Proteome Composition of Bovine Amniotic Membrane and Its Potential Role in Corneal Healing

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Purpose. To investigate the protein profile of bovine amniotic membranes (bAM) and to determine putative associations between protein composition in bAM and known corneal healing pathways.

Methods. The bAM were acquired from normal full-term births (n = 10), processed, and stored at −80°C for two days. Subsequently, the frozen membranes were thawed at room temperature and prepared for proteomic exploration using high-resolution liquid chromatography–mass spectrometry, followed by bioinformatics analysis. Recently identified corneal healing pathways were contrasted with protein profiles and pathways present in bAM.

Results. The analyses identified 2105 proteins, with an interactive network of 1271 nodes (proteins) and 8757 edges (interactions). The proteins with higher betweenness centrality measurements include microfibril-associated protein 4, HSD3B1, CAPNS1, ATP1B3, CAV1, ANXA2, YARS, and GAPDH. The top four pathways in Kyoto Encyclopedia of Genes and Genomes were ribosome, metabolic pathway, spliceosome, and oxidative phosphorylation. The bAM and cornea shared abundant proteins, genome ontology, and signaling pathways.

Conclusions. The high-throughput proteomic profile of the bAM demonstrated that numerous proteins present in the cornea are also present in this fetal membrane. Our findings collectively demonstrate the similarity between bAM and the cornea's protein composition, supporting our hypothesis that bAM can be used to treat corneal diseases.

Keywords: amniotic membrane, bovine, cornea, protein, proteome, tissue proteomics

Complicated corneal ulcerations have complex corneal signaling pathways[1] and inappropriate inflammatory responses[2] because of inadequate protein interactions.[3,4] Amniotic membrane (AM), the innermost layer of the placenta,[5] has been used for the treatment of challenging corneal ulcers[6] and other ocular disorders (e.g., perforation, chemical or thermal burns, keratomalacia) because of its biocompatibility, antiproteolytic, antiangiogenic, anti-inflammatory, antifibrotic, antiscarring, antibacterial and re-epithelializing properties.[5–7] Although the exact mechanism by which AM improves the corneal healing process is unclear, it has been suggested that it is due to interactions of cytokines, growth factors, and protease inhibitors.[8,9] In recent years, advanced proteomic techniques coupled with bioinformatics have allowed investigators not only to explore the protein composition of various biological samples but also to assess the protein interactions and pathways.[10,11]

Topical medications can be applied to improve corneal healing by manipulating the corneal microenvironment, including the signaling pathways of the wound.[1,12] To understand how the bovine AM (bAM) can influence the corneal healing process and improve corneal ulceration, the purpose of this study was to investigate the protein profile of the bAM, identify the proteins previously associated with positive corneal healing, and evaluate the protein/protein interactions and significant signaling pathways.

Material and Methods

Amnion Collection

Ten placentas (n = 10) were acquired from normal full-term, live births from healthy multiparous cows then placed on ice and transported to the investigators' laboratory. The bovine fetal membranes are formed by the chorion, the allantois (a sac-like structure, serving an extra-fetal bladder urine deposit, not present in primates or rodents), and the amnion. The AM partially fuses the chorion, forms the chorioamnion, and partially fuses the allantois forming the allan-

tochorion.[13] The allantois attaches to the chorion creating the chorioallantois present in all domesticated mammals.[13] On-field, the AM not attached to either allantois or chorion was collected, and if a piece of allantoamnion or chorioamnion was grossly detected (i.e., based on differences in tissue color from white and smooth appearance (amnion) to red and velvety in appearance (chorion or allantois), these segments were removed before transportation to the laboratory. This approach was pursued to ensure that the AM protein composite was determined and avoid confounding effects of the other fetal membranes. Additionally, the AM segment not fused to chorion or allantois represents the largest portion of the bovine AM. For the preparation, the bovine amniotic membrane was washed three times with PBS (Life Technologies, Carlsbad, CA, USA), then submerged in PBS containing 1% antibiotic-antimycotic solution (Life Technologies) for three hours. Amniotic membranes then were sectioned (4 × 4 cm) and placed in individual 50 mL tubes containing 50 µg/mL gentamicin and Hank's balanced salt solution (Corning CellGro, Corning, NY, USA). Tubes were stored at −80°C for two days, then thawed at room temperature, and prepared for proteomic analysis.

Protein Extraction

Frozen tissue was thawed and then homogenized with 6 M guanidinium chloride at a ratio of 1 g tissue per 4 mL buffer. After gross debris was cleared by centrifugation for 15 minutes at 3000*g*, triethylammonium bicarbonate, tris(2-carboxyethyl)phosphine, and 2-chloroacetamide were added to the supernatant. The samples were heated at 95°C for 20 minutes to promote the reduction of disulfide bonds and alkylation of cysteine residues. Protein concentrations were measured by BCA assays (Pierce; Thermo Fisher Scientific, Waltham, MA, USA). A 100 µg aliquot of protein from each sample was then digested with 1 µg of LysC (Wako Chemicals USA, Richmond, VA, USA) at 25°C for four hours, followed by 2 µg of trypsin (Pierce) in a CEM Discover microwave reactor at 55°C for 30 minutes. The resulting peptides were desalted with C18 spin columns (Glygen Corporation, Columbia, MD, USA) and lyophilized.

Liquid Chromatography–Mass Spectrometry Analysis (LC-MS/MS)

Dried peptides were suspended in 0.1% FA in 5% ACN, and 500 ng from each sample was injected into an Ultimate 3000 UHPLC coupled to a Q Exactive HF-X Orbitrap mass spectrometer (Thermo Fisher Scientific). The peptides were chromatographically separated using a 25 cm Acclaim PepMap 100 C18 column (75 um ID, 2 µm particle size). Mobile phase A was 0.1% FA, and mobile phase B was 0.1% FA in 80% ACN. The separation gradient was 5% to 35% B over 80 minutes, followed by 35% to 50% B over 10 minutes, all at a flow rate of 300 nL/min.

The mass spectrometer was run in a data-dependent mode so that a full MS scan from 350 to 1500 m/z (120,000 resolution, 3e6 AGC, 50 ms IT) was followed by HCD fragmentation (30 NCE) of the 15 most abundant species. The isolation window for precursors was 1.3 m/z, and MS/MS scans were acquired at 15,000 resolution. The AGC target for MS/MS scans was 5e4, the max IT was 30 ms, and the dynamic exclusion time was 60 seconds.

Protein Identification

The LC-MS data were analyzed with the MaxQuant software (v.1.6.5.0) against the *Bos taurus* database from Uniprot (37,511 entries; accessed November 2019) and a database of common contaminants. Tryptic digestion was specified with a maximum of two missed cleavages. Other search criteria included a fixed modification of cysteine carbamidomethylation and variable modifications of methionine oxidation and N-terminal acetylation. Mass tolerances were set to 6 ppm and 10 ppm for precursor and fragment matches, respectively. Protein, peptide, and site false discovery rates were all set to 0.01. Any proteins matching the reverse decoy or contaminant databases were removed before further bioinformatics analyses. MaxQuant was also used for label-free quantitation so proteins could be ranked by abundance.

Bioinformatic Analysis

The predicted function of the proteins presented was analyzed using Uniprot annotation. The PANTHER gene expression analysis tool (pantherdb.org; v15.0) was used to classify the proteins and their genes (genome ontology (GO) classification). The biological database STRING (http://string-db.org; v11.0) was used to retrieve the predicted interactions of the identified genes and establish connections between the nodes with the highest confidence (0.900). Cytoscape (v3.8.1) and the plugin Bingo (v3.0.4) were used to analyze the network properties with the GO Biological process option and $P < 0.01$. NetworkAnalyzer (v4.4.6) was used to calculate betweenness centrality values. Signaling pathways were analyzed using PANTHER and Kyoto Encyclopedia of Genes and Genomes (KEGG).

Results

In total, 2105 unique proteins were identified in the bovine amnion samples by LC-MS/MS, and the genome ontology annotation was available for 1472 of the bovine amnion proteins. The highest scored genome ontology (GO) terms for molecular function were binding (GO:0005488), catalytic activity (GO:0003824), and structural molecule activity (GO:0005198). For biological process (BP), the highest scored GO terms were cellular processes (GO:0009987), metabolic processes (GO:0008152), biological regulation (GO: 0065007), and cellular component organization or biogenesis (GO:0071840). For cellular localization (CL), the most frequently found GO were cell part (GO:0044464), cell junction (GO:0030054), and organelles (GO:0043226). When we analyzed the protein classes in the samples, the top categories were metabolite interconversion enzyme (PC00262), nucleic acid-binding protein (PC00171), membrane traffic protein (PC00150), and protein modifying enzymes (PC00260).

The 50 most abundant proteins found in the bAM were described in Table 1. When evaluating all the proteins present in the bAM using an interactive network, 1271 nodes (proteins) and 8,757 edges (interactions) were identified (Fig. 1), and the clusters with the highest degree of BP association were generated (Figs. 2 and 3). The proteins expressed in the bAM with regard to their properties and functions and that are also present in the 500 most abundant proteins in the cornea[14] are described in Table 2. Proteins identified in the bAM with relevance for the corneal stroma are included in Table 3.

Table 1. The 50 Most Abundant Proteins Expressed in the Bovine Amniotic Membrane (n = 10)

Protein Name	Gene ID	Notes
Actin, cytoplasmic 2	ACTG1	ATP binding, angiogenesis, positive regulation of wound healing, regulation of transepithelial transport
Actin-depolymerizing factor	GSN	Actin filament and calcium ion binding
AHNAK nucleoprotein	AHNAK	Structural molecular activity conferring elasticity/regulation calcium channel
Alpha-actin 4	ACTN4	Calcium-binding/ protein transport/
Annexin	ANXA1	Cytoskeleton, inflammatory response, insulin secretion, keratocyte differentiation, regulation of IL-1, positive regulation wound healing, response to drugs
Annexin A2	ANXA2	Calcium channel activity, biomineral tissue development, protease binding
Biglycan	BGN	Extracellular matrix binding, glycosaminoglycan binding
Clathrin heavy chain 1	CLTC	Autophagy, cell division
Collagen alpha-1(I) chain	COL1A1	Extracellular matrix structural constituent, platelet-delivery growth factor binding, blood vessel development, collagen fibril organization
Collagen type VI alpha 2 chain	COL6A2	Collagen degradation, signaling of PDGF, integrin cell surface interaction, assembly of collagen fibrils, ECM proteoglycans function
Collagen type VI alpha 1	COL6A1	Collagen degradation, signaling of PDGF, integrin cell surface interaction, assembly of collagen fibrils, ECM proteoglycans function
Collagen type VI alpha 3	COL6A3	Cell adhesion
Decorin	DCN	Collagen binding, negative regulation of angiogenesis
Desmoplakin	DSP	Cell adhesion, a scaffold protein, wound healing
Elongation factor 1-alpha 1	EEF1A1	GTPase activity, cellular response to epidermal growth factor stimulus
Envoplakin	EVPL	Epidermis development/wound healing/filament binding
Fibronectin	FN1	Acute-phase response, cell adhesion, regulation of cell shape
Filamin A	FLMA	Acting filament binding, protein stabilization, wound healing, spreading of cells
Filamin B	FLNB	Acting binding/cell differentiation
Fructose-bisphosphate aldolase	ALDOA	Glycolytic process
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	playing a role in glycolysis and nuclear functions
Heat shock cognate 71 kDa protein	HSPA8	ATPase activity
Heat shock 70 kDa protein 1B	HSPA1B	ATPase activity and binding, heat shock protein binding
Heparan sulfate proteoglycan 2	HSPG2	Calcium-binding/tissue development
Histone H2A	H2AC6	DNA binding, chromatin organization
Histone H2B	H2BC15	DNA binding, nucleosome assembling
Histone H3.3	H3-3A	DNA binding
Keratin, type I cytoskeletal 19	KRT19	Organization of myofibers
Keratin, type II cytoskeletal 8	KRT8	Scaffold protein binding
Keratin, type II cytoskeletal 7	KRT7	Stimulates DNA synthesis in the cell
KRT15 protein	KRT15	Scaffold protein binding, structural molecule activity
Lactotransferrin	LTF	Iron binding transport proteins
Laminin A/C	LMNA	Cellular response to hypoxia, negative regulation of extrinsic apoptotic signaling pathway, regulation of protein stability
Mimecan	OGN	Growth factor
Myosin heavy chain 9	MYH9	Actin cytoskeleton organization, angiogenesis, cell adhesion
Periplakin	PPL	Structural molecule and wound healing
Phosphoglycerate kinase 1	PGK1	ADP and ATP binding, gluconeogenesis, negative regulation of angiogenesis, epithelial cell differentiation
Plectin	PLEC	Acting binding
Protein-glutamine gamma-glutamyl transferase 2	TGM2	Catalytic activity of cross-linking proteins
Protein disulfide-isomerase	P4HB	Acting and integrin-binding, cellular response to interleukin-7
Protein S100	S100A11	Facilitates the differentiation and the cornification of keratinocytes
Pyruvate kinase	PKN	Catalytic activity/ATP binding/glycolytic process/positive regulation of plasmatic translation and angiogenesis
Spectrin alpha	SPTAN1	Calcium ion binding
Transforming growth factor-beta-induced protein ig-h3	TGFBI	Extracellular matrix binding, glycosaminoglycan binding, angiogenesis, extracellular matrix organization, cell adhesion, cell population proliferation.
Triosephosphate isomerase	TPI1	catalytic activity
Tubulin beta-4B chain	TUBB4B	Microtube-based process, RNA binding
Vimentin	VIM	RNA and keratin filament binding
2-phospho-D-glycerate hydro-lyase	ENO1	Catalytic activity
14-3-3 protein theta	YWHAQ	Adapter protein

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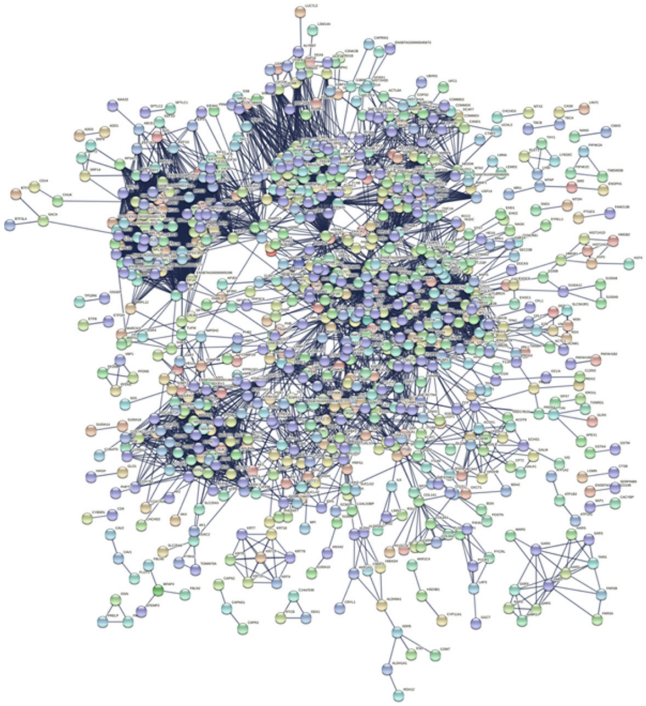

Figure 1. Interactive network of the proteins identified on bovine amniotic membranes.

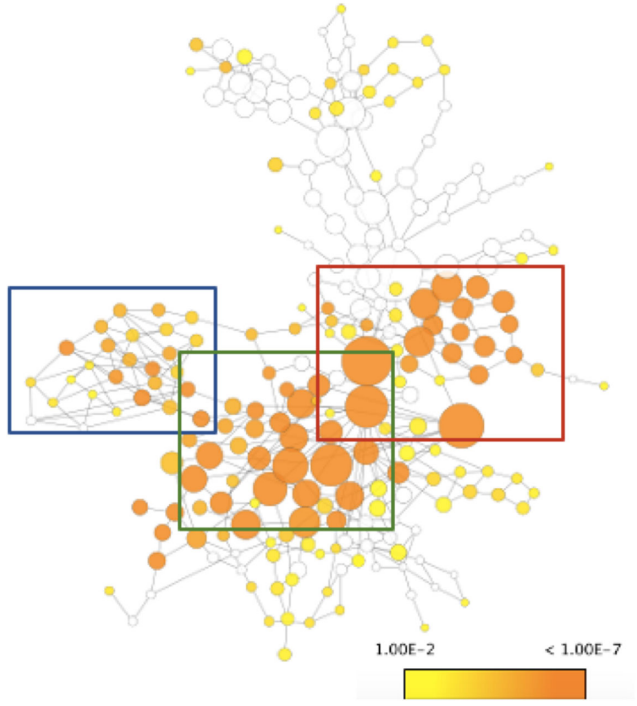

Figure 2. Clusters with the highest degrees of biological process association; (a), (b), and (c) are shown separately with magnification as following.

When evaluating the betweenness centrality, the proteins with higher measurements include microfibril-associated protein 4, 3 beta-hydroxysteroid dehydrogenase (HSD3), Calpain small subunit 1 (CAPNS1), Sodium/potassium-transporting ATPase subunit beta-3 (ATP1B3), caveolin-1 (CAV1), annexin A2 (ANXA2), tyrosine-tRNA ligase (YARS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Numerous signaling pathways were revealed in the bAM. In the KEGG pathway analysis, the top four pathways incorporate ribosome, metabolic pathway, spliceosome, and oxidative phosphorylation. Bovine amniotic membrane and cornea shared 30 pathways in common using the KEGG database (Table 4). In the PANTHER database, the top pathways outlined are described in Table 5, and the selected pathways identified in the bAM with relevance for corneal function include Integrin signaling pathway, Cytoskeletal regulation by Rho GTPase, Ubiquitin proteasome pathway, WNT signaling pathway, epidermal growth factor (EGF) receptor signaling pathway, and Muscarinic acetylcholine receptor.

Discussion

In this study, high-throughput proteomic analyses, coupled with bioinformatics, was used to identify proteins expressed in the bAM to investigate its potential contribution to the corneal healing process. Herein, we have identified 2105 unique proteins in the bAM. This number is remarkably more extensive than that reported by other studies involving equine AM (eAM), where authors reported 149 and 220 numbers of unique proteins.[15–17] The difference in values between the AM of these two species could be real, or because the equine database for proteins is significantly more limited compared with the bovine database. In allantoic and amniotic fluids, 112 proteins were described in horses,[18] whereas in human amnion membrane (hAM), 61 proteins were identified in de-epithelialized amnion using 2D gel electrophoresis.[19] The bAM expressed all the proteins identified in common in the eAM reported by McCoy et al.,[16] Galera et al.,[15] and Hopkinson et al.,[18] except for fibulin 1, keratin 6B, keratin 14, laminin subunit 4, major allergen equ c 1-like, and hemoglobin beta. Keratins, originated from amnion epithelial cells, were highly expressed in the top 50 most abundant proteins in the bAM. Although they were not all the same type as expressed in the eAM and hAM, the keratins identified herein were types 1, 2, 7, 15, and 19. In addition, bAM also was rich in fibulins 2, 5, and 7 (i.e., a group of extracellular proteins), but not in fibulin 1 as described in eAM and hAM.

A recent study identified 4824 proteins in the human cornea.[14] Of interest, 46% of the 500 most abundant proteins present in the human cornea were also found in the bAM in the present study (Table 2). The bAM also expressed the top five most abundant proteins presented in the human cornea: keratocan, decorin, lumican, TGF-β–induced protein ig-h3, and albumin.[14] Some of these proteins (lumican, keratocan, and decorin) are members of the proteoglycan and small leucine-repeat proteoglycan families, which are important for keeping the natural stability of the cornea[15] and responsible for numerous functions, including corneal transparency and wound-healing modulation response.[14,20] TGF-β–induced protein ig-h3, included in the top five proteins expressed in the cornea,[14] is also present in eAM, bAM, and hAM, and its mutation is associated with corneal dystrophy.

The AM is composed of a single layer of cuboidal epithelium cells bathed by the amniotic fluid.[13,21] This epithelium is supported by a basement membrane rich in collagen. Beneath the latter, a collagen-elastin–rich extracellular matrix is infiltrated with fibroblasts, mesenchymal stromal

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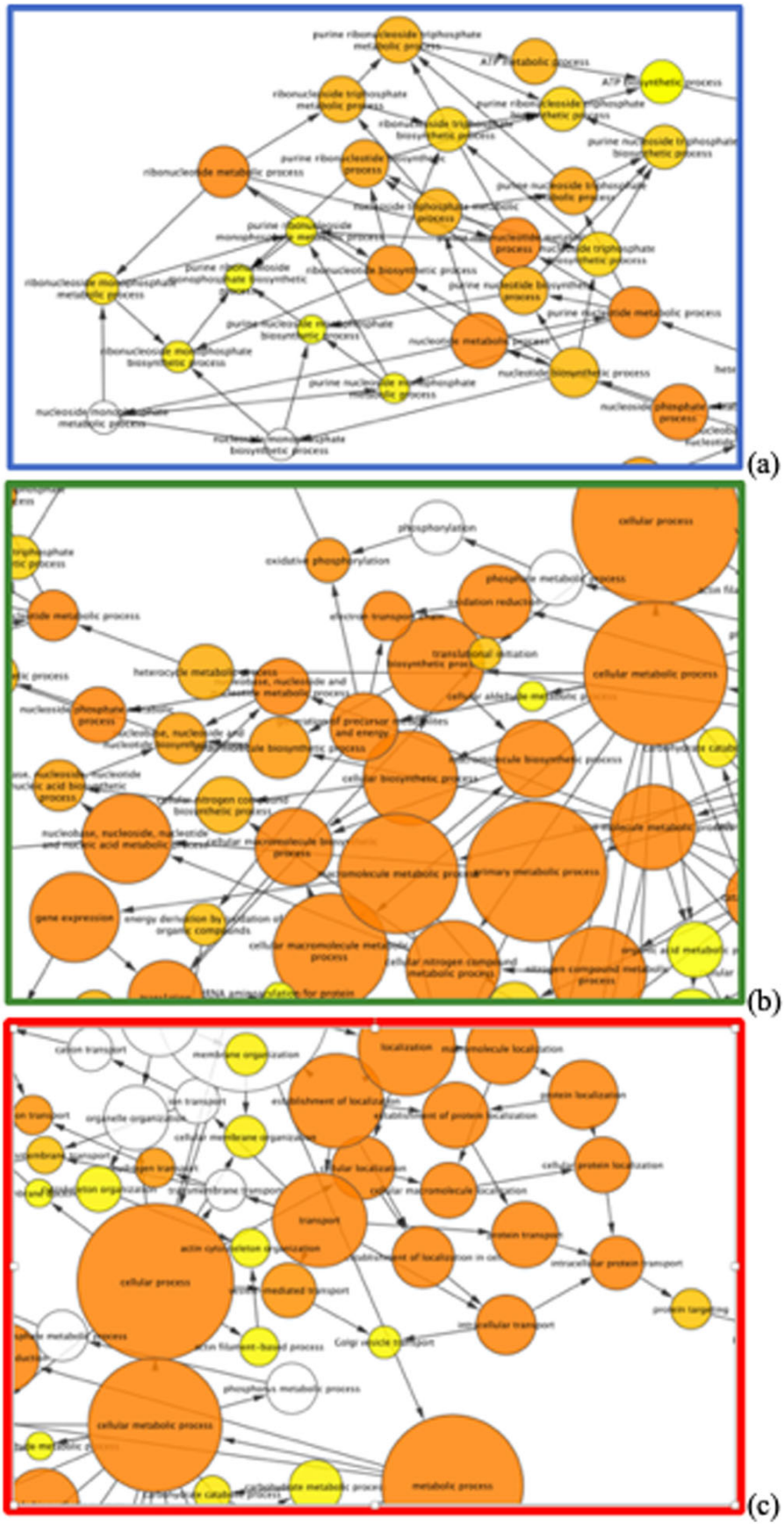

Figure 3. (a), (b), and (c) denote the areas of Figure 2 with magnification and visible biologic process.

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Table 2. Proteins Expressed in Both BAM and Cornea Regarding the Biological Properties and Functions

Inflammation and Immune Response Function	Metabolism Function	Support and Cell Adhesion Function	Development and Maintenance Function
Immune response:	TCA cycle and respiratory electron transport:	Extracellular matrix structural constituent:	Transport activity:
ANP32A, AZGP1, C1QB, C3, CLU, DCP, DDT, HPR, LBP, LGALS1	ATP5H, COX4I1, COX6C, MDH2	BGN, COL12A1, COL1A1, COL1A2, COL6A1, COL6A3, DCN, DPT, ECM1, EFEMP1, FBLN5, FMOD, FN1, KERA, LUM, PCOLCE, THBS1, THBS3, THBS4	CRIP1, FABP5, GC, LTF, SAA1, SLC25A6, TF, TMED10, TTR, VAT
Cytokine activity:	Glycolysis:	Structural constituent of the cytoskeleton:	Growth factor activity:
MIF, IL1RN	GPI, PGK1, PKM, TPI1	ACT2, ACTB, ACTG1, ACTN4, ACTR3, CAPZB, GSN, KRT15, KRT17, KRT18, KRT19, KRT4, KRT7, MSN, MYL6, TPM4, TUBA1A, TUBA4A, TUBB, TUBB2B, TUBB6, VIM	OGN
ER-phagosome pathway:		Protease inhibitor activity:	Transcription factor activity:
CALR, PDIA3, PSMA1, PSMA5, PSMB1, RPS27A		CST3, CSTB, PEBP1, SERPINA3, SERPINB1, SERPINB5	ILF2
MHC class I and II receptor activity:		Transparency:	
ANP32A, SET		KERA, LUM, CRYAB, CRYGD, CRYZ	

Proteins expressed in the bovine amniotic membrane, regarding their biological properties and functions, are also present in the most 500 abundant proteins found in the cornea by Subbannaya et al.[13]

A list of abbreviations is presented in the supplementary material (S. 1).

Table 3. Summary of the Proteins Expressed in the Bovine Amniotic Membrane and in the Corneal Stroma[15]

Categories	Subcategories	Proteins Specific in the Cornea and Expressed in the BAM
Stromal gel-like material	Proteoglycans	Mimecan, prolargin, decorin (DCN), biglycan (BGN), lumican (LUM), keratocan (KERA), fibromodulin (FMOD)
	Glycoproteins	Fibronectin (FN), laminin (LN), alpha-1-antitrypsin
	Enzymes	Glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, Glutathione S-transferase P, alpha-1-antitrypsin
	Binding proteins	Protein S100-A4, fatty acid-binding protein, integrin
	Serum protein	Serotransferrin
Network of filaments	Collagens	Collagens types V and VI,
	Non-collagenous proteins	Elastin, keratin, vimentin, FN

cells, and tissue macrophages ("Hoffbauer cells").[13,21] The corneal stroma also consists of a collagen-rich extracellular matrix with proteoglycans, enzymes, binding proteins, and serum proteins that modify the structure and function of the collagen fibrils.[15] Proteins components of the extracellular matrix were highly expressed in our samples, corroborating with a previous proteomic study of eAM.[16] On the basis of the previously known composition of the AM, it is not surprising that collagens were some of the most abundant proteins in bAM. In addition to the list of important proteins for the extracellular matrix, dermatopontin was expressed in our bAM samples, as well as in eAM,[16] but not in the hAM.[19] This protein is expressed abundantly in the stromal cellular matrix and interacts with proteoglycans to maintain the stromal shape.[22]

When we focus on the 50 most abundant proteins present in the bAM, we can identify proteins important for the normal corneal function, such as annexin A2. This protein presents many biological activities and is profusely expressed in the cornea. Its role in the cornea appears to be associated with epithelial migration during wound healing.[23] Another protein expressed in the bAM and habitually found at the supra layer limbal epithelial cells is S100A11, which is involved in cell membrane organization, ion channel modulation, and keratinocyte differentiation.[24]

Two groups of proteins expressed in our study deserve extra attention, including laminin A/C (LMNA; present among the 50 most expressed proteins in the bAM) and integrins (the subfamilies expressed in the bAM include: integrin alpha-3, integrin α-V, and integrin β-6). Laminin

Table 4. List of the Bovine Amniotic Membrane KEGG Pathways Also Observed in the Human Cornea[14]

KEGG Pathway	False Discovery Rate
Ribosome	1.28e-28
Metabolic pathway	9.07e-23
Spliceosome	1.41e-20
Oxidative phosphorylation	4.34e-20
Carbon metabolism	9.78e-13
Protein processing in endoplasmic reticulum	9.79e-11
Proteasome	5.42e-10
Regulation of actin cytoskeleton	9.69e-10
Phagosome	1.30e-09
Biosynthesis of amino acids	3.01e-09
Endocytosis	1.34e-08
Citrate cycle (TCA cycle)	6.99e-08
Glycolysis/ Gluconeogenesis	5.03e-07
Focal adhesion	5.66e-07
RNA transport	5.66e-07
Tight junction	6.49e-07
Valine, leucine and isoleucine degradation	6.80e-06
Lysosome	9.41e-06
Amino sugar and nucleotide sugar metabolism	0.00011
Apoptosis	0.00043
Fatty acid degradation	0.00045
Adherens junctions	0.0013
Glutathione metabolism	0.0025
mRNA surveillance pathway	0.0024
Platelet activation	0.0111
Purine metabolism	0.0129
PI3K-Akt signaling pathway	0.0155
Propanoate metabolism	0.0171
Insulin signaling pathway	0.0426
ECM-receptor interaction	0.0449

Table 5. List of Pathways Generated in PANTHER in Order of Abundance of Proteins Identified in the Bovine Amniotic Membrane

PANTHER Pathway	Term
Integrin signaling pathway	P00036
Inflammation mediated by cytokine	P00031
Cytoskeletal regulation by Rho GTPase	P00016
Ubiquitin proteasome pathway	P00060
WNT signaling pathway	P00057
EGF receptor signaling pathway	P00018
Heterotrimeric G-protein	P00027
Apoptosis signaling pathway	P00006
Angiogenesis	P00005
Muscarinic acetylcholine receptor	P00043
T-cell activation	P00053
Thyrotropin-releasing hormone signaling pathway	P04394

is one of the major glycoproteins present in the basement membrane, with abundant distribution along the ocular surface, which functions as cell-matrix adhesion and matrix cell signaling support.[25,26] The integrins, consisting of alpha and beta subtypes, are proteins that function as transmembrane receptors, facilitating cell–extracellular matrix adhesion, including the ligand laminin.[27] A study analyzing human corneal epithelial cell adhesion to placental laminin mediated by α-integrin concluded that these cells are capable of rapid adhesion to laminin isoforms not usually resident in the corneal basement membrane.[24] The reason AM transplantation can be advantageous for ocular surface reconstruction can be explained in part by the presence of the external laminin expressed in the AM and applied directly on the cornea.[26]

The fetal membranes express several growth factors responsible for proliferation, migration, differentiation, and re-epithelization during normal pregnancy.[21] These same factors regulate the extracellular matrix modeling during pregnancy[21] and are likely the same ones responsible for the beneficial properties of the AM in the various areas of medicine. Several growth factors and cytokines with importance for the cornea[25] were found expressed in the bAM include EGF, insulin-like growth factors, platelet-delivered growth factors, thymosin-β4, IL-1, opioid growth factor, and TGF-β. Normally, growth factors and cytokines are difficult to detect in samples deep-frozen for an extended period of time[28] and in processed amnions,[17,19] emphasizing the reason that fresh bAM or cryopreserved for a short period of time could be positive for corneal wound healing because it can preserve several properties beneficial to corneal healing, including growth factors and cytokines.

Traditionally, AM has been extensively used in human and veterinary ophthalmology as a fresh preparation, or cryopreserved (e.g., deep-frozen, freeze-dried, or extended in media containing glycerol or propylene glycol and commercial media such as Optisol-GS) or lyophilized.[29–31] More recently, the AM homogenate has been gaining popularity as an alternative biological approach to treat challenge ophthalmic diseases.[1,32] Studies comparing AM homogenate and transplanted AM showed equivalent results.[33] The homogenate can be used as a fresh preparation or, more commonly, after being deep-frozen.[32,33] Herein the AM was deep-frozen for two days before homogenization. There have been no comprehensive proteomics studies comparing the effects of the various techniques in preserving all the desirable features of AM in human and veterinary medicine. A recent study using identical AM cryopreservation protocol to the present study reported no detrimental effects of dual freezing-thawing cycles on protein concentrations (i.e., EGF, TGF-β1, HGF, bFGF, hyaluronic acid, and laminin), histological and ultrastructure, transparency, and biomechanical properties of the hAM kept in the freezer in a short period of time[34]; however, AM kept for periods longer than seven days at −80°C presented low concentrations of cytokines.[17,28] The findings of this recent study suggest that the short period of the deep-freezing method used herein does not significantly affect the protein composition of AM.

Another group of proteins with fundamental meaning to the corneal healing process is the protease inhibitors class. The disequilibrium between proteases and protease inhibitors in the cornea is involved in many ocular diseases, and it may represent a target for therapeutic approaches.[35] Some proteinase inhibitors found in this study that are also expressed in the human cornea[14] include cystatin-C, cystatin-B, phosphatidylethanolamine-binding protein 1, serpin A3, serpin B1, maspin, and tissue inhibitor of metalloproteinase 2.

The functional annotations of the proteins (GO categories) of the bAM were identical to the EAM[17] when comparing molecular function and biological process. The cellular localization was similar for the cell part and organelle. Although they are different species, similar results in GO were expected because both amnions perform the

same function in the body. In the cornea, protein binding, structural molecule activity, and regulation of biological process categories are high in all three layers,[36] and these three categories were also found in the bAM.

The betweenness centrality is a way of detecting the amount of influence a node has over the flow of information in the network,[37] and in this study, it was used to identify the most connected protein nodes in the BAM. Proteins with high betweenness centrality measures include microfibril-associated protein 4, 3 beta-hydroxysteroid dehydrogenase (HSD3), Calpain small subunit 1 (CAPNS1), Sodium/potassium-transporting ATPase subunit beta-3 (ATP1B3), caveolin-1 (CAV1), annexin A2 (ANXA2), tyrosine-tRNA ligase (YARS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). From this group, GAPDH was also identified with high betweenness centrality in the cornea,[14] and it is known for having multiple functions related to glycolysis, initiation of apoptosis, and transcription of genes involved in antiapoptotic pathways and cell proliferation in the nucleus.[38]

Exploring the signaling pathways with the KEGG database, we observed that the top four pathways in the bAM were also shared in the cornea,[14] and 30 of the 33 signaling pathways in the cornea[14] were also present in the bAM. The PANTHER classification system also demonstrated several pathways in the bAM that could contribute to the corneal function. It is known that several medications can modulate the signaling pathways that are found to be defective in certain diseases.[4,39] Still, to our knowledge, limited studies demonstrate the use of biological tissue as medication and how the same acts in these signaling pathways. Consequently, it is not clear how the similarity of the signal pathways in the bAM and cornea could improve the healing process, but we speculate that the synergism of these same pathways found in the bAM and the cornea could remodel this signal and alter the behavior of the cells, reversing the disease.

When using AM grafts to treat corneal disease, it might be advantageous to use the mesenchymal side or the epithelial side of AM based on the protein composition. Knowing the origin of AM proteins could potentially guide ophthalmologists in opting to use one side versus another. Unfortunately, up to now, research in this field is still at its early stages, and most proteins found in the AM have not been specifically linked as being primarily secreted by mesenchymal or epithelial cells. Previously, it was thought that vimentin, one of the top 50 most abundant proteins in bAM, was expressed by amnion mesenchymal cells, but a recent study showed that vimentin and cytokeratin-18 are coexpressed by amnion epithelial and mesenchymal cells.[21] Additional research should address the origin of proteins present in the AM and potentially allow clinicians to decide whether to face the mesenchymal side or epithelial side of AM onto the affected area could be beneficial.

In conclusion, this study explored the proteomic profile of bAM, correlating the findings with corneal proteomic studies in the literature. Many of the expressed proteins in the bAM were also present in the cornea, and several of these proteins are involved in diverse functions as predicted with gene ontology, signaling pathways analysis, and clusters of protein/protein interactions. Understanding the molecular mechanism of the bAM could help us clarify the possible beneficial effect that it could offer during the corneal healing process and improve its therapeutic use in ophthalmic diseases.

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