Biochemical characterization of pure dehydrated binate amniotic membrane: role of cytokines in the spotlight

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Aim: Placental allografts used for tissue regeneration differ in membrane compositions and processing techniques. A uniquely folded dehydrated binate amniotic membrane (DBAM) was biochemically characterized to evaluate its potential role in wound healing. **Methods:** Histology, Luminex-based immunoassay and standard *in vitro* cell biology techniques were employed. **Results:** Histological staining confirmed that the DBAM was chorion free with epithelial cell layer of the respective amnion membranes facing outward. DBAM had quantifiable levels of relevant cytokines that induced proliferation and migration while bolstering secretory activity of the cells. DBAM retained biological efficacy at a broad range of temperatures. **Conclusion:** Cytokines in DBAM stimulate bone marrow stromal and stem cells that may lead to tissue regeneration and wound healing in a clinical setup.

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Wound healing implies a complete physical and functional restoration of damaged tissue through cellular homeostasis, inflammation, proliferation and tissue remodeling [1–3]. These biological processes are further regulated by extracellular signaling pathways involving cytokines, growth factors and membrane receptors [4,5]. Although wound healing is a natural physiological process, chronic wound management often requires clinical intervention to engineer a biochemical environment that will aid in the normal healing process. Chronic wounds like diabetic and venous ulcers pose a significant burden to the US healthcare system, affecting around 5 million patients and costing an estimated US\$25 billion annually [6,7]. Different clinical modalities are currently available to address and modulate the complex pathophysiology of chronic wounds [8]. They include biophysical modalities like electro-physical stimulation, bioengineered therapy based on recombinant growth factors, and application of platelet rich plasma and stem cell-based therapy [9,10]. However, given the inherent complexity of the wound healing process, a multifaceted approach that can trigger an efficient signaling response is more likely to achieve therapeutic benefits, rather than merely replenishing growth factors or targeting individual functions like angiogenesis or proliferation.

One such approach is the use of placental membranes, the earliest reported biomaterials used for wound repair. Placental membranes combine a demonstrated advantage of high clinical efficacy with a comparatively low treatment cost [11]. Placental membranes have their origin in extraembryonic tissue. This tissue is composed of a fetal component (the chorionic plate) and a maternal component (the decidua) [12–14]. The fetal component includes amniotic membrane (AM) or amnion, a thin membrane that surrounds the embryo and creates the amniotic sac, which separates the fetus from the endometrium [13–16]. AM is made up of three distinct layers: the epithelial monolayer, a basement membrane and the avascular stromal matrix.

AM was first used clinically for therapeutic application in skin grafting in 1910 [17]. Early literature indicates widespread use of fetal membranes in ophthalmic reconstructive surgeries and conjunctival defects [18]. AM allografts have also been extensively used in fornicular reconstruction, repair of conjunctival membrane and corneal

perforation [19–22]. Another popular application of AM is in periodontal surgeries, either as temporary or permanent graft [23–25]. Many clinical case studies reported use of amnion tissue as a biological dressing in treatment of skin loss due to second- and third-degree burns, and open infected wounds and ulcers [26–28]. Similarly, human-derived placental tissues have been shown in randomized clinical trials and *in vitro* studies to be effective for healing chronic wounds through their ability to release active cytokines and influence cell migration, proliferation and angiogenesis. Further, AMs provide a biological barrier for the growth of fibrous tissues, which limits scarring. The fetal origin of the amniotic tissue makes it immune-privileged, providing a perfect scaffold for enhanced wound repair, along with significantly reduced inflammatory pain and fibrogenic scarring [29–31].

The amniotic epithelial cells are a rich source for biologically active factors like cytokines, which in most circumstances remain bound to the extracellular matrix even after decellularization and exposure to various drying techniques [32,33]. These growth factors play a vital role in wound healing and tissue regeneration. Thus, it is critical to maintain the stability and biological activity of these cytokines and growth factors when amnion tissue-based products are prepared [5,34–36].

Amnion allografts commercially available for clinical use are either dehydrated or cryopreserved to maintain the growth factor composition and to increase stability and shelf life of the product [37–40]. Different preservation techniques have significant impact on biochemical composition and physical properties of amnion allografts and thus can affect the clinical efficacy of these products. Dehydrated AM showed higher growth factor retention and bioavailability when compared with cryopreserved membrane allograft. In biological assays too, dried AM was superior than cryopreserved amnion tissue and possessed a higher wound healing capacity [39]. Most translational and clinical studies reporting biological/clinical effects of placental allografts in chronic wound healing, however, have predominantly utilized the dehydrated human amnion/chorion composite membranes [41–43]. The comparative studies between dehydrated human amnion/chorion composite membranes and amnion-only allografts are largely confined to relative cytokine concentrations, but do not evaluate the biological efficacy of the products *in vitro*. Amnion-only membranes that are commercially available and evaluated are either single-layer or double-layer allografts with an intact epithelial cell layer facing inward [44,45].

Here, we studied an amniotic allograft whose histological staining revealed an architectural organization that uniquely combines the high tensile strength of pure amnion with the biological efficacy of the partially disrupted epithelial cell layers, which face outward to be in direct contact with the wound. Given the structure–function relationship of processed tissue allografts in general, and the absence of any previous comprehensive study of a similarly organized AM, it is highly relevant to characterize this dehydrated binate AM (DBAM) in terms of biological functions to assess its wound healing potential. We quantified the array of cytokines and growth factors present in DBAM homogenates. We further evaluated, using bone marrow stromal and mesenchymal stem cells as *in vitro* model systems, whether the biological efficacy of DBAM is regulated by these cytokines. Stability of the DBAM was studied by thermal preconditioning. Taken together, our data provide compelling evidence that the DBAM contains quantifiable levels of cytokines, which induce cell proliferation, migration and secretion of endogenous cytokines. The wound healing property of DBAM is potentially derived from its ability to influence these cellular processes.

Materials & methods

Cells & tissue culture media

Bone marrow stromal cells (HS5; CRL-11882) and human bone marrow-derived mesenchymal stem cells (BM-MSCs; PCS-500-012) were purchased from ATCC (VA, USA) and grown in the recommended culture media and culture conditions. HS5 cells from passage 3–6 and BM-MSCs from passages 3 and 4 were used for experiments.

Processing of dehydrated binate amnion membrane

Dehydrated binate amnion membrane (DBAM) was obtained from the manufacturer, Burst Biologics (ID, USA). Amnion layer was processed with the unique method developed by the manufacturer to retain the optimum biological activity. Broadly, the process included the following steps. First, placental tissues were procured from donors during full-term, healthy cesarean sections from informed births, as per the US FDA's Good Tissue Practice. Then, the procured tissues were transferred on wet ice within 24 h to the processing facility and stored at -20°C until processing. The tissues were thawed at room temperature and washed in distilled water to remove residual blood clots and mucus and cut in $4\text{--}16\text{ cm}^2$ pieces. The amnion layer was physically separated from the chorion layer and then further processed to remove bacterial contamination. Two amnion layers were fused to expose the

epithelial cell layer outward, desiccated under controlled conditions, and then sterilized by γ irradiation. Each donated placenta was also tested for infectious diseases and bacterial contamination prior to processing.

Histochemistry

DBAM patches obtained from three different donors were fixed in 10% formalin, embedded in paraffin, sectioned into 5 μ m thick cross sections, and stained by Premier Laboratory (CO, USA). Tissues were stained with hematoxylin and eosin (H&E) for cell nuclei and Masson's trichrome for collagen. Stained tissue sections were then imaged by Leica Aperio and analyzed.

Preparation of DBAM homogenized extracts

DBAM from individual donors was homogenized in a tissue grinder and extracted in phosphate-buffered saline (PBS) at a concentration of 10 mg tissue/ml. The crude homogenates were centrifuged at 5000 $\times g$, 4°C for 15 min, supernatant collected and sterile filtered before using in tissue culture. For heat-inactivation studies, DBAM homogenate was boiled at 95°C for 5 min and then subsequently used for downstream assays. For thermal preconditioning, DBAM was shelved at the indicated temperature for 48 h before homogenization.

Cytokine quantification

Cytokines were measured in the DBAM homogenate or the conditioned media by Luminex xMAP technology/Magpix™ (Thermo Fisher Scientific, MA, USA) as per the manufacturer's guideline. Cytokine concentration in the DBAM homogenate, was normalized to per mg of DBAM obtained from six different donors. Cells were treated for 36 h with basal media (reduced growth factor and 0.2% serum) or the 1 mg/ml DBAM homogenate and the concentrations of the secreted cytokines were quantified. Baseline values were determined from the concentration of cytokines in the basal media or the diluted DBAM aliquoted at the initiation of the treatment.

Cell proliferation assay

Cells were plated (3000 cells/well of 96-well plate) overnight, serum starved for 6 h and then treated for 48 h with complete media, basal media (reduced growth factors and 0.2% serum), or with the DBAM homogenates (diluted in basal media to reach a final concentration of 1–2 mg/ml). Cell proliferation was measured by CyQUANT Cell Proliferation Kit (Thermo Fisher Scientific Cat. #C7026) as per the manufacturer's guidelines. Live cells were also imaged by Calcein-AM (Thermo Fisher Scientific) staining.

Transwell migration assay

Transwell membranes were coated with 5 μ g/ml of fibronectin (Sigma-Aldrich, MO, USA) and left for 18 h for surface adsorption. Serum starved cells were resuspended in basal media (reduced growth factors and 0.2% serum) and 2.5×10^4 cells were added to the upper chamber of a 24-well, 8 μ m pore membrane. Complete media, basal media or DBAM diluted in basal media was added to the lower chamber as a chemoattractant. After 16 h, cells migrated to the lower surface of transmembrane were stained with crystal violet stain, imaged and quantified.

Thermal conditioning of DBAM

Processed DBAM was incubated for 48 h at indicated temperatures as determined using temperature data loggers (Pharma Watch, ID, USA). After conditioning, all samples were kept at ambient conditions until processed.

Data analysis & statistics

All the experiments were repeated using DBAM from three to six donors, each time at least in triplicate. Data are expressed as mean $\pm$ standard deviation or standard error of the mean. Dunnett's test was performed to compare more than two groups. Student's t-test was used for statistical analysis between two groups. Statistical significance was set at $p < 0.05$ (*).

Results

Histological composition of the DBAM

Histological architecture of the dehydrated AM is critical for its biological efficacy. The histological parameters of the AM are known to be sensitive to different methods of sterilization and processing, often affecting the integrity

Figure 1. Histological images of dehydrated binate amniotic membrane. Binate patches ($n = 3$ donors, shown on left) were embedded in resin, cross sectioned into $5\ \mu\text{m}$ thickness, and then stained with hematoxylin & eosin (A) or trichrome (B). Hematoxylin (purple) stains the cell nuclei, and eosin (pink) stains the protein. For trichrome staining, blue stain represents collagen, and red stain represents keratin and other fibrous proteins. Representative images are captured at low and high magnification (scale bar = $20\ \mu\text{m}$).

of the amniotic epithelial layer and dissolution of the connective tissue layers [46]. This prompted us to study the histological composition of the DBAM (Figure 1). Hematoxylin and eosin (Figure 1A) staining of the DBAM demonstrates that amniotic epithelial cell layers are partially intact and show clearly stained nuclei that face outward in the dual-layered amnion patch. Several studies have reported that the paracrine factors released from the amniotic epithelial cell have the potential to regenerate a wide range of tissues, including hepatic and cardiac tissues [47]. In addition to epithelial cells, the main fibrous skeleton of the AM is formed by a compact layer of stroma, followed by the fibroblastic layer containing mesenchymal cells, secreting different types of collagens. Collagen is well tolerated and bioabsorbable when administered. It has homeostatic properties and promotes migration of adjacent autogenous connective tissue and epithelial cells over its surface [48]. In this study, trichrome staining of the DBAM demonstrates that fibrous proteins such as collagen and keratin are present (Figure 1B). Hematoxylin and eosin, and trichrome staining therefore confirm that DBAM is completely free from chorion and has two AMs fused together with their respective epithelial cell layer facing outward. After assessment of the structural features of the binate amnion membrane, we determined if rehydration and homogenization of the DBAM in saline solution could release the cytokines and growth factors.

Table 1. Concentration of growth factor, chemokine and cytokine in the homogenized dehydrated binate amniotic membrane. The content of cytokines and growth factors in the Binate patch was analyzed by MAGPIX multiplexing system.

Cytokines/growth factors	Mean value in DBAM (pg/mg)	Values reported in literature (pg/mg)	Function	Ref.
G-CSF	134.85 ± 85.33	0–2	Stimulates the bone marrow to produce granulocytes and stem cells and release them into the bloodstream	[44]
VEGF- α	54.68 ± 9.20	0–94	Stimulates endothelial cell migration, proliferation and survival; potent stimulator of angiogenesis	[44]
EGF	50.11 ± 21.31	70–85, 8	Acts as a potent mitogenic factor that plays an important role in cell growth, proliferation and differentiation	[44,51]
SDF-1 α	11.97 ± 0.12	20–26	Plays a major role in cell trafficking and homing of CD34(+) stem cells that aid in regeneration	[44]
PDGF-BB	0.77 ± 0.16	22–142	Potent chemoattractant and activator of neutrophils and monocytes; increases the synthesis of phospholipids, cholesterol esters, glycogen and prostaglandins; and modulates LDL receptor binding	[44]
IL-8	0.75 ± 0.08	–	Chemotactic factor that attracts neutrophils, basophils and T cells, but not monocytes	–
TGF- α	0.44 ± 0.26	–	Activates a signaling pathway for cell proliferation, differentiation and development	–
IL-10	0.21 ± 0.02	0–2.18	An anti-inflammatory cytokine with pleiotropic immunoregulatory role	[44]

The Binate patch was homogenized in the PBS and concentrations for each factor were normalized to the dry weight of the patch. Data are represented as mean values ± standard error of the mean. N = 6. Cytokine concentration per mg of the dry weight of single layer amniotic membrane allografts as previously reported in literature is included.
DBAM: Dehydrated binate amniotic membrane; LDL: Low-density lipoprotein; pg: Picogram.

Cytokines & growth factors associated with DBAM

Cytokines and growth factors are key signaling molecules those can execute complex cellular processes such as migration, proliferation, differentiation and vascularization. To evaluate the biological efficacy of the DBAM, we first determined if there were any quantifiable amounts of the cytokines associated with DBAM. These factors were eluted by homogenization of the hydrated membrane in phosphate-buffered saline solution at neutral pH. Concentration of the growth factors and cytokines was determined by MagpixonTM multiplex assay. Concentrations of six representative growth/chemokine factors (G-CSF, VEGF- α , EGF, SDF-1 α , TGF- α and PDGF-BB) and two representative interleukins (IL6 and IL10) were determined in the homogenized samples and normalized to the initial dry weight of the DBAM. The EGF, TGF- α , PDGF-BB and VEGF are important contributors to wound healing and angiogenesis [40]. G-CSF promotes the mobilization and differentiation of stem cells, and anti-inflammatory interleukins modulate response to aid in pain relief [49,50]. Our Luminex-based assay was sensitive enough to detect concentrations (Table 1) ranging from 0.21 picograms (pg)/mg (IL10) to 134.85 pg/mg (G-CSF), extracted from the DBAM.

Amniotic allografts are often applied as a localized membrane barrier directly at the site of an injury, where relatively small concentrations of these growth factors and cytokines can significantly modulate the microenvironment to induce signaling pathways for the wound healing process. Research has shown that a combination of two or more growth factors/cytokines is merited over the use of a single component, as evident from the failure of a Phase III clinical trial of PDGF-BB formulation in the treatment of chronic diabetic foot ulcers [52,53]. Given the broad array of cytokines and growth factors associated with DBAM, it may provide a possible alternative to cytokine and growth factor-based therapy in promoting wound healing, in addition to its role as a scaffold and barrier. However, a mere presence of these growth factors and cytokines does not guarantee the biological efficacy of the product. Thus, to determine if the cytokines and growth factors eluted from the DBAM can induce biological effect, we subsequently treated the human bone marrow stromal cells and BM-MSCs with the homogenate obtained from the DBAM.

Effects of DBAM on cell proliferation

The proliferative or new tissue formation phase is critical for effective wound healing and tissue repair. This prompted us to determine the effects of DBAM on cell proliferation. Homogenized DBAM/Binate patch was used

Figure 2. Effects of the dehydrated binate amniotic membrane on cell proliferation *in vitro*. Bone marrow stromal cells (A) and bone marrow mesenchymal stem cells (B) were treated either with basal media (reduced growth factors) or with basal media supplemented with the homogenized extract of the Binate patch/dehydrated binate amniotic membrane (2 mg/ml) from three to five different donors. Cells growing in complete media (10% Fetal Bovine Serum; FBS) served as positive control. Relative proliferation was measured by fluorescence quantification of the total cellular DNA (CyQUANT assay) and is represented as fold change with respect to the growth observed in basal media after 48 h (left panel). Live cells were also stained with the cell-permeant Calcein-AM fluorescent dye and imaged at 40 $\times$ magnification to visualize the number of viable cells in randomly chosen fields. Representative images are shown. The scale bar represents 100 μm (right panel). Experiments were repeated thrice, at least in triplicates and $p < 0.05$ (*) was considered statistically significant.

to treat bone marrow stromal cells (HS5) and BM-MSCs (Figure 2). Both cell lines were grown in their respective basal media (reduced serum) for 48 h with or without supplementation of the DBAM homogenate (2 mg/ml). Cells grown in the complete media (10% fetal bovine serum) served as positive control for the experiment. Cell proliferation was monitored quantitatively and qualitatively using fluorometric reading of DNA concentration and Calcein-AM staining of viable cells, respectively. HS5 cells demonstrated a 3.6-fold increase in cell proliferation when compared with the cells maintained in basal media (negative control, Figure 2A). It was interesting to note that the effect of the DBAM was more pronounced, even when compared with that of the positive control. Similar effects of the DBAM homogenates on cell proliferation were observed in BM-MSCs, which reported an approximately 2.2-fold increase over the negative control (Figure 2B). Calcein-AM stained HS5 and BM-MSCs visualized under a fluorescence microscope (Figure 2A & B right-hand panels) revealed that viability was significantly higher in cells cultured in the DBAM homogenate supplemented basal media than in the nonsupplemented control. The spectrofluorometric data and micrograph clearly indicate that a 2 mg/ml concentration of DBAM homogenate was sufficient to support growth and promote proliferation of the stromal cells and mesenchymal stem cells derived from bone marrow. The proliferative effect of the DBAM homogenate was abolished when it was subjected to heat denaturation prior to treatment. In both the HS5 and BM-MSCs cell lines treated with the heat denatured DBAM homogenate, the rate of proliferation was comparable to that of the nonsupplemented control (Supplementary

Figure 1). Extreme heat denatures the thermolabile PDGF, EGF and G-CSF, which are potent regulators of cell proliferation, resulting in loss of the proliferative effect of DBAM homogenate. Therefore, the biological effect of the DBAM homogenate may be exerted by the growth factors and cytokines associated with the allograft. After establishing a positive effect of the DBAM homogenate on cell proliferation, it was imperative to study its role on cell migration, another important aspect of the wound healing process.

Effects of DBAM on cell migration

The repair process involves both cell migration and proliferation, the kinetic of these two mechanisms being extensively regulated by chemokines and other signaling molecules. Migration of cells to the site of the injury in response to physical and chemical cues in their microenvironment is strongly associated with cell proliferation [54]. Loss of contact inhibition and physical tension at cell adhesion structures trigger migration response and lead to activation of membrane-associated kinases. This pathway leads to increased membrane permeability for calcium [5]. Chemokine profiling of the DBAM homogenate has revealed the presence of VEGF- α , SDF-1 α , and IL6 and IL10 (Table 1) that promote cell migration and homing [5]. The envisaged liaison between chemokines associated with DBAM and their effect on cell migration was determined by modified Boyden chamber assay with HS5 and BM-MSCs (Figure 3). Cells were cultured overnight in the basal media (reduced growth factors) and then re-plated on the upper membrane of a cell culture transwell insert. After 18 h, cell migration toward the lower chamber of the transwell insert – which contained basal media supplemented with homogenized extracts of DBAM/Binate patch (2 mg/ml) – was 4.6-fold higher in HS5 and 3.3-fold higher in BM-MSCs, in comparison to migration toward the basal media alone. Cell migration in response to the complete media supplemented with 10% FBS served as a positive control for this assay (Figure 3).

Effects of DBAM on modulating secretory profiles

While DBAM positively regulates proliferation and migration of stromal and mesenchymal stem cells derived from bone marrow, it is also possible that the cytokines and growth factors in the DBAM homogenate can trigger signaling pathways that reprogram the transcriptional and translational landscape of the target cells. The cells stimulated by DBAM homogenate secreted a distinct subset of cytokines and growth factors. Such amplification of cell signal through a feedforward mechanism may be instrumental in reducing healing time, while maximizing regenerative potential. HS5 and BM-MSCs secrete detectable levels of cytokines and growth factors even under basal unstimulated condition. G-CSF and IL6 secreted by the HS5 cells can promote cell proliferation and differentiation of CD34⁺ cells. HS5 supports proliferation of hematopoietic progenitor cells when cocultured in serum-deprived media with no exogenous factors. Conditioned media derived from mesenchymal stem cells has been shown to stimulate the recruitment of bone marrow endothelial cells and stromal cells to promote angiogenesis, callus formation and fracture healing in a diabetic rat model [46]. We hypothesized that the BM-MSCs and HS5 cells when treated with the DBAM homogenate would be stimulated to secrete more growth factors and cytokines into the conditioned medium. The growth factors and cytokines secreted from the HS5 and BM-MSCs in response to the DBAM treatment were determined after normalization of the respective cytokines present in the DBAM homogenate used to stimulate the cells. We see a significant increase in production of the IL4, IL10, G-CSF, TGF- α and EGF by the HS5 conditioned with the DBAM/Binate patch extracts when compared with the unstimulated controls (Figure 4A). A similar stimulatory effect from the DBAM treatment was also evident in mesenchymal stem cells (Figure 4B), with significantly higher expression of SDF1- α , IL-4, IL-6, IL-8 and VEGF-A. Interestingly, for certain growth factors like G-CSF, TGF- α and EGF, synthesis was several folds higher in BM-MSCs conditioned with DBAM compared with nearly undetectable levels in the unstimulated cells. Expression of these cytokines can regulate tissue repair via several signaling pathways, such as Smad and extracellular signal-regulated kinase 1/2 pathways [1,55,56]. Thus, our data unequivocally establish that DBAM, in addition to upregulating cell proliferation and migration, also influence the signaling network at large by stimulating the cellular secretion of cytokines, which may have a sustained effect on tissue repair and wound healing.

Effect of storage temperature on DBAM

Evaluation of the DBAM establishes the importance of cytokines and growth factors that stimulate target cells to proliferate, migrate and induce the secretion of cytokines. However, the protein factors that are critical to the biological efficacy of the dehydrated patch are temperature sensitive. So, we evaluated the biological efficacy of DBAM/Binate patch that has been preconditioned for 48 h at different temperatures. Three sections of a single

Figure 3. Effects of the dehydrated binate amniotic membrane on cell migration *in vitro*. Bone marrow stromal cells (A) and bone marrow mesenchymal stem cells (B) were cultured overnight in basal media with reduced growth factors. Cells were re-plated in the upper chamber of a cell culture insert with permeable membrane. Basal media or basal media supplemented with homogenates of the Binate patch/dehydrated binate amniotic membrane (2 mg/ml) was added to the lower chamber of the insert. Following an incubation period of 18 h, the cells those migrated through the membrane were stained, imaged (right panel) and counted (left panel). Quantification of the images was performed by ImageJ (right panel). Scale bar = 200 μ m. Experiments were repeated thrice in triplicates and $p < 0.05$ (*) was considered statistically significant.

patch (donor) were preconditioned at -20°C , 37°C and 65°C . We chose to measure two representative cytokines and one representative biological function (cell proliferation). Tm values are known to correlate with chemical degradation kinetics for wild-type G-CSF [57]. G-CSF was also present in significant concentration (Table 1) and is responsible for multiple biological functions including cell proliferation and angiogenesis. So, we were interested to know if G-CSF concentration is impacted by thermal preconditioning. Additionally, there are previous reports on the effect of storage temperatures on interleukins and Luminex analyses of the frozen and refrigerated pairs exhibited no significant differences in levels of IL-4 when measured simultaneously [58]. Therefore, IL-4 was a good candidate to study the effect of thermal preconditioning along with G-CSF. No significant variation in cytokine concentration was noted in the homogenates extracted from the DBAM sections subjected to different temperatures (Figure 5A). We further evaluated the influence of the temperature on the biological efficacy of DBAM. We performed our cell proliferation assay with the same homogenates that were used to determine the cytokine concentration. Cells were treated with the homogenate obtained from the patch sections preconditioned at different temperatures. However, homogenates from the preconditioned patch were equally effective in inducing cell proliferation in HS5

Figure 4. Quantification of the cytokines secreted in response to dehydrated binate amniotic membrane treatment. Bone marrow stromal cells (A) and bone marrow mesenchymal stem cells (B) were treated for 36 h with basal media or basal media supplemented with Binate patch/dehydrated binate amniotic membrane homogenate (2 mg/ml). Concentration of the secreted cytokines was determined by Magpix™ multiplex analyzer and reported as picograms per milliliter, after accounting for the net concentration of those factors in the initial homogenate and normalizing to the cell proliferative effect over 48 h. Experiments were repeated thrice in duplicates and $p < 0.05$ (*) was considered statistically significant.

and BM-MSCs cells (Figure 5B) compared with the untreated control cells. Our data indicate that the binate amnion patch maintains its stability at a flexible range of temperatures that prolongs its shelf life.

Figure 5. The biological activity of the dehydrated binate amniotic membrane subjected to thermal preconditioning. Biological activity of DBAM in terms of cytokine content (A) and effect on cellular proliferation rate (B) of bone marrow stromal cells and bone marrow mesenchymal stem cells were quantified by Magpix™ multiplexing system and CyQUANT assay, respectively. DBAM patch obtained from the same donor was conditioned at three different indicated temperatures for 48 h. DBAM was homogenized as described previously and the extracts were used for cytokine quantification or for the cell proliferation assay. The cells were cultured for 48 h in the absence or presence of 2 mg/ml homogenized extract of Binate patch/DBAM to determine cell proliferation. Data are presented as fold change $\pm$ standard deviation relative to basal media (reduced growth factors). Experiments are performed in triplicates with N = 3 donors, and $p < 0.05$ (*) was considered statistically significant.

DBAM: Dehydrated binate amniotic membrane; MSC: Mesenchymal stem cell.

Discussion

Biomechanical and biochemical studies provide ample evidence that the placental membrane functions as an optimal scaffold, as well as a barrier for reducing fibrosis and improving healing rate for chronic wounds [59–61]. Placental membranes currently available for clinical applications significantly vary in their method of preparation, preservation and storage, ranging from cryopreserved or freeze dried to completely dehydrated allografts. Even the dehydrated placental grafts available from various vendors differ largely in their structural composition, orientation of membrane constituents and overall processing, all of which deeply impact their mechanism of action in tissue regeneration and wound healing. Biochemical studies on allografts with amnion-chorion layers, single-layered amnion or the dual AM with the epithelial layer buried inside [38,51,62] have been previously conducted to determine their relative cytokine concentration, assess their biological function and to attribute a structure–function relationship to these allografts [44].

The AM is approximately 20% of the thickness of the amnion/chorion composite layer, but dominates the mechanical responses of the bilayer, with both stiffness and strength being ten-times greater than the chorion layer [63]. Hence, it is the AM that contributes to the strength of the amnion-chorion composite layer. Increased stiffness enhances the scaffold strength of amnion, which is an important consideration in tissue engineering [64]. Histopathological analysis has revealed that re-epithelialization of the wounds occurs preferentially on the basement/epithelial side of the AM [65]. In ophthalmic model, Hao *et al* has suggested that AM applied with the epithelial cell surface in direct contact with the wound can deliver a higher concentration of regenerative factors to the damaged tissue surface [66]. Research has shown that even when the cell viability of the amnion layer is compromised because of dehydration, the growth factors and cytokines remain associated with the membrane and sterilization with γ rays does not have any significant negative effect on their content [46]. It has been reported that removal of the epithelium eliminated nearly all important growth factors [67] and the native, intact AM contains higher levels of EGF, KGF, HGF and bFGF compared with epithelium denuded AM [68]. Therefore, several lines of research have established that amniotic epithelium contains cytokines that play a crucial role in the microenvironmental niche of progenitor cells. To combine the merits of the higher tensile strength of amnion tissue compared with chorion and to exploit the beneficial effects of the epithelial surface in wound healing, we designed a dehydrated, pure binate AM (DBAM), with the amniotic epithelial cells facing outward (Figure 1). This binate design also eliminated the need to determine orientation of the allograft during clinical applications.

Several cytokines including TNF- α , NGF, brain-derived neurotrophic factor (BDNF), noggin and activin are known to be secreted from amniotic epithelial cells [69,70]. Previous research that specifically compared between the single- and double-layered (epithelial cells facing inward) amnion grafts processed by the same manufacturer showed that the cytokine concentrations were generally higher in the dual-layer grafts, with certain cytokines like EGF and TGF- β enriched by nearly fivefold compared with the single-layered amnion graft [44]. Dual-layered amniotic graft accounted for only 40–50% of the overall cytokines in comparison to the amnion/chorion composite layer grafts. In contrast, EGF and bFGF were significantly higher in the dual amnion grafts compared with the composite grafts [44]. This study was conducted with amniotic grafts obtained from one to two donors, and cytokine concentrations were presented mostly as relative rather than absolute values, making it difficult to compare the previous findings with our current study, which uses a binate AM with the epithelial cell layer exposed rather than being buried inside. Nevertheless, these data reflect that the profile and concentration of cytokines associated with the chorion and amnion layers do not necessarily correlate to the relative thickness of these layers, but also depend on additional factors like the inherent cellular properties and composition of these layers. Eight different cytokines with direct relevance to wound healing were quantified in the DBAM (Table 1). While a direct comparative analysis of dual amnion allografts with epithelial cell layers either buried or exposed would have made this study more comprehensive, values derived from the published literature on similar AMs show that DBAM is significantly enriched with certain cytokines like G-CSF, but low in others like PDGF-BB (Table 1).

The mere presence of a cytokine or growth factor cannot be used as evidence of its bioavailability and bioactivity after clinical application. Based on the *in vitro* model system, we can only infer a plausible mechanism of action of the DBAM in wound healing. However, the delicate balance of the pro- and antifactors, and the complicated interplay among these factors, will ultimately determine the potential for wound healing. A more in-depth study needs to be conducted to garner a deeper understanding of the mechanistic action of DBAM.

The results from *in vitro* proliferation and migration studies support the biological efficacy of DBAM homogenates (Figures 2 & 3). Abolition of the proliferative effect of the homogenate upon heat denaturation (Supplementary Figure 1) can be attributed to the involvement of the heat labile components of the DBAM, including cytokines and growth factors, in regulating various cellular processes. These cytokines and growth factors may initiate a feedforward response, thus significantly altering the molecular milieu of the tissue microenvironment and tipping the balance in favor of rapid wound healing. We have carefully chosen two representative model cell systems: effector bone marrow stromal cells and progenitor bone marrow-derived primary mesenchymal stem cells. Both cell lines participate in regeneration and wound healing [71]. In addition to the first tier of response by stromal cells and BM-MSCs, we have demonstrated that bone marrow stromal cells and BM-MSCs are stimulated upon DBAM homogenate pretreatment to secrete more cytokines and growth factors (Figure 4), providing the second tier of response. This represents an ongoing multidirectional communication, both primary and secondary, with several effector cells playing vital roles in mediating the process of wound healing. This finding is in tune with previous reports by Alcaraz *et al.* who have shown in a chronic wound model using keratinocytes how the AM influences cell signaling and genetic programs to antagonize TGF- β -mediated pathways that negatively impact epithelialization of the wound [72]. AM favored proliferation and migration of keratinocytes that could allow chronic wounds to recover from their nonhealing state and progress into epithelialization [73].

While cryopreservation is generally considered a better method to maintain the structural and functional properties of the biological tissues, the logistical advantage of the dehydrated membrane is notable, as it reduces transportation and storage cost and ensures a wider reach, especially effective in developing countries and hospitals with a remote setup. Evidence suggests that even the dehydrated AM has distinct advantageous in several clinical conditions [22,74]. Moreover, meticulous optimization of the manufacturing process can lead to preservation of biochemical properties at par with the traditionally cryopreserved membrane [39]. We demonstrate that careful processing of the DBAM retained its biological efficiency even when preconditioned at temperatures between -20 and 65°C (Figure 5).

Despite being one of the oldest natural scaffolds used for regenerative tissue engineering and having a vast literature on the use of the AM, there are several issues like donor variability and well-defined application criteria that need to be addressed in future. Although there are multiple clinical studies underway, it is critical that the outcome data are published to further support the use of these products in different wound healing environments. These studies will give us further insight in establishing and optimizing clinical applications and understand the impact that donor variability may have on clinical outcomes. Clinical studies with proper and adequate controls will

be conducted with the DBAM in various fields, including chronic diabetic and venous ulcers, severe ophthalmic conditions and burn injuries.

Conclusion

Binate AM with epithelial layers facing outward presents two distinct benefits: high tensile strength and direct contact of wound to the epithelial cell surface. Our study indicates that the cytokines and growth factors present in the native AM were preserved in the dehydrated placental membrane. Using *in vitro* model system, we provided insight into the possible mechanism of DBAM, which justifies its current clinical applications, especially related to wound healing. Multicenter, prospective, randomized and comparative trials should be considered in future to further advance our understanding on the unique biological properties of the amniotic allografts, and its structure–function relationship in wound healing and tissue regeneration.

Future perspective

Placental membrane draws great interest as a source of tissue allografts for regenerative medicine. Several clinical studies reinforce the advantages of using AM allografts as a biological dressing to achieve scar-free wound healing. However, it is interesting to note that the composition (amnion and/or chorion), architecture and processing technique used for manufacturing these allografts significantly impact its molecular composition and thus its biological efficacy. Therefore, tissue allografts including amnion preparations should be evaluated in different *in vitro* model systems to understand the precise mechanism of action. The importance of understanding the correlation of histological, biomechanical and biochemical composition of a Binate patch to the application criteria is paramount in advancing the current dogma of why these placental allografts have reported broadly effective clinical outcomes. With this insight, more opportunity exists for application and will undoubtedly provide both the translational researchers and clinical communities with better tools to address complex health issues. We demonstrated that the binate amnion allografts uniquely folded with epithelial cell layer facing outward have higher cytokine content. These cytokines are readily bioavailable, bioactive and stimulate physiological processes associated with wound healing. Thus, apart from being used as a surgical dressing, AM products with such unique architecture should be further explored as an adjunct treatment for large wounds, burn, skin damage and ocular tissue damage surgeries to promote tissue regeneration.

Summary points

- Placenta has long been known for its regenerative properties. However, with improvement in processing technologies, the dehydrated amniotic membrane is now being utilized in clinical applications for treating chronic wounds like venous and diabetic ulcers.
- Previous research on dehydrated placental membranes has been restricted to the products derived from composite tissue of the placenta comprised of the inner layer of amnion cells fixed to collagen-rich mesenchymal cells of the chorion; single layer of amnion/chorion; or dual amniotic layer with intact epithelial surfaces facing inward.
- Here we investigated a uniquely processed dehydrated membrane composed of two amnion layers (binate), with the partially disrupted epithelial cell surfaces facing outward. It was hypothesized that this unique structural architecture of the dehydrated binate amniotic membrane (DBAM) would increase bioavailability of the cytokines and contribute to the regenerative potential.
- Histochemical staining confirmed the unique structural organization of the DBAM. Important growth factors and cytokines were identified in quantifiable amounts in the homogenate derived from DBAM. The cytokines retained biological activity as the DBAM homogenate could significantly induce cell proliferation and migration, as well as bolster the secretory activity of the mesenchymal stem and stromal cells. DBAM stored at -20 and 65°C retained its biological activity.
- The data conclusively established that the cytokines and growth factors in DBAM are thermostable, bioactive and bioavailable to the host cells. These cytokines participate directly and indirectly in the process of wound healing. The mechanistic insight gained from this study will help to better interpret the clinical efficacy of this DBAM.
- Future investigations should include comparative analysis of similar products to decipher the roles of structural organization and biological function of the amnion and/or chorion layers for tissue regeneration and wound healing. Prospective and randomized trials must also be conducted to expand the scope of clinical application of the DBAM through informed outreach.

Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/rme-2018-0085

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Ethical conduct of research

Tissue was procured from consenting adults and manufactured in the US FDA-registered facility following all necessary regulatory guidelines as per the FDA's Good Tissue Practice.

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