balancing the disadvantage of lower retention of growth factors and protein amounts in respect to cryopreservation [12]. Moreover, lyophilized AM graft could successful treat venous leg ulcers, crash injury, arterial insufficiency, immunologic skin disease, and snake bite refractory to one month traditional therapy [13].

In this study, we report the successful application of a commercial lyophilized, powdered, amniotic membrane (AMX) to a patient with a diabetic ulcer resistant to other treatments. We further investigate AM effect on proliferation and angiogenic properties of amniotic fluid stem cells (AFS). Moreover, we performed a proteomic analysis of different AMX lots.

2. Materials and methods

2.1. Lyophilized amniotic membrane (AMX)

AMX (Amniotic Membrane eXtract, US Patent: US7871646B2) was obtained from the Italian licensee Keera (Arcugnago, Italy) and consists in pure lyophilized amniotic membrane obtained with a proprietary process of freeze-drying and soft milling of the fresh tissue. The lyophilized membrane is reconstituted right before use with balanced salt solution or normal saline solution (0.9%) and used topically, dropwise.

2.2. Patient

AMX was used in the treatment of a skin diabetic leg ulcer of a 77-year old woman. The patient history comprises an 8-year clinical history of diabetes mellitus type II, hypertension, and dyslipidemia. Initial lesion size was 10 mm x 5 mm. Prior to AMX, the ulcer was treated with serial debridement, topical antibacterial ointments, and various silver dressings with no signs of closure. AMX was administrated at the dose of 2–3 drops (150–200 µl) of a solution of 5 mg/mL of normal saline 4–6 times a day for 2 months and the wound was kept dressed between the applications. The study was conducted in accordance with the principles of the Helsinki Declaration as revised in Fortaleza 2013. The use of AMX, even without surgery, for the Italian regulation, resembled an amniotic membrane graft that is approved for wound treatment (disease-related group 86.66). The patient was informed about the benefits and possible risks before the beginning of the study.

2.3. Amniotic fluid stem cells

Amniotic fluid stem cells (AFS) samples were obtained from leftover samples of routinely performed amniocentesis (16–18 weeks of gestation). Healthy donors signed informed consent approved by the local committee (Azienda Ospedaliera of Padova Pr. N. 32887/451 P).

Samples were processed in accordance with the previous described method [14]. Briefly, cells from amniotic fluid were seeded on glass coverslips and adherent cells were then positively selected for CD117 by using the MACS CD117 MicroBead Kit from Miltenyi Biotec (Bergisch Gladbach, Germany). Chang Medium C Lyophilized (Irvine Scientific, Santa Ana, CA, USA) before selection was used. For expansion, the medium was minimum essential medium alpha (Gibco, now part of Thermo Fisher Scientific, Waltham, MA, USA) containing 20% Chang medium, 15% fetal bovine serum (Gibco), antibiotics and L- Glutamine (2 mM final). Cells were maintained under standard 20% O_2, 5% CO_2, and 95% relative humidity.

2.3.1. Cell proliferation

6000 cells/well were seeded on 96 well plate and the number of viable cells were counted in Burker chamber after 3, 6, 9 and 12 days. Three different AMX lots (Lot 1,2,3) and concentrations (1, 0.5, 0.1%) were tested.

2.3.2. In vitroendothelial differentiation

To test the endothelial potential of AFS cells we used the endothelial cell tube formation assay over Matrigel™Basement Membrane Matrix (BD Biosciences, East Rutherford, NJ, USA). AFS cells were detached from the original expansion culture and seeded in EGM-2 (endothelial growth medium-2) medium (Lonza, Basel, Switzerland) at a concentration of 15,000 cells/cm^2 over the solidified coating. ImageJ software coupled with Carpentier G Angiogenesis Analyzer was used [15].

2.4. ELISA quantification of NGF, TGF, NT-3, and IL1-ra

Resuspended AMX was centrifuged 13,000 xg for 5 min and total protein content was quantified (see below) on the supernatant. Nerve growth factor, NGF (Promega Corp., Madison, WI, USA), transforming growth factor alpha, TGF-alpha (R & D Systems, Minneapolis, MN, USA), and neurotrophin-3, NT-3 (Promega), and interleukin 1 receptor antagonist, IL1-ra (Abcam, Cambridge, UK) were quantified by commercially available ELISA. Growth factors were quantified in 3 different lots of AMX.

2.5. Proteomic analysis of lyophilized membrane AMX

2.5.1. Sample preparation

We analyzed 3 different lots of lyophilized membrane. To mimic AMX behavior, membrane was reconstituted and proteins were solubilized using only normal saline solution.

Protein content was determined with the method of Lowry. Equal amounts (20 µg) of proteins from the 3 different lots were diluted 1:1 with Laemli solution and loaded into a SDS-PAGE (Miniprotean TGX 4–15%, Biorad, USA), the electrophoretic process was allowed to proceed until all proteins formed a tight band at the top of the gel. The gel was then stained with colloidal Comassie blue G250 (0.25% in methanol, acetic acid, and water 45:10:45) and destained with water. The 3 gel bands were excised and subjected to in-gel reduction/alkylation and trypsin digestion as detailed previously [16].Peptides were extracted from the gel by 3 changes of 50% acetonitrile/0.1% formic acid, dried under vacuum and stored at −20 °C.

2.5.2. LC–MS/MS and data analysis

Samples were suspended in 30 µL of 3% acetonitrile/0.1% formic acid analyzed using a nano-HPLC Ultimate 3000 (Dionex, Thermo Fisher Scientific, USA) coupled online with a LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific). Three µL of each sample were loaded onto a 10 cm pico-frit capillary column (75 um I.D., 15 um tip, New Objective) packed in-house with C18 material (Aeris Peptide 3.6 μm XB-C18, Phenomenex, Italy) and peptides were separated using a linear gradient of acetonitrile/0.1% formic acid, from 3% to 40% in 50 min at a flow-rate of 250 nL/min. The analysis was conducted in a data dependent mode: a full MS scan (in the 300–1700 m/z range) was performed at high resolution (60,000) in the Orbitrap, followed by MS/MS scan of the 10 most abundant ions acquired in the linear ion trap with CID fragmentation.

Raw files were analyzed using the software package Proteome Discoverer (version 1.4, Thermo Fisher Scientific) interfaced to a Mascot search engine (version 2.2.4, Matrix Science). The search was done against the Human section of the Uniprot database (version 20150401, 90,411 sequences) using the following parameters: trypsin as digesting enzyme with up to 1 missed-cleavage allowed; precursor and fragment tolerance 10 ppm and 0.6 Da respectively; carbamidomethylation of cysteine residues as fixed modification and oxidation of methionine residues as variable modification. The Precursor Ions Area Detector node and the Percolator algorithm (both implemented into the Proteome Discoverer package) were used in the workflow of protein identification. The results were filtered with a false discovery rate (FDR) $\leq$ 0.01 and only proteins that were identified with at least 2 unique peptides with high confidence (99%) were considered as positive hits. Proteins were grouped into protein families according to the principle of maximum parsimony.

3. Results

3.1. Diabetic ulcer treatment

At 2-month follow-up, the ulcer was 60% less than the initial wound (10 × 2 mm) and it remained steady at 3-month follow-up. At 4-month follow-up the lesion started to show signs of full-area re-epithelization: left edge was markedly less sloping, healthy pink granulation tissue is present with no sign of necrotizing tissue, and the whole wound is less swollen and inflamed (Fig. 1). Patient reported a consistent ease of the pain starting from the first weeks of treatment.

3.2. Proliferation and endothelial differentiation of AFS cells

To evaluate whether different lots of AMX possess same proliferation characteristics, three distinct preparations were tested at three concentrations on hAFS cells. Each lot showed peculiar proliferation influence, however 0.1% AMX concentration was identified as the best condition similar to the control (Fig. 2).

It is known that hAFS cells are able to differentiate toward endothelial cells, both *in vitro* and *in vivo*. To evaluate whether AMX enhanced endothelial properties of the cells, matrigel tube assay was performed. Both cell culture, with or without AMX, were prone to form endothelial tubules with similar characteristics of branches and mesh area (Fig. 2). Human umbilical vein endothelial cells (HUVEC) were used as internal control (data not shown) (Fig. 3).

3.3. ELISA quantification of NGF, TGF, NT-3, and IL1-ra

In three lots of AMX we measured 2 neurotrophic factors (NGF, NT-3), TGF-alpha, and IL1-ra. Results are showed in Table 1.

3.4. Proteomic analysis

We analyzed the protein content of AMX to identify the possible mediators that determine its *in vivo* effects. We confidently identified 207 different proteins, 125 of which were in common between all 3 lots. All identified proteins are reported in Table A (supplementary material).

In our specific study, we focused on some proteins emerged from proteomic data that could have been crucial in ulcer healing and *in vitro* angiogenesis (see Table 2 for references):

Decorin is a small leucine-rich proteoglycan. Decorin deficiency causes impaired angiogenesis in the injured cornea of decorin-null mice. Even if this seems to support a stimulatory role of decorin in the angiogenic response, there are studies supporting the opposite view. Decorin suppresses angiogenesis in tumors and the overexpression of decorin retards corneal neovascularization. Whether decorin activity will be pro- or antiangiogenic appears to depend on the physiological or pathological condition of the tissue.

Lumican: is a keratin-sulfate proteoglycan that localizes macrophages to the cornea after damage and recruits neutrophils in lipopolysaccharide-induced mice keratitis. It promotes the secretion of pro-inflammatory cytokines through TRL-4 pathway. Lumican is also important in mediating Fas-Fas ligand interactions. It has been demonstrated that the administration of lumican purified by AM promotes corneal epithelial wound healing.

Calreticulin: it has been shown to favor cell adhesion, focal adhesion disassembly, cell migration, and phagocytosis when locate on the cell

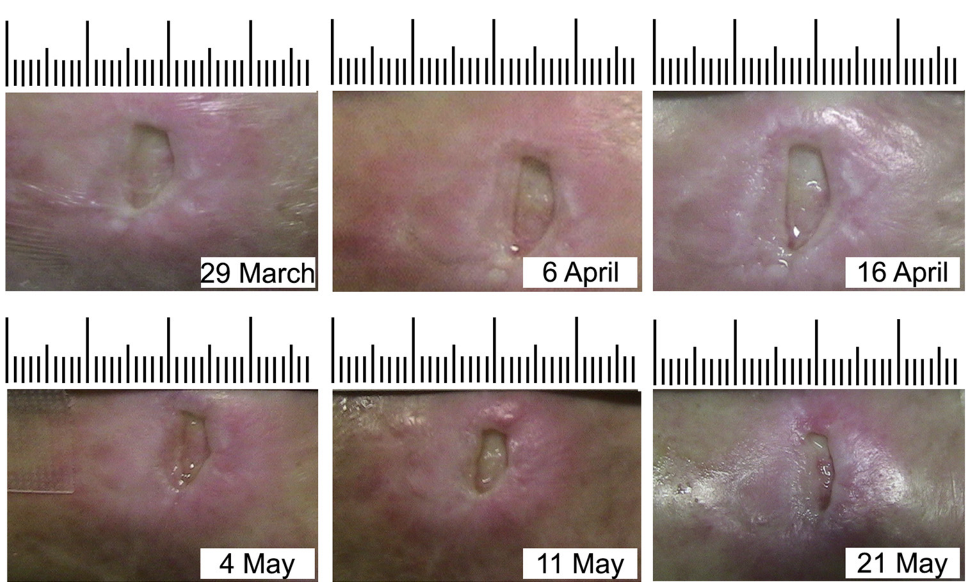

Fig. 1. AMX in a patient.
AMX effect in the patient with chronic ulcer. Re-epithelization appeared after 2 months treatment.

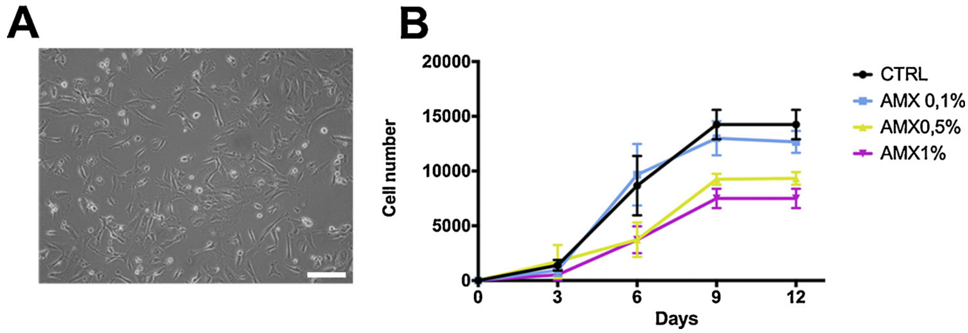

Fig. 2. AFS cells.
Gross appearance of AFS cells (A) and growth curve of cells. Three different lots were tested together with three AMX concentrations. Scale bar: 100µm

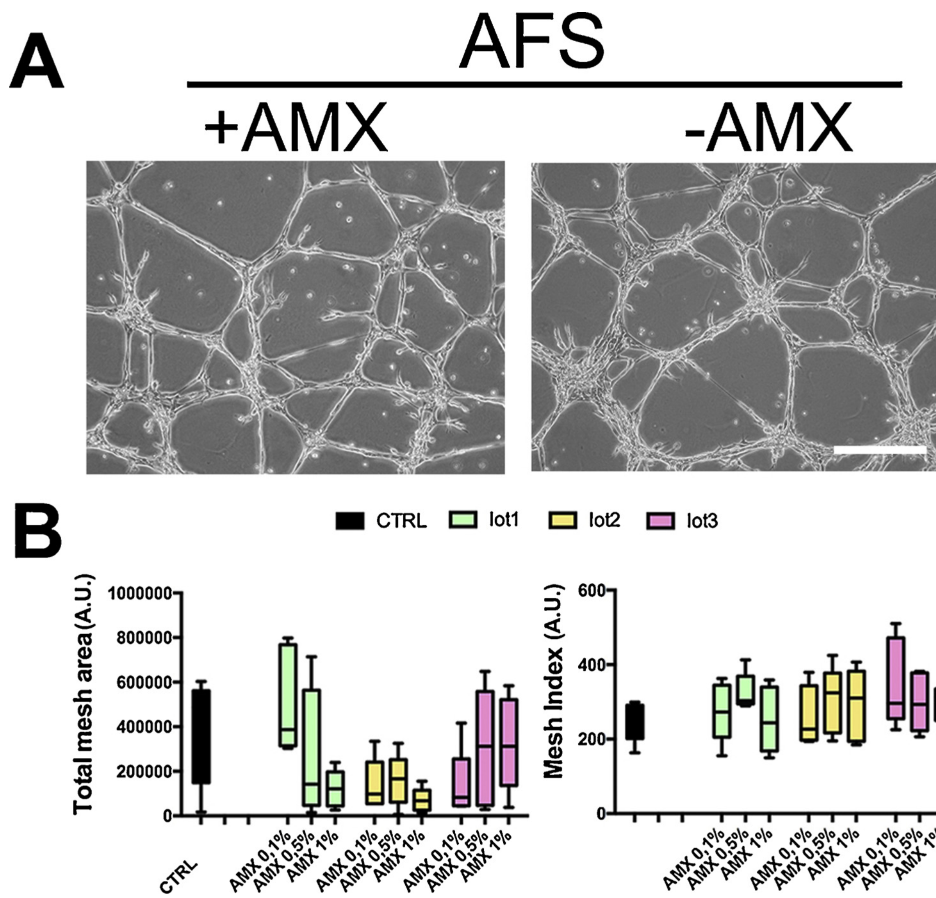

Fig. 3. *In vitro*angiogenic assay.
Representative picture of tubule formation of AFS cells with (A) and without (B) AMX. In C total mesh area and branches of the capillary like structure are measured. No statistical differences with the control (HUVEC). Scale bar: 100µm

surface. Calreticulin treated wounds show an increased rate of epithelialization thanks to the facilitated migration of keratinocytes and a strong induction of granulation tissue.

Annexin A1: is an anti-inflammatory mediator, which regulates neutrophilic recruitment during inflammation, limiting an excessive neutrophil accumulation. It induces neutrophil apoptosis and their phagocytosis by macrophages. Indeed, AnxA1 contributes to tissue homeostasis by inducing macrophage reprogramming toward a resolving phenotype.

Versican: Versican shows both pro- or anti-inflammatory activities. These activities seem to depend on the context in which versican is presented to cells and the temporal and spatial availability of the versican molecule during tissue remodeling and inflammation. Versican is able to bind to a wide variety of receptors and other inflammatory components and to regulate their availability and activity.

Serum amyloid P-component: reduces neutrophil adhesion to matrix proteins, inhibits the differentiation of monocytes into fibrocytes, attenuates profibrotic macrophages, activates the complement pathway, and promotes phagocytosis of cell debris.

4. Discussion

In this study we reported the successful use of lyophilized, powdered, AM to treat a refractory diabetic ulcer with a simple daily dropwise application. We further characterized *in vitro* the effect of AMX with cellular and proteomic analyses. For the first time, with this work, we demonstrated the clinical usefulness of topic AMX application in a chronic wound. This pathological environment is characterized by

Table 1
Quantification of growth factors and IL1-ra. NGF: nerve growth factor; TGF-alpha: transforming growth factor alpha; NT-3: neurotrophin-3; IL1-ra: interleukin 1 receptor antagonist. Data are expressed as mean ± SD and were obtained from 3 replicates.

Analyte	Concentration
Total Proteins	0.14 ± 0.04*
NGF	60 ± 20**
TGF-alpha	108 ± 32**
NT-3	179 ± 36**
IL1-ra	6078 ± 911**

* mg/mL.
** pg/mg proteins.

dysfunction of growth factor, cytokine and angiogenic factor signaling thus leading to a delayed or inappropriate healing.

The usefulness and clinical interest on the use of AM in wound healing principally resides in AM particular low immunogenicity. Kubo et al. demonstrated that AM graft survives longer when transplanted on the limbal area, intracorneal space and under the kidney capsule than the skin graft [17], suggesting that AM is an immuno-privileged tissue despite the presence of HLA antigens. This thesis is supported by the presence of immunoregolatory factors such as FAS ligand [18] in AM tissue.

The rationale of using AM in diabetic ulcers relies on the common aspects of corneal and diabetic neurotrophic lesions where a dysregulation of the cellular and extracellular environment could land to persistent defects exposing the organ to infection (skin) and scarring (cornea and skin).

Clinical effects of AM depend on anti-inflammatory, anti-fibrotic proprieties, capability to promote re-epithelization, reduce pain, and protect wounds with also antibacterial action [19]. Regarding angiogenesis modulation, AM displays different effects, which make it an appropriate candidate for different clinical situations. Ophthalmological employment of AM results in the inhibition of corneal neovascularization, which allows the maintenance of ocular surface transparency. AM suppresses TGF-β signaling system which promotes the differentiation of myofibroblasts in corneal, limbal, conjunctival, and pterygium tissues [20], thus explaining AM antiscarring effects. The application of the supernatant of AM epithelial cells on a corneal mice model of induced neovascularization and inflammation show that AM epithelial cells secrete soluble mediators, which suppress corneal neovascularization and reduce the activation of MHC class II and APCs in the epithelium [21]. Pigment epithelium-derived factor (PEDF), which localizes in the basement membrane of amniotic membrane, is reported to play a major role in inhibiting endothelial cell growth in the cornea [22]. Finally, AM epithelium promotes the re-epithelization of ocular surface expressing growth factors such as epidermal growth factor and keratinocyte growth factor, hepatocyte growth factor, and basic fibroblast growth factor [23]. The basement membranes of AM and conjunctiva share the same structural proteins: laminin and collagen VII. Therefore AM might act as a substrate promoting migration of epithelial cells [24].

On the other hand, the capability of AM in promoting new vessel formation in skin wounds, enhances the healing process [25]. Zhu et al. demonstrated that AM epithelial cells promote angiogenesis in a hyperoxia neonatal lung injury model and inhibit it in a bleomycin-induced pulmonary fibrosis model which is associated with neovascularization [26]. Lyophilized AM demonstrated to induce angiogenesis *in vivo* in a murine subcutaneous implant model [27]. The ability of AM to induce angiogenesis is supported by the presence of pro-angiogenic factors including angiogenin, angiopoietin-2 (ANG-2), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), heparin binding epidermal growth factor (HB-EGF), hepatocyte growth factor (HGF), platelet derived growth factor BB (PDGF-BB), placental growth factor (PlGF), and vascular endothelial growth factor (VEGF) [27]. Koob et al. demonstrated that dehydrated human amnion/chorion allografts induce proliferation and production of angiogenic growth factors in human microvascular endothelial cells and migration of HUVECs [27]. Nevertheless, the molecular mechanisms that lead to AM angiogenic modulation are still poorly understood.

AMX is a lyophilized form of amniotic membrane that retains the characteristic of traditional lyophilized AM grafts but has the advantage of the topical administration without surgery. This permits long period of treatment and repeated applications widening the spectrum of AM applications in pathologies. AMX demonstrated to promote faster healing of primary human corneal epithelial cells under mechanical and oxidative stress thus suggesting its corneal acute injury treatment potential [28]. AMX has been mainly employed in the treatment of ocular disease but in this study, supported by the usefulness of the fresh AM in wounds, we used AMX on a chronic skin ulcer unresponsive to routine treatments.

In our patient AMX reduced the ulcer by 60% in 2 months of multiple daily applications, while easing the perceived pain. Pain reduction is in line with what was described for AM application in the eye [29]. Multiple daily applications are not a compliance-friendly mode of administration but since AMX seems to be effective for chronic wound healing, a more concentrated preparation or a topic ointment could be prepared to reduce the number of applications. More studies are needed

Table 2
Main proteins and their function of the water-soluble fraction of AMX. Proteins are in order of relative abundance.

Protein	ID	Function	References
Transforming growth factor-beta-induced protein ig-h3	Q15582	Involved in cell adhesion, migration, and proliferation. Crucial in wound healing	[32]
Decorin	P07585	Modulates angiogenesis in response to the environment	[33,34,35]
Annexin A2	P07355	Promotes fibrinolysis, angiogenesis, and cell migration	[36]
Vimentin	P08670	Promote angiogenesis and it plays a role in neurons regeneration	[37]
Lumican	P51884	Stimulates immune response and wound healing in the cornea	[38]
Mimecan	P20774	Cell proliferation modulator. Essential for corneal matrix transparency	[39]
Thrombospondin-1	P07996	Anti-inflammatory mediator and angiogenesis inhibitor	[40]
Calreticulin	P27797	Promotes wound healing through epithelialization	[41]
Annexin A1	P04083	Inflammation mediator	[42]
Periostin	Q15063-7	Promotes wound healing *in-vitro*	[43]
Galectin 3	P17931	Promotes cell migration and interactions. Promotes angiogenesis in tumors	[44]
Versican	E9PF17	Modulates inflammatory response during tissue remodelling	[45]
Dermatopontin	Q07507	Enhances the biological activity of transforming growth factor beta 1. Promotes re-epithelialization	[46]
Galectin 1	P09382	Promotes angiogenesis and endothelial proliferation	[47]
Serum amyloid P-component	P02743	Inhibit scar formation through cell-response modulation	[48]
Aminopeptidase N	P15144	Promotes angiogenesis	[49]
Transgelin-2	X6RJP6	Inhibit angiogenesis	[50]

to assess the optimum concentration of AMX for wound healing.

Placenta is bathed by the amniotic fluid where the stem cells reside and this is the fetal environment that recently has been appointed as fetal stem cell niche [15]. Following these premises, we planned to test in hAFS cells the effect of the innermost part of the placenta under the lyophilized form (AMX), to study whether proliferation and angiogenic potential would be enhanced or reduced.

It is well known that hAFS are broadly multipotent stem cells and retain high capacity to proliferate and differentiate in cells of mesodermal origin such as osteocytes, adipocytes, chondrocytes, and muscle cells [30]. Schiavo and colleagues proved the pro-angiogenic characteristics of AFS cells both *in vitro* and *in vivo*, the latter in two different models of endothelial damage [15]

Consequently, in this work we studied the angiogenic potential of AFS cells cultured with AMX. Cells with and without AMX formed endothelial tubules with similar characteristics suggesting that the addition of the pro-angiogenic AMX on AFS cells still exerts a strong action toward endothelial differentiation keeping at high level the angiogenic differentiation ability.

Proteomic analyses confirmed the presence in AMX of angiogenic factors but we could not establish a direct effect from a specific protein to wound healing or angiogenesis; *in vivo* inhibition or synergic effects could take place. We speculate a synergic effect toward the enhancement of endothelium formation. We found solubilized intracellular proteins released from the cells after lyophilization. Some of these proteins could have unexpected function in the extracellular environment [31].

Furthermore, we quantified the presence of two neurotrophic (NGF and NT-3) and inflammatory (TGF-alpha, and IL1-ra) factors to confirm that proliferation and anti-inflammatory mediators present in the fresh membrane are maintained in the lyophilized form of the membrane. All the quantifications were successful but we did not further investigate the effects of each single factor to AMX overall effect.

The limits of the study are on the single patient report and on the lack of evidence of a direct action of the detected proteins; future experiments will be developed to shed light on this aspect.

Conclusions

In conclusion, we described for the first time the successful use of AMX lyophilized amniotic membrane to ameliorate the pain and extent of a chronic diabetic ulcer. The effect could be ascribed to a strong angiogenesis and epithelium stimulation that was confirmed in the *in vitro* model of angiogenesis. Multiple wound healing-involved proteins were detected but further studies are needed to assess the specific role of these proteins.

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EG is the inventor and retains the rights of the AMX patent (US7871646B2) “Use of a human amniotic membrane composition for prophylaxis and treatment of diseases and conditions of the eye and skin”. All the other authors declare no conflict of interests.

Declaration of Competing Interest

EG is the inventor and retains the rights of the AMX patent (US7871646B2) “Use of a human amniotic membrane composition for prophylaxis and treatment of diseases and conditions of the eye and skin”. All the other authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.wndm.2019.100171.

References

[1] C. Malhotra, A.K. Jain, Human amniotic membrane transplantation: different modalities of its use in ophthalmology, World J. Transplant. 4 (2014) 111–121.
[2] A. Gheorghe, M. Pop, M. Burcea, M. Serban, New clinical application of amniotic membrane transplant for ocular surface disease, J. Med. Life 9 (2016) 177–179.
[3] A. Tamhane, R.B. Vajpayee, N.R. Biswas, R.M. Pandey, N. Sharma, J.S. Titiyal, R. Tandon, Evaluation of amniotic membrane transplantation as an adjunct to medical therapy as compared with medical therapy alone in acute ocular burns, Ophthalmology. 112 (2005) 1963–1969.
[4] G.D.J.Y. Siu, A.L. Young, L.L. Cheng, Long-term symptomatic relief of bullous keratopathy with amniotic membrane transplant, Int. Ophthalmol. 35 (2015) 777–783.
[5] C.L. Insausti, A. Alcaraz, E.M. Garcia-Vizcaino, A. Mrowiec, M.C. Lopez-Martinez, M. Blanquer, A. Pinero, M.J. Majado, J.M. Moraleda, G. Castellanos, F.J. Nicolas, Amniotic membrane induces epithelialization in massive posttraumatic wounds, Wound Repair Regen. 18 (2010) 368–377.
[6] M. Rainer Kesting, K.-D. Wolff, C. Philipp Nobis, N. Hagen Rohleder, Amniotic membrane in oral and maxillofacial surgery, (n.d.).
[7] G.M. Caputo, P.R. Cavanagh, J.S. Ulbrecht, G.W. Gibbons, A.W. Karchmer, Assessment and management of foot disease in patients with diabetes, N. Engl. J. Med. 331 (1994) 854–860.
[8] B. Werber, E. Martin, A prospective study of 20 foot and ankle wounds treated with cryopreserved amniotic membrane and fluid allograft, J. Foot Ankle Surg. 52 (2013) 615–621.
[9] C.M. Zelen, T.E. Serena, G. Denoziere, D.E. Fetterolf, A prospective randomised comparative parallel study of amniotic membrane wound graft in the management of diabetic foot ulcers, Int. Wound J. 10 (2013) 502–507.
[10] A.A. Mohammadi, H.G. Johari, S. Eskandari, Effect of amniotic membrane on graft take in extremity burns, Burns. 39 (2013) 1137–1141.
[11] M. Litwiniuk, T. Grzela, Amniotic membrane: new concepts for an old dressing, Wound Repair Regen. 22 (2014) 451–456.
[12] M.T. Rodríguez-Ares, M.J. López-Valladares, R. Touriño, B. Vieites, F. Gude, M.T. Silva, J. Couceiro, Effects of lyophilization on human amniotic membrane, Acta Ophthalmol. (Copenh) 87 (2009) 396–403.
[13] J. Forbes, D.E. Fetterolf, Dehydrated amniotic membrane allografts for the treatment of chronic wounds: a case series, J. Wound Care 21 (292) (2012) 294–296 290.
[14] M. Pozzobon, M. Piccoli, A.A. Schiavo, A. Atala, P. De Coppi, Isolation of c-Kit+ human amniotic fluid stem cells from second trimester, Methods Mol. Biol. 1035 (2013) 191–198.
[15] G. Carpentier, M. Martinelli, J. Courty, I. Cascone, Angiogenesis analyzer for ImageJ, 4th ImageJ User Dev. Conf. (2012), pp. 198–201.
[16] G. Arrigoni, M.A. Pagano, S. Sarno, L. Cesaro, P. James, L.A. Pinna, Mass spectrometry analysis of a protein kinase CK2β subunit interactome isolated from mouse brain by affinity chromatography, J. Proteome Res. 7 (2008) 990–1000.
[17] M. Kubo, Y. Sonoda, R. Muramatsu, M. Usui, Immunogenicity of human amniotic membrane in experimental xenotransplantation, Investig. Ophthalmol. Vis. Sci. 42 (2001) 1539–1546.
[18] R. Runic, C.J. Lockwood, L. LaChapelle, B. Dipasquale, R.I. Demopoulos, A. Kumar, S. Guller, Apoptosis and Fas expression in human fetal membranes, J. Clin. Endocrinol. Metab. 83 (1998) 660–666.
[19] Y.P. Talmi, L. Sigler, E. Inge, Y. Finkelstein, Y. Zohar, Antibacterial properties of human amniotic membranes, Placenta. 12 (1991) 285–288.
[20] S.B. Lee, D.Q. Li, D.T. Tan, D.C. Meller, S.C. Tseng, Suppression of TGF-beta signaling in both normal conjunctival fibroblasts and pterygial body fibroblasts by amniotic membrane, Curr. Eye Res. 20 (2000) 325–334.
[21] K. Kamiya, M. Wang, S. Uchida, S. Amano, T. Oshika, N. Sakuragawa, J. Hori, Topical application of culture supernatant from human amniotic epithelial cells suppresses inflammatory reactions in cornea, Exp. Eye Res. 80 (2005) 671–679.
[22] C. Shao, J. Sima, S.X. Zhang, J. Jin, P. Reinach, Z. Wang, J.X. Ma, Suppression of corneal neovascularization by PEDF release from human amniotic membranes, Investig. Ophthalmol. Vis. Sci. 45 (2004) 1758–1762.
[23] N.J. Koizumi, T.J. Inatomi, C.J. Sotozono, N.J. Fullwood, A.J. Quantock, S. Kinoshita, Growth factor mRNA and protein in preserved human amniotic membrane, Curr. Eye Res. 20 (2000) 173–177.
[24] K. Fukuda, T. Chikama, M. Nakamura, T. Nishida, Differential distribution of subchains of the basement membrane components type IV collagen and laminin among the amniotic membrane, cornea, and conjunctiva, Cornea. 18 (1999) 73–79.
[25] H. Niknejad, G. Paeini-Vayghan, F.A. Tehrani, M. Khayat-Khoei, H. Peirovi, Side dependent effects of the human amnion on angiogenesis, Placenta. 34 (2013) 340–345.
[26] D. Zhu, R. Muljadi, S.T. Chan, P. Vosdoganes, C. Lo, J.C. Mockler, E.M. Wallace, R. Lim, Evaluating the impact of human amnion epithelial cells on angiogenesis, Stem Cells Int. 2016 (2016) 4565612.
[27] T.J. Koob, J.J. Lim, M. Massee, N. Zabek, R. Rennert, G. Gurtner, W.W. Li, Angiogenic properties of dehydrated human amnion/chorion allografts: therapeutic potential for soft tissue repair and regeneration, Vasc. Cell. 6 (2014) 10.
[28] D.V. Dudok, I. Nagdee, K. Cheung, H. Liu, L. Vedovelli, E. Ghinelli, K. Kenyon,

S. Parapuram, C.M. Hutnik, Effects of amniotic membrane extract on primary human corneal epithelial and limbal cells, Graefes Arch. Clin. Exp. Ophthalmol. 43 (2015) 443–448.
[29] J. Liu, H. Sheha, Y. Fu, L. Liang, S.C. Tseng, Update on amniotic membrane transplantation, Expert Rev. Ophthalmol. 5 (2010) 645–661.
[30] J.M. Gimble, F. Guilak, M.E. Nuttall, S. Sathishkumar, M. Vidal, B.A. Bunnell, In vitro differentiation potential of mesenchymal stem cells, Transfus. Med. Hemother. 35 (2008) 228–238.
[31] P.N. Goodfellow, C.J. Barnstable, W.F. Bodmer, D. Snary, M.J. Crumpton, Expression of HLA system antigens on placenta, Transplantation. 22 (1976) 595–603.
[32] J. Cha, T. Kwak, J. Butmarc, T.A. Kim, T. Yufit, P. Carson, S.J. Kim, V. Falanga, Fibroblasts from non-healing human chronic wounds show decreased expression of βig-h3, a TGF-β inducible protein, J. Dermatol. Sci. 50 (2008) 15–23.
[33] E. Schönherr, C. Sunderkötter, L. Schaefer, S. Thanos, S. Grässel, Å. Oldberg, R.V. Iozzo, M.F. Young, H. Kresse, Decorin deficiency leads to impaired angiogenesis in injured mouse cornea, J. Vasc. Res. 41 (2004) 499–508.
[34] D.S. Grant, C. Yenisey, R.W. Rose, M. Tootell, M. Santra, R.V. Iozzo, Decorin suppresses tumor cell-mediated angiogenesis, Oncogene. 21 (2002) 4765–4777.
[35] H. Jarvelainen, A. Sainio, T.N. Wight, Pivotal role for decorin in angiogenesis, Matrix Biol. 43 (2015) 15–26.
[36] K.A. Hajjar, The biology of Annexin A2: from vascular fibrinolysis to innate immunity, Trans. Am. Clin. Climatol. Assoc. 126 (2015) 144–155.
[37] J.M. Dave, K.J. Bayless, Vimentin as an integral regulator of cell adhesion and endothelial sprouting, Microcirculation. 21 (2014) 333–344.
[38] L.-K. Yeh, W.-L. Chen, W. Li, E.M. Espana, J. Ouyang, T. Kawakita, W.W.-Y. Kao, S.C.G. Tseng, C.-Y. Liu, Soluble lumican glycoprotein purified from human amniotic membrane promotes corneal epithelial wound healing, Invest. Ophthalmol. Vis. Sci. 46 (2005) 479–486.
[39] S. Deckx, S. Heymans, A.P. Papageorgiou, The diverse functions of osteoglycin: a deceitful dwarf, or a master regulator of disease, FASEB J. 30 (2016) 2651–2661.
[40] P.R. Lawler, J. Lawler, Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2, Cold Spring Harb, Perspect. Med. 2 (2012) a006627.
[41] L.I. Gold, P. Eggleton, M.T. Sweetwyne, L.B. Van Duyn, M.R. Greives, S.M. Naylor, M. Michalak, J.E. Murphy-Ullrich, Calreticulin: non-endoplasmic reticulum functions in physiology and disease, FASEB J. 24 (2010) 665–683.
[42] M.A. Sugimoto, J.P. Vago, M.M. Teixeira, L.P. Sousa, Annexin A1 and the resolution of inflammation: modulation of neutrophil recruitment, apoptosis, and clearance, J. Immunol. Res. 2016 (2016) 8239258.
[43] C.G. Elliott, J. Wang, X. Guo, S. Xu, M. Eastwood, J. Guan, A. Leask, S.J. Conway, D.W. Hamilton, Periostin modulates myofibroblast differentiation during full-thickness cutaneous wound repair, J. Cell. Sci. 125 (2011) 121–132.
[44] T. Funasaka, A. Raz, P. Nangia-Makker, Galectin-3 in angiogenesis and metastasis, Glycobiology. 24 (2014) 886–891.
[45] T.N. Wight, I. Kang, M.J. Merrilees, Versican and the control of inflammation, Matrix Biol. 35 (2014) 152–161.
[46] V.R. Krishnaswamy, P.S. Korrapati, Role of dermatopontin in re-epithelialization: implications on keratinocyte migration and proliferation, Sci. Rep. 4 (2014) 7385.
[47] V. Lthijssen, A.W. Griffioen, Galectin-1 and -9 in angiogenesis: a sweet couple, Glycobiology. 24 (2014) 915–920.
[48] N. Cox, D. Pilling, R.H. Gomer, Serum amyloid P: a systemic regulator of the innate immune response, J. Leukoc. Biol. 96 (2014) 1–5.
[49] Y. Luan, W. Xu, The structure and main functions of aminopeptidase N, Curr. Med. Chem. 14 (2007) 639–647.
[50] Y. Xiao, Y. Li, J. Han, Y. Pan, L. Tie, X. Li, Transgelin 2 participates in lovastatin-induced anti-angiogenic effects in endothelial cells through a phosphorylated myosin light chain-related mechanism, PLoS One 7 (2012) e46510.