Standardized reporting of amnion and amnion/chorion allograft data for wound care

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Abstract

Background: The favorable biological and mechanical properties of the most common components of the placenta, the amnion and chorion, have been explored for regenerative medical indications. The use of the combination of amnion and chorion has also become very popular. But, published data from placental tissues in their final, useable form is lacking. During treatment with membrane product, the tissue is usually sterile, intact and laid on a wound or treatment area. The factors available to the treatment area from the applied product need to be elucidated and presented in a relatable form. Current reporting for eluted growth factor results are typically expressed per milliliter, which is not informative with respect to the area of tissue covered by the actual membrane and may differ among techniques.

Methods: To address this inconsistency, amnion or amnion/chorion were isolated from human placentas and processed by a proprietary procedure. The final dry, sterilized product was evaluated for structural components and growth factor elution. Growth factors were quantified by multiplex panels and ELISAs and the values normalized to specific area and elution volume of finished product. This information allows extrapolation to all membrane sizes and affords cross-study comparisons.

Results: Analysis of membrane supernatants show that dehydrated, sterilized amnion and amnion/chorion elute factors that are conducive to wound healing, which are available to recipient tissues. Importantly, these measurable factors eluted from dehydrated, sterilized membranes can be reported as a function of available factors per square centimeter of tissue.

Conclusions: The standardized characterization of dehydrated, sterilized amnion and amnion/chorion as delivered to recipient tissues permits understanding and comparison of the products across various graft sizes, types, and eluate volumes. Further, reporting this data as a function of cm^2 of dehydrated tissue allows extrapolation by independent scientists and clinicians.

KEYWORDS
amnion, chorion, dehydrated, growth factor, placenta

1 | INTRODUCTION

The medical benefits of placental membranes have become increasingly apparent over the past several years. A variety of membrane products and combinations have been developed for applications ranging from cosmetic to invasive surgery. This is not surprising for the many established beneficial attributes characterized for multiple membrane products. Some are ideal for protection from external insults (e.g., contamination) while others provide an extensive array of growth factors. Although there is a vast array of information collected on amnion and amnion/chorion membranes, most reports are collected from fresh or cryopreserved tissues. Unprocessed tissue may be more advantageous to utilize, but unprocessed tissue cannot be used for clinical application due to transmittable disease risk and storage time before end use. Hence, not currently Food and Drug Administration (FDA) cleared for use. Cryopreserved tissue is stored in and absorbs cryopreservant, usually dimethyl sulfoxide, which is accepted, but not desirable, for human applications. The few studies conducted on dehydrated or freeze-dried membranes utilize rehydrated, macerated, and/or centrifuged extracts. In the course of treatment with membrane product, the tissue is usually sterile, intact, and laid on a wound or treatment area. The factors present which are available to the treatment area from the applied product need to be elucidated and presented. We report here the properties of dehydrated, sterilized amnion (dsAM) and amnion/chorion (dsAC) membranes standardized to square centimeter of tissue and available to the recipient. This information adds to the ever-expanding understanding of why dehydrated placental membranes are efficacious and presents a quantitative aspect to the reporting that may be extrapolated for both use and comparison.

The placenta is the organ that develops in the uterus shortly after implantation of a fertilized egg, attaching the egg to the wall of the uterus.[1] The placenta is composed of parenchyma, chorion, amnion, and umbilical cord which connects to the fetus. During fetal development, the amnion and chorion (AC) come together to form the chorio-amniotic membrane that encases the amniotic fluid and the fetus. The most common components of the placenta under exploration for regenerative use are the amnion, chorion, amniotic fluid, and the umbilical cord.

The fetal membrane (amniotic sac) full thickness measures approximately 0.25 mm at term. This includes the layers of the innermost amnion and layers of the outer chorion. The amnion is between 0.02 and 0.05 mm thick. It is avascular with no nerve or lymph. Hence, it receives nutrients through diffusion. The amnion is in direct contact with the amniotic fluid. Amnion has three layers: the epithelial layer, the thick basement membrane, and the avascular mesenchymal tissue. These contain type I and type III–VII collagen and high concentrations of proteoglycans and glycoproteins along with fibronectin and laminin. There are two main cell types in amnion: amniotic epithelial cells and amniotic mononuclear mesenchymal cells. The functions of amniotic membrane include physical protection of the fetus, protection from bacterial infection, regulation of pH, and secreting growth factors and other molecules. These serve antimicrobial and anti-inflammatory functions.

John Davis was the first to report (1910) the use of amnion membrane as a surgical material in skin transplantation. He showed that in skin grafting, amniotic membrane resulted in better outcomes than xenograft or cadaveric dressings.[2] Since then, human amniotic membrane has been used widely in regenerative medicine due to continued discovery of its favorable biological and mechanical properties.[3–6] Numerous applications benefit from these properties. For example, amnion membrane for management and treatment of skin burns is well documented[7–17] and has been utilized in over 200 clinical trials. Similarly, amniotic membrane has shown advantageous outcomes when used for chronic wounds,[18–20] urinary system issues,[21,22] dental and oral treatments,[23–25] ophthalmic indications,[26–32] and orthopedic treatments.[33–35] Many other applications for amniotic membrane have been studied. Of particular interest is its documented antimicrobial activity.[36–39]

The chorion is the outer layer in contact with the mother's cells. It forms tail-like structures called the chorionic villi which provide blood and nutrients from the mother to the baby. The AC are separated by a jellylike matrix (chorionic fluid) until the two membranes "fuse" at 11–13 weeks postconception. The chorion is 3–4 times thicker than the amnion and has three layers: the intermediate/reticular layer (contacts the amnion), the basement membrane, and the trophoblast layer. The reticular layer contains type I, type III, type IV, type V, and type VI collagen.[40] The basement membrane contains type IV collagen along with fibronectin and laminin.[41] Chorion by itself is not commonly used in medicine for regenerative applications. But the combination of AC has become very popular. When AC are used together, the chorion, which is thicker, is reported to be responsible for 75% of the growth factors present.[42] Various preparations focus on exploiting the available growth factors and adding strength to AC. These include lamination,[43] cryopreservation,[44,45] dehydration,[43] and freeze drying.[46] Each processing and preservation method results in the optimization of different properties of the membrane. Some focus on retaining cellular viability[45] while others focus on providing an optimal substrate for protection and endogenous cell adhesion. Still others prefer to provide natural growth factors.

Like AM, AC in various forms have been used for numerous applications. The primary application to date is for wound healing.[43,45,47] For example, in 2015 Zelen et al.[48] reported that use of laminated dehydrated human AC resulted in better and faster healing in wound care compared to other products. Other reports include AC in dental and oral treatments,[46,49,50] urogenital system issues,[51–53] and orthopedic treatments.[54,55] As seen with AM, there is documented antimicrobial activity from AC.[56,57]

Characterization of AM and AC in its native state is reported. But this information does not properly describe the membranes as delivered products for medical use. As mentioned previously, the processing method has a substantial effect on the properties of final product membranes. Furthermore, the methods of characterization may not be appropriate for the indicated end use. For example, using the digested, preprocessed membrane to release all proteins gives us a clear look at the potential of the membrane, but does not allow us

to determine the factors available to a patient. In addition, grinding or homogenizing a membrane that is ready for use, to extract all factors present for analysis does not clearly indicate what would be released when the membrane is placed intact on a wound. Finally, growth factor results are typically expressed per milliliter, which is not informative with respect to the actual membrane. To address this lack of information, we characterized dehydrated AM (dsAM) and dehydrated AC (dsAC) from the perspective of factors eluted from a specific area (per square centimeter) of finished product, as would be eluted to patient tissue. This information can be applied to all sizes and compared to all other data produced in this manner. We additionally present the histological properties of dsAM and dsAC and the localization of some structural proteins.

2 | MATERIALS AND METHODS

2.1 | Amnion and amnion with chorion

Donated human placentas were acquired from accredited Gift of Life tissue recovery agencies (e.g., Telegen) after planned cesarean sections with informed consent. All donations and processing were completed in accordance with FDA Good Tissue Practices and American Association of Tissue Banks standards. Donors were screened for medical issues, social issues, and communicable diseases, as well as infectious diseases, including human immuno-deficiency virus, human T-lymphotropic virus, hepatitis B and C, syphilis, and cytomegalovirus. Additionally, grafts are terminally sterilized by electron beam sterilization (Steri-tek).

Amnion or amnion with chorion were isolated from the placenta and processed with a proprietary procedure at BioStem Technologies® which involves several gentle cleaning stages followed by dehydration under specific drying conditions. The resulting dehydrated amniotic membrane (Vendaje®; Biostem Technologies) or dehydrated amnion/chorion (Vendaje AC®; Biostem Technologies) was cut to preferred sizes, packaged, and sent out for E-beam sterilization. The final sterilized products were used for all tests. Dehydrated amnion/chorion is processed intact without separation of layers.

2.2 | Histology

Histology was performed by HistoWiz Inc. (histowiz.com) using a Standard Operating Procedure and fully automated workflow. Samples were processed, embedded in paraffin, and sectioned at 4 µm. Immunohistochemistry was performed on a Bond Rx auto-stainer (Leica Biosystems) with enzyme treatment (1:1000) using standard protocols. Antibodies used were rat monoclonal F4/80 primary antibody (eBioscience; 14-4801; 1:200) and rabbit anti-rat secondary (Vector; 1:100). Bond Polymer Refine Detection (Leica Biosystems) was used according to the manufacturer's protocol. After staining, sections were dehydrated and film coverslipped using a

TissueTek-Prisma and Coverslipper (Sakura). Whole slide scanning (x40) was performed on an Aperio AT2 (Leica Biosystems). Four separate lots of dehydrated amniotic membrane and four separate lots of dehydrated amnion/chorion were sent in biopsy cages with 50% paraformaldehyde/PBS. Stains include H&E for structure and nuclei, Van Gieson for elastin and collagen, Alcian blue for polysaccharides, and immunohistochemistry for collagen I, collagen III, fibronectin, and laminin.

2.3 | Growth factors

To determine the elution of growth factors from the membranes, 8 mm biopsy punches from five separate lots of dehydrated, sterilized amniotic membrane (dsAM) and dehydrated, sterilized amnion/chorion (dsAC) were placed in 500 µl DPBS at 37°C for 24 h. For the hyaluronic acid (HA) assay, dsAM, and dsAC were placed in 1 ml DPBS at 37°C for 3 days. The supernatant was collected and stored at -80°C until use. A 30-plex cytokine assay (Bio-Plex Pro; Bio-Rad) was performed on three separate lots of dsAM and dsAC. The growth factors included in the 30-plex panel are listed in Table 1. A custom 5-plex panel (Bio-Plex Pro; Bio-Rad) was designed to determine the concentration of hepatocyte growth factor (HGF), IL-1 receptor antagonist (IL-1ra), basic fibroblast growth factor (bFGF), interleukin 10 (IL-10), and platelet derived growth factor B homodimer (PDGF-BB). Assays were performed with a wash station and analyzed on a Bio-Plex 200 multiplex system (Bio-Rad) with high-throughput fluids. All samples were run in duplicate. Results were obtained using 5-parameter logistic standard curves and a plate blank. Vascular endothelial growth factor receptor 1 (VEGFR1) was assessed with the Human VEGFR1 ELISA Kit (Invitrogen #BMS268-3). HA was assessed with the Hyaluronan Immunoassay (R&D Systems #DHYALO) and vascular endothelial growth factor (VEGF) was assessed with the Human VEGF ELISA Kit (Invitrogen #KHG0111). All ELISAs were calculated from a 4-parameter logistic standard curve.

2.4 | Calculation

The 8 mm punches have an area of 0.503 cm.^{2} The average pg/ml of growth factor was multiplied by the milliliters of eluate. This total GF content in the eluate was divided by 0.503 to reveal the pg of growth factor in 1 cm^2 of tissue. This can be utilized for any volume of eluate used and any sample punch size.

$$\frac{\text{GF}\left(\frac{\text{pg}}{\text{ml}}\right) \times \text{ml}}{0.503\ sqcm} = \text{pg of growth factor per cm}^2.$$

2.5 | Statistical analysis

For protein experiments a two-tailed, unpaired Student's t-test was used with $p < 0.05$ considered significant (Prism; GraphPad).

TABLE 1 Cytokines included in the 30-plex cytokine assay (Bio-Plex Pro)

Cytokine/ chemokine	Name (also called)
EGF	Epidermal growth factor
Eotaxin	
FGF2	Basic fibroblast growth factor (bFGF)
G-CSF	Granulocyte colony-stimulating factor (CSF3)
GM-CSF	Granulocyte-macrophage colony-stimulating factor (CSF2)
HGF	Hepatocyte growth factor (SF)
IFNα	Interferon alpha
IFNγ	Interferon gamma
IL-1β	Interleukin 1 beta
IL-1RA	Interleukin-1 receptor antagonist
IL-2	Interleukin 2
IL-2R	Interleukin-2 receptor
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-7	Interleukin 7
IL-8	Interleukin 8
IL-10	interleukin 10 (CSIF)
IL-12/IL-23p40	Interleukin 12
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-17a	Interleukin 17
MCP-1	Monocyte chemoattractant protein-1
TNF-α	Tumor necrosis factor alpha (cachexin)
VEGF	Vascular endothelial growth factor
CCL3	Macrophage inflammatory protein-1α (MIP-1α)
CCL4	Macrophage inflammatory protein-1β (MIP-1β)
CCL5	Regulated on activation, normal T-cell expressed, and secreted (RANTES)
CXCL9	Monokine induced by gamma interferon (MIG)
CXCL10	Interferon gamma-induced protein 10 (IP-10)

The initial 30-plex analysis had a sample size of 3, hence was reported as mean with standard deviation (s.d.) directly from the Bioplex Manager software. Subsequent analysis' had sample sizes of 5, permitting us to utilize mean with standard error of the mean (SEM). Use of s.d. or SEM is indicated on all figures.

3 | RESULTS

To address accurate quantification of factors eluted from dehydrated, sterile amniotic membrane (dsAM), and amnion/chorion membrane (dsAC) to patient tissue, we characterized dsAM and dsAC from the perspective of factors eluted from a finished product. Two multiplex panels and several individual ELISAs were used on membrane supernatants. For verification that processing and sterilization had not adversely affected the structural integrity of the membranes, we assessed the histological properties of dsAM and dsAC in their final state.

3.1 | Histology and localization of structural factors

Since one of the main benefits of using placental tissues for healing and regeneration is the existence of numerous structural components that promote endogenous cell migration and proliferation, total glycosaminoglycans (GAGs) were visualized with Alcian blue, elastin and total collagens with Van Gieson, and nuclei against extracellular matrix (ECM) by Hematoxylin and eosin (H&E) staining. H&E staining clearly shows the retention of structure for dsAM (Figure 1A) and dsAC (Figure 1B) after dehydration and sterilization. Alcian Blue staining shows GAGs distributed evenly below the basement membrane in dsAM (Figure 1C) and strongly between the two basement membranes in dsAC (Figure 1D), also strongly evident in the lower portion of the chorion. Van Gieson indicates collagen distribution throughout the dsAM (Figure 1E) with elastic fibers distributed sparsely. In the dsAC (Figure 1F) collagen is also seen throughout and elastin more strongly dispersed below the chorionic basement membrane.

We then evaluated the localization of fibronectin, collagen I, collagen III, and laminin. There was a robust overall distribution of fibronectin in dsAM (Figure 2A) and dsAC (Figure 2B) and its presumed binding to other ECM proteins (e.g., collagen). Although expressed throughout, collagen I was strongly expressed in the dsAM (Figure 2C) below the basement membrane, and in intermediate layer of the dsAC (Figure 2D). Less intense expression was also seen throughout the chorion below the trophoblast layer. Collagen III was lightly expressed in the dsAM with the strongest expression in the intermediate layer of the dsAM (Figure 2E) and dsAC (Figure 2F) along the connective tissue tendrils. There was minimal expression of laminin in the dsAM (Figure 2G) which may be an effect of the processing. In contrast, laminin was expressed throughout the dsAC (Figure 2H) with even distribution in the compact layer of the amnion. It is conceivable that keeping the layers together during processing protects the laminin within the tissue.

3.2 | Growth factors

It is widely accepted that fresh amniotic membrane and chorion are rich in growth factors. Furthermore, it is known that processed and
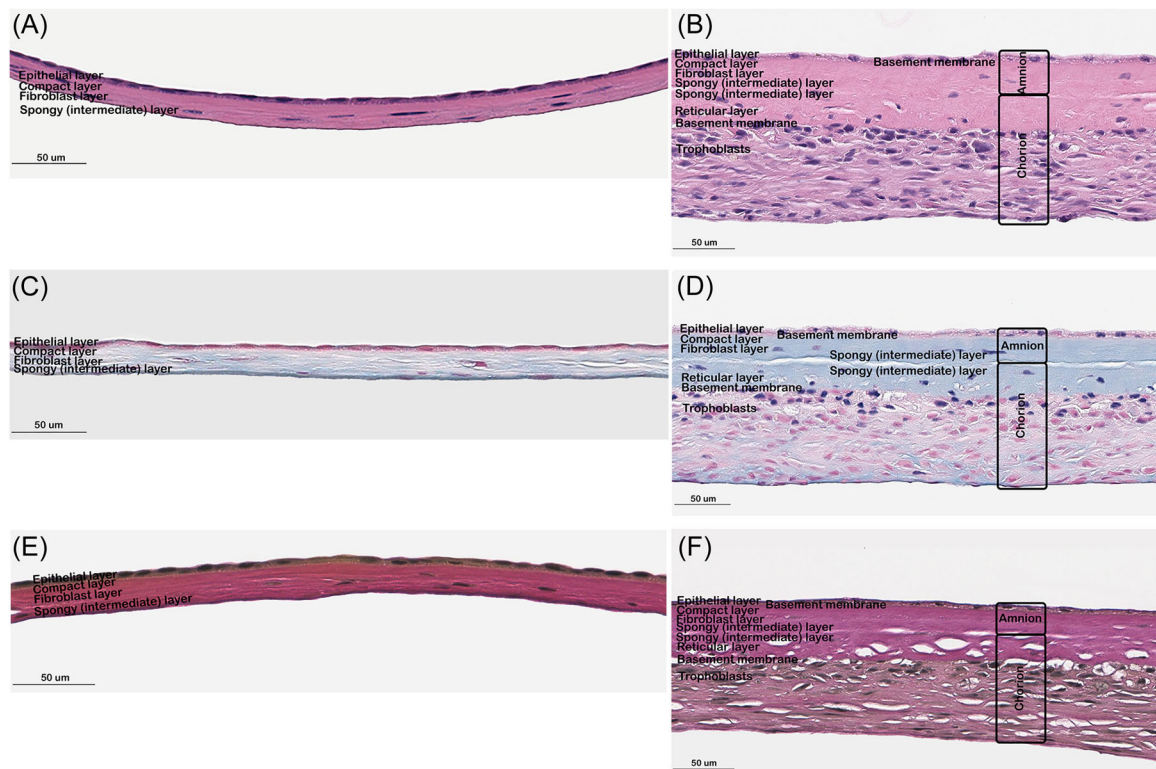
FIGURE 1 Staining of dsAM and dsAC show retention of structural components after dehydration and sterilization. H&E staining of (A) dsAM and (B) dsAC shows the extracellular matrix as pink with purple nuclei. Alcian blue staining on (C) dsAM and (D) dsAC shows localization of glycosaminoglycans apically between the basement membranes. Van Gieson staining on (E) dsAM and (F) dsAC shows strong pink total collagen between the basement membranes with intermittent elastin located in the trophoblast layer. The yellow cytoplasm can be seen in the dsAM slide between the epithelial cells. dsAC, dehydrated AC; dsAM, dehydrated AM; H&E, hematoxylin and eosin.

dehydrated placental tissues retain much of these growth factors. What is not known is the growth factors that are available to the tissue when applied to the patient. Furthermore, processed and dehydrated AC, which has not been separated, has not been reported previously. By utilizing the eluate from dsAM and dsAC, we determined what factors are delivered to patient tissue upon application of these placental membranes. Our initial study tested a broad range of cytokines for the purpose of highlighting highly expressed factors. For this, a 30-plex analysis was performed that included desired and undesired factors. Of the 30 cytokines tested, the most highly expressed factors in dsAM (Figure 3A) were HGF and IL-1ra (200.74 and 56.17 pg/cm,^{2} respectively) and dsAC (Figure 3B) highly expressed IL-1ra and CXCL8 (680.48 and 206.04 pg/cm,^{2} respectively). Results were calculated and reported by BioPlex Manager software as average of 3 lots with standard deviation. Notably, many inflammatory factors were not detectable in dsAM or dsAC. (Figure 3A,B) These include IFNa, IFNg, IL-15, IL-17a, IL-5, IL-6, GM-CSF, IL-12, CXCL10, and TNFa.

Based on these results, we tested five growth factors (HGF, IL-1ra, bFGF, IL-10, and PDGF-BB) with a more specific 5-plex. We further tested for bFGF and IL-10, even though they were not significantly expressed, because they have immunoregulatory and anti-inflammatory properties which could provide a substantial effect at low concentration on regeneration and wound healing. The sample

size was increased to 5 lots for increased accuracy and calculation. This provided a sample size appropriate for additional error calculation. Raw results were collected by BioPlex Manager software and the standard deviation was divided by the square root of the sample size for SEM. The 5-plex confirmed strong elution of IL-1ra and HGF from dsAM (Figure 4A) and dsAC (Figure 4B), with absence of IL-10. In addition, due to the increased specificity and reduced cross-reactivity of using only five antibodies, we obtained consistent results that shed light on the variability between the five donors. We identified that PGDF-BB and FGF-2 were also expressed in dsAM and dsAC (Figure 4A,B). To expand on the available factors eluted from these membranes, we tested the eluate for VEGF, VEGFR1, and HA separately from five donors by ELISA. VEGF was not detectable in dsAM (Figure 5A) or dsAC (Figure 5B). VEGFR1 was eluted from dsAM (Figure 5A) and, to a much greater extent, from dsAC (Figure 5B). In addition, HA was highly expressed by both dsAM (Figure 5C) and dsAC (Figure 5D).

4 | DISCUSSION

Published data from placental tissues in their final, useable form is lacking. Additionally, transferrable data that may be used for calculation by others in understanding the true composition of birth
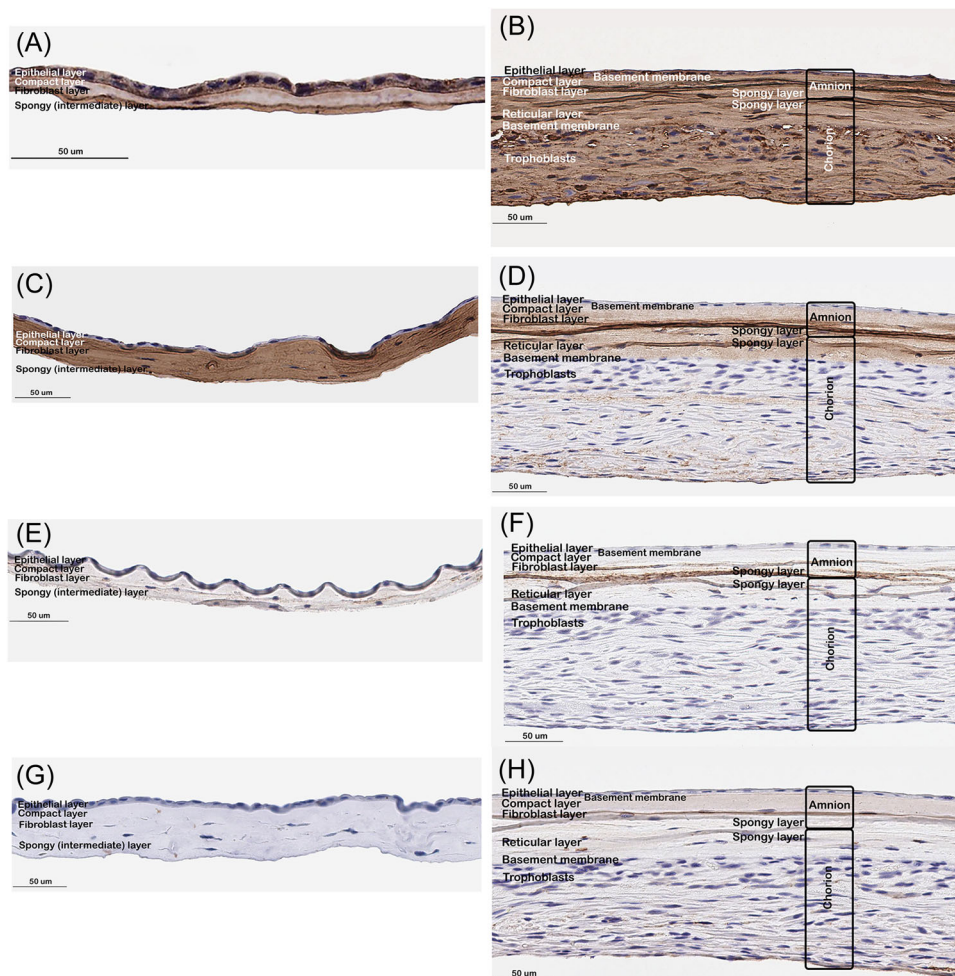
FIGURE 2 Fibronectin, collagen, and laminin are differentially localized in dsAM and dsAC. Antibody-specific staining of dsAM and dsAC: Brown fibronectin staining on (A) dsAM and (B) dsAC shows generalized localization of fibronectin with higher concentrations around collagen fibers. Brown collagen I staining on (C) dsAM and (D) dsAC shows localization of collagen I between the basement membranes with strong staining in the intermediate spongy layer. Brown collagen III staining on (E) dsAM and (F) dsAC reveals expression in the intermediate spongy layer. (G) Brown laminin staining on dsAC shows expression throughout with stronger staining in the compact layer of the amnion, and along the fibroblast layer. dsAC, dehydrated AC; dsAM, dehydrated AM.
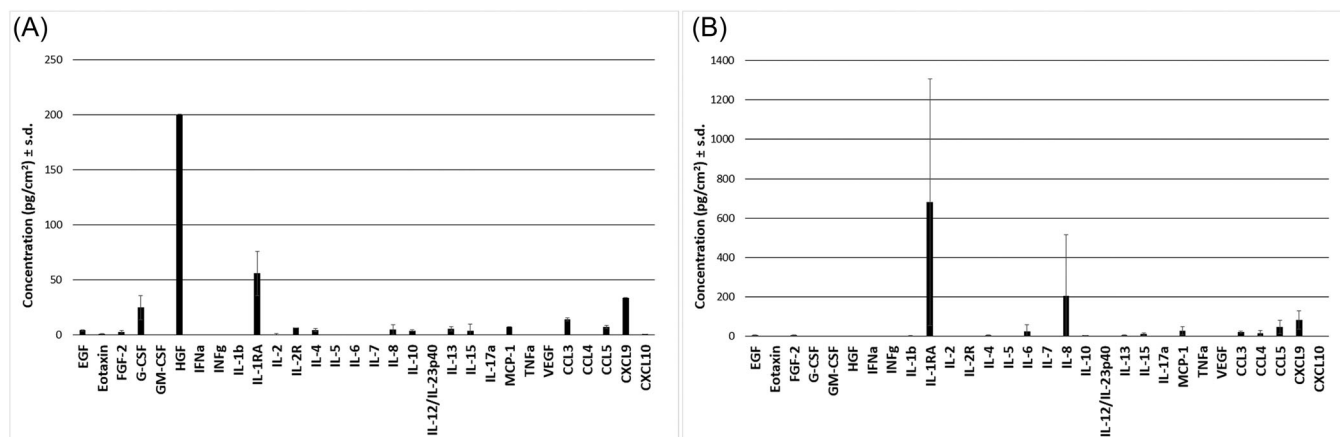

FIGURE 3 Multiplex analysis of cytokines and growth factors eluted from amnion and amnion/chorion membranes. (A) Levels of 30 cytokines released from dehydrated amnion over 24 h. (B) Levels of 30 cytokines released from dehydrated amnion/chorion over 24 h. See Table 1 for abbreviations. Results are from n = 3 biological samples each performed in duplicate.
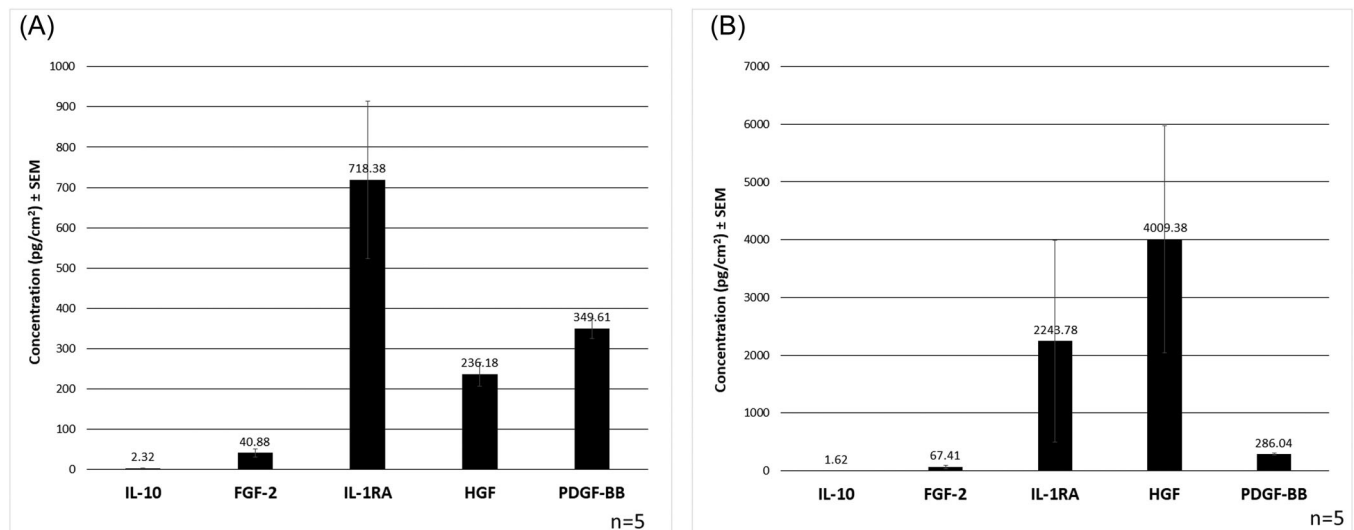
FIGURE 4 Strong elution of cytokines from amnion and amnion/chorion membranes. (A) Levels of five specific cytokines released from dehydrated, sterilized amnion over 24 h. (B) Levels of five specific cytokines released from dehydrated, sterilized amnion/chorion over 24 h. Interleukin 10 (IL-10), basic fibroblast growth factor (FGF-2), interleukin 1 receptor antagonist (IL-1ra), platelet-derived growth factor BB (PDGF-BB). $n = 5$ in duplicate.

tissue grafts is missing. For example, using the digested, preprocessed membrane does not allow us to determine the factors available to a patient from a sterilized, dehydrated membrane. Publications to date utilize enzymatic digestion of dehydrated membranes followed by homogenization to extract all the factors present.[58–62] This does not clearly indicate what would be released when the membrane is placed intact on a wound. In this study, specific areas of sterilized, dehydrated membranes are placed in physiologic buffer at physiologic temperature. This allows for calculations based on release of factor from the final membrane under physiologic conditions.

In addition, growth factor results have been expressed as percentage of eluted to retained,[62] as a function of weight,[59,60,62] in log based charts ranging from 10 to 100,000[61] and per milliliter,[58] which is not informative with respect to the actual membrane. Reporting as a function of weight does not help the end user understand the contents of the membrane, as they are unlikely to have a scale or be able to weigh the membrane in a surgical environment. Log-based charts with multiple factors over a broad range make it nearly impossible to elucidate the quantity of any single factor. Reporting factors per milliliter cannot be translated to the membrane that the end user is handling. Additionally, any amount of buffer or enzyme could be used, which makes it difficult to interpret or apply the results. By presenting the data per cm^2 of sterile dehydrated tissue, the user may apply the data to larger or smaller grafts and understand the eluate-able concentration available to the patient from the membrane that is in their possession. McQuilling et al.[61] is the only group to report per cm^2, although still uses digestion of the membranes.

In this study we present standardized characterization of dehydrated, sterilized amnion and amnion/chorion as delivered to recipient tissues. Reporting this data as a function of cm^2 of

dehydrated tissue allows a more thorough understanding of the range of available factors and permits extrapolation by independent scientists and clinicians, hence we suggest such standardization as general practice.

Histology verified that sterilized dehydrated amnion and amnion/chorion was structurally intact and retained distribution of several ECM proteins. GAGs are distributed evenly between the two basement membranes and appear in the lower portion of the chorion. This is important as GAGs play a role in all phases of wound healing. For example, GAGs assist in stabilization and activation of growth factors, presentation of chemokines to chemokine receptor-expressing immune cells, as well as promoting keratinocyte migration to the wound.[63]

Fibronectin was strongly distributed and appears to colocalize with other ECM proteins (e.g., collagen). This is beneficial for a membrane since fibronectin plays a crucial role in wound healing[64–66] and is responsible for protection of the underlying tissue. Collagen I was strongly expressed in the dsAM and intermediate layer of the dsAC. Expression was also seen in the trophoblast layer, although less intense. This is the most common collagen used in the wound healing process. Collagen III is closely related to type I collagen in terms of location and manner of synthesis, although collagen III is laid down first, followed by remodeling with collagen I. Collagen III was the most strongly expressed in the intermediate layer of the dsAC along the connective tissue tendrils. The retention of fibronectin, collagen I and collagen III in the dehydrated, sterilized membranes is a promising result for the provision of natural substrate for endogenous cell infiltration and remodeling. Total collagen stained positively throughout, indicating a favorable matrix for wound repair. Elastin is not present in adults unless induced by injury but is abundant in in fetal tissue and was shown here to be present in dsAM and dsAC.
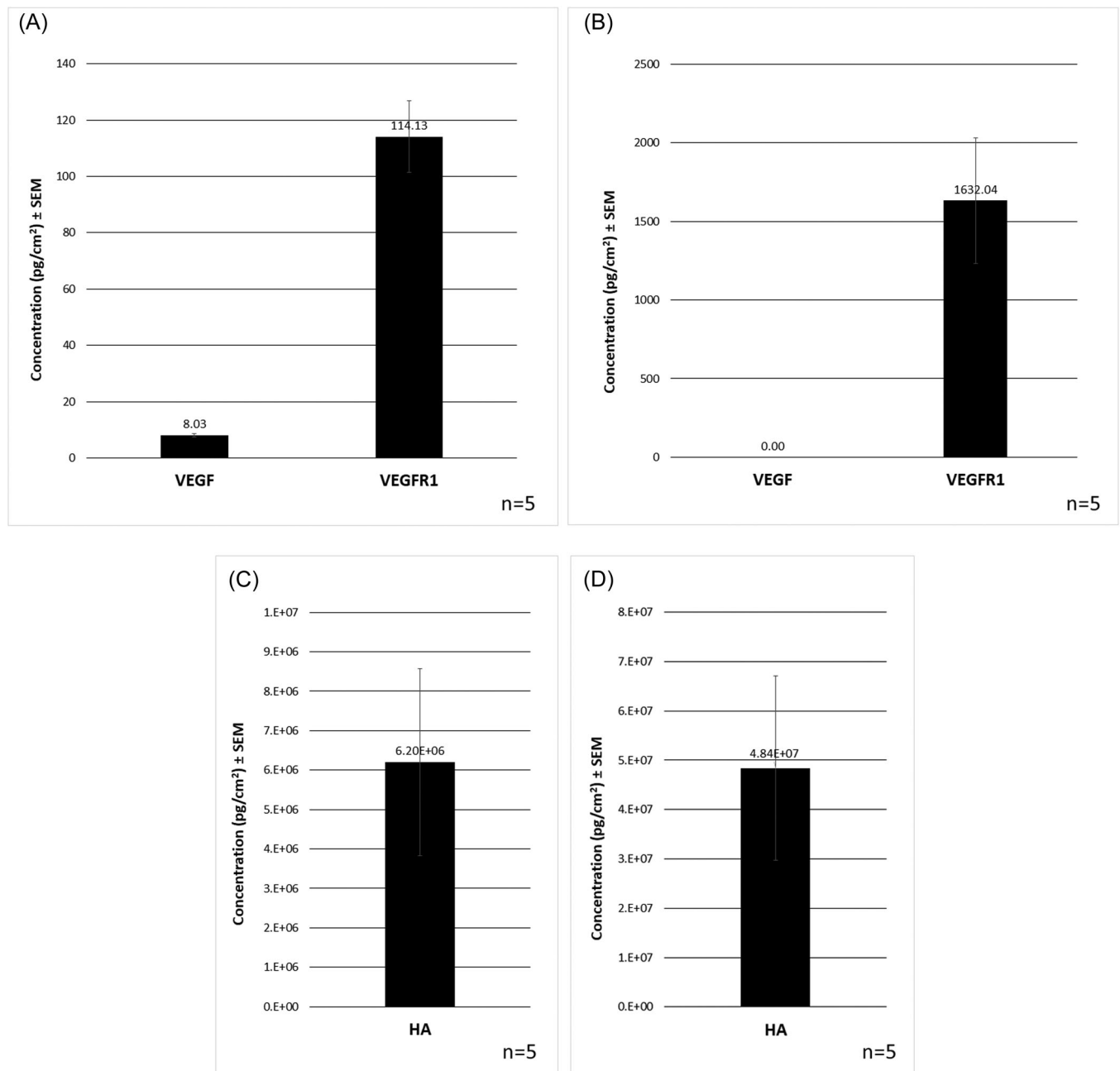
FIGURE 5 VEGF, VEGFR1, and HA are eluted from amnion and amnion/chorion membranes. Levels of VEGF and VEGFR1 released from (A) dehydrated amnion or (B) amnion chorion over 24 h. $n = 5$ in duplicate. Levels of HA released from (C) dehydrated amnion or (D) amnion chorion over 3 days. $n = 5$ in duplicate. HA, hyaluronic acid; VEGF, vascular endothelial growth factor; VEGFR1, vascular endothelial growth factor receptor 1.

Elastin induces cell migration and proliferation, matrix synthesis, and protease production but is aberrantly expressed during wound healing in disorganized networks.[67] By providing elastin contained within the dsAM or dsAC, favorable biological responses should be activated including monocyte chemotaxis,[68] fibroblast migration,[69] proliferation,[70] and MMP-1 expression.[71]

Laminin was expressed in the compact layer of the amnion of the dsAC sample. Because laminin is an important part of the basement membrane, we expected to see strong staining in these areas. It is possible that the dehydration and sterilization process affected laminin. This warrants further investigation since laminin influences cell differentiation, migration, and adhesion[72] and has been demonstrated to be involved in regulating core cell behaviors required for wound repair.[73] One early study demonstrated that direct application of a preparation of human placenta laminins to a superficial rat skin resulted in an enhanced rate of reepithelialization.[74] As it is desirable to retain laminin in placental grafts, further studies are needed to determine the effect of dehydration and sterilization on laminin within AM and AC.

Several regenerative and anti-inflammatory (e.g., HGF and IL-1ra) cytokines were eluted from dsAM and dsAC, as well as the absence of inflammatory cytokines (e.g., IFNα/ɣ and TNF-α). As the growth factor panel utilized was not comprehensive, there are likely more factors present that are important in the healing and regenerative process. For example, TGF-β1 was not included in the panel, which is known for regulation of inflammatory processes, particularly in the gut.[75] Going forward, our standardized protocol will include a more expansive panel, for example, TGFβ and TIMPs, followed by verification via individual assays.

To accurately quantify some specific growth factors known to promote regeneration and/or modulate inflammation, we conducted a more specific assay. Results confirmed the elution of IL-1ra, PDGF-BB, and HGF from dsAM and dsAC. As expected, dsAC eluted a higher concentration of these factors. The presence of the factors stated above, coupled with the absence of inflammatory factors, partly explains the efficacy observed when these tissues are used in wound healing. IL-1ra blocks the inflammatory IL-1 receptors, resulting in less inflammation, PDGF-BB could be promoting proliferation and directing migration of mesenchymal cells and fibroblasts[76] and HGF may be contributing to cell growth and enhancing wound healing.[77] FGF-2 was also eluted reducing tissue death and encouraging cellular repair.[78] Not surprisingly, IL-10 was not detected in the membrane eluate. Although it is anti-inflammatory, it is not highly expressed in the placenta.[79] Thus, it is not surprising that it was not detected in the eluate from the placental tissues. Similarly, VEGF is not highly expressed in the placenta[79] and was not eluted above background in our study. However, VEGFR1 is highly expressed in the placenta[79] and was eluted by both dsAM and dsAC. The presence of VEGFR1 would partially explain the accelerated wound healing in patients receiving dsAM or dsAC. VEGFR1 has been shown to help wound healing in diabetic foot ulcers and other wound models.[80–82] In addition, HA was strongly eluted from both dsAM and dsAC and contributes to wound repair through tissue regeneration, inflammation response, and angiogenesis.[83–85]

It is not known how much of these factors are taken up by recipient tissues, hence the functional outcomes are not clear as yet. Unprocessed tissue may be more advantageous to utilize, but unprocessed tissue cannot be used for clinical application due to transmittable disease risk and storage time before end use. Hence, not currently FDA cleared for use. The purpose of this study is to highlight the need for standardized data collection and reporting in the field of dry membrane allografts. When applied to clinical use, the end user will know that the factors reported to be available to the recipient, relate to the square centimeters of final product membrane provided. This allows for more informed clinical decisions, application and reporting.

5 | CONCLUSION

It is clear from the data presented here, and by others, that dsAM and dsAC contain factors that are conducive to wound healing. We show that these factors are truly eluted to recipient tissues, accounting for their efficacy. Our informed analysis sheds light on the benefit of collecting data in a standardized fashion and reporting data per square centimeter as a standardized reporting method. This will have a significant impact on the field of regenerative allograft medicine by providing information that can be extrapolated to other membrane sizes prepared by identical methods for clinical use. Standardization is also critical for accurately comparing data when optimizing processing methods applicable to both in vitro and clinical studies.

AUTHOR CONTRIBUTIONS
Taylor J. Sabol, MS assisted in the design and administration of this study and the writing and review of the manuscript. Grace S. Tran, BA was responsible for the processing of all tissues and review of the manuscript. Wendy W. Weston, PhD designed and administrated the study, and assisted with the writing and review of the manuscript. Jason Matuszewski, BS assisted in the design of the study and review of the manuscript.

CONFLICT OF INTEREST
Taylor Sabol, MS, Grace Tran, BA, and Wendy W. Weston, PhD are employed by Biostem. Jason Matuszewski, BS is CEO of Biostem.

DATA AVAILABILITY STATEMENT
The authors confirm that the data supporting the findings of this study are available within the article. All authors have read and approved the final version of the manuscript. Wendy W. Weston had full access to all of the data in this study and takes complete responsibility for the integrity of the data and the accuracy of the data analysis.

TRANSPARENCY STATEMENT
The lead author (Wendy W. Weston, PhD) affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

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