Proteomic analysis and cell viability of nine amnion-derived biologics

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INTRODUCTION: Amnion products have been used in hand, ankle, and foot orthopedics to decrease inflammation and pain[1], prevent fibrous adhesions[2], and serve as scaffolding for epithelial cells, reducing scarring and wound healing time[3]. Variable claims have been made about the growth factor/protein content and cell viability of these products[4]. Our objective was to determine the full proteome of amnion-derived products using bottom-up shotgun proteomics, quantify lumican concentration in 48-hour sample eluates using ELISA, and examine cell viability with confocal microscopy.

METHODS: Nine samples from four manufacturers (Fig 1) were lysed, stored overnight at -20C, thawed, and centrifuged. Supernatants were subjected to liquid chromatography tandem mass spectrometry. Results were compared against a UniProt database for *Homo sapiens* containing 134,000 entries, quantified by MaxQuant software, and sorted by possession of ≥3 unique peptides. Relative protein abundance was determined by dividing the intensity by the sum of all intensities. For Lumican ELISA (R&D Systems), liquids were reconstituted according to manufacturer specification, sheet samples were incubated in PBS at 37°C for 48 hours. To determine cell viability, products were stained with calcein AM (live) and ethidium homodimer (dead) and imaged using confocal microscopy. Equine MSCs and fresh bovine amnion served as positive controls.

RESULTS: 677 unique proteins were detected between the nine products. The majority of proteins identified were ECM constituents such as keratin, collagen, and albumin (Fig 1). Lumican, a small leucine-rich proteoglycan (SLRP), was found in relatively high abundance in all samples. No growth factors were identified. Lumican ELISA indicated that dehydrated sheet or lyophilized/particulate liquid products eluted more protein, as did liquid products containing more than one type of tissue (Fig 2). No live cells were identified using confocal microscopy when compared to live controls (Fig 3).

DISCUSSION: ECM proteins are the primary contents of amnion-derived products. No growth factors were detected using LC-MS/MS which detects proteins at femtomole and 0.6 Dalton thresholds. Growth factors such as TGF-β and IGF-I are approximately 25kDa. 10 femtomoles of TGF-β converts to 0.25 nanograms, indicating that even small amounts of TGF-β or similarly sized proteins would have been detected, if present. The SLRP Lumican was present in all samples. SLRPs have been implicated in the upregulation of TGF-β and IGF-I, as well as in protecting collagen fibril formation.

SIGNIFICANCE: Our results suggest that amnion-derived products do not enact their positive effects by serving as a source of live cells or growth factors. The presence of other proteins, such as Lumican, may account for some of the biological properties of amnion-derived products.

REFERENCES: [1]Hanselman *FAI* 2015 [2]Kim *J Hand Surg Eur. Vol* 2010 [3]Zelen *Int Wound J* 2013 [4]Koob *Am J Sports Med.* 2014

Sample ID per Fig 1 (total proteins detected)

	UCsh1 (310)	UCsh2 (135)	AMsh1 (336)	AMsh2 (337)	AMUCsh3 (214)	AMUCliq3 (192)	AFliq1 (132)	AFliq2 (93)	AMAFliq4 (113)
1	Keratin, type II, 6A	Collagen VI	Collagen VI	Collagen VI	Collagen VI	Collagen VI	Albumin	Albumin	Albumin
2	Keratin, type I, 14	Fibrinogen	Collagen XII	Collagen XII	Collagen XII	Tenascin C	α -1 Antitrypsin	α-1 Antitrypsin	Annexin
3	Annexin	Keratin, type II, 8	Periostin	Tenascin C	Annexin	Collagen XII	Hemoglobin	Vitamin D Binding Protein	Transthyretin
4	Keratin, type II, 8	Keratin Type I, 18	Tenascin C	Mimecan	Lumican	Lumican	Complement C3	Complement C3	Collagen VI
5	Collagen VI	Keratin, type II, 6A	Mimecan	Hemoglobin	TGFβ-induced ig-h3	Mimecan	Vitamin D Binding Protein	Ceruloplasmin	Hemopexin
6	Keratin, type I, 17	Vimentin	Lumican	Decorin	Mimecan	TGFβ-induced ig-h3	Ceruloplasmin	Transthyretin	GAPDH
7	Lumican	Transglutaminase	Vimentin	Lumican	Tenascin C	Periostin	Transthyretin	Hemopexin	Alpha-1-B glycoprotein
8	Keratin, type I, 18	Annexin	Transgelin	Periostin	Keratin, Type II, 8	Filamin A	Hemopexin	Zymogen granule protein 16	Filamin B
9	Mimecan	Osteoglycin	Filamin A	TGFβ-induced ig-h3	Keratin, Type I, 14	Keratin, type I, 9	Apolipoprotein A-I	Angiotensinogen	Collagen XII
10	Vimentin	Lumican	Actinin	Perlecan	Plectin	Matrilin 2	Angiotensinogen	Bone marrow proteoglycan	Plectin

Fig 1. Ten most abundant proteins in amnion-derived samples. Keratin and collagen were the predominant proteins in sheet (sh) products. Albumin was the most abundant protein in all amniotic fluid (AF) samples. Lumican was among the ten most abundant proteins in 6 of 9 samples and present in all products tested. 677 unique proteins were identified between all nine samples. Manufacturers (mfr): 1=Arthrex, 2=MiMedX, 3=Amniox, 4=Integra. Samples: UCsh1=umbilical cord sheet mfr 1; UCsh2=umbilical cord mfr 2; AMUCliq3=amniotic membrane/umbilical cord liquid mfr 3; AMsh1=amniotic membrane sheet mfr 1; AMUCsh3=amniotic membrane/umbilical cord sheet mfr 3; AFliq1=amniotic fluid liquid mfr 1, AFliq2=amniotic fluid mfr 2; AMAFliq4=amniotic membrane/amniotic fluid mfr 4.

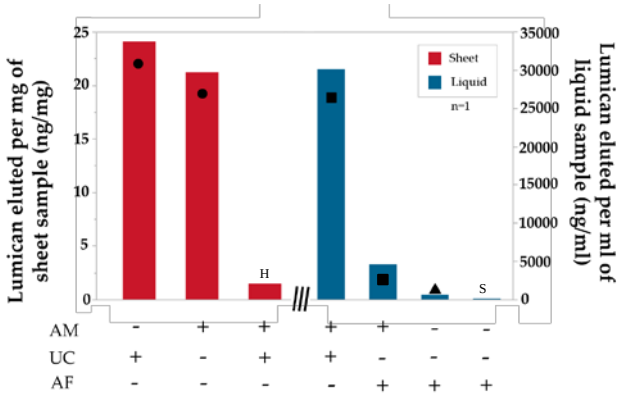

Fig 2. Lumican elution at 48 hours. Dehydrated sheet samples eluted 10 times more lumican than hydrated sheet samples. Particulate and lyophilized liquid samples containing more than one type of amniotic tissue eluted 67 and 10 times more lumican, respectively, than samples containing only amniotic fluid. AM=amniotic membrane, UC=umbilical cord, AF=amniotic fluid, ● =dehydrated sheet, H = hydrated sheet, ■ =particulate liquid, ▲ =lyophilized liquid, S = standard amniotic fluid.

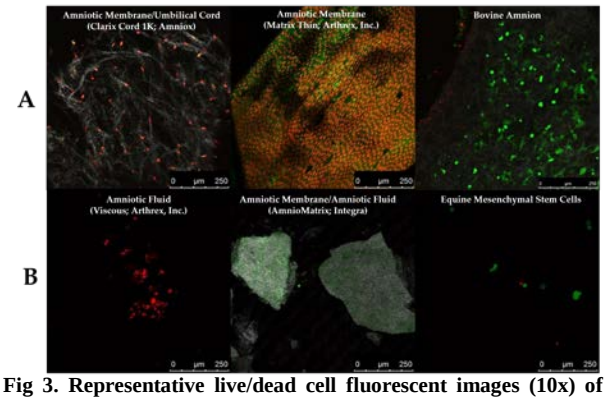

Fig 3. Representative live/dead cell fluorescent images (10x) of A) sheet and B) liquid amnion-derived products. Samples were stained with calcein AM (green/live), ethidium homodimer (red/dead), and collagen reflectance (grey), and imaged using confocal microscopy. There are no live cells in any of the products compared to the positive controls of bovine amnion and equine mesenchymal stem cells. Green coloration seen in samples was determined to be autofluorescence at higher magnifications.