Cryopreserved Amniotic Fluid/Membrane/Wharton's jelly graft bioassay; Indications for use in regenerative therapeutics

ARTICLE INFO

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Keywords:
Placenta
Cryopreserved, Wharton's jelly, Amniotic fluid,
Amniotic membrane

ABSTRACT

Rheo a cryopreserved amniotic fluid, Wharton's jelly, ECM allograft, minimally manipulated via the AnuSureTech proprietary process, maintains the functions of placental tissue containing extracellular matrix and bioactive cytokines (growth factors, chemokines). It is the combination of the amniotic fluid, Wharton's jelly, and extra-cellular matrix (ECM), cytokines, their immunomodulatory effect and their anti-inflammatory, immunomodulating, tissue regenerative attributes that make their use in regenerative medicine highly effective and safe, providing significant value in pain control, orthopedic pathologies, organ system pathologies. The bioassay results presented in this paper demonstrate that the AnuSureTech process is a significant advancement in placental tissue processing providing high levels of cytokines, growth factors, cellular components and ECM after processing and sterilization.

1. Introduction

The purpose of this whitepaper is to highlight the background, science and potential uses of human cryopreserved amniotic fluid, Wharton's jelly, and amniotic ECM combination allograft. The BioStem Life Science team has developed novel proprietary techniques (ANUsureTECH) that are well within minimal manipulation guidelines set forth by the FDA in November 2017, yet yield significantly higher concentrations of cellular components, growth factors, chemokines, cytokines, exosomes and miRNA than reported (via literature and marketing materials) by other tissue banks in the placental tissue arena.

This white paper will be focused on the growth factors (GF) and cellular components found in Rheo, a BioStem Life Sciences cryopreserved Amniotic fluid, Wharton's jelly, ECM product. BioStem Life Sciences continues to assay its placental tissue product line and will follow up with analysis of cellular components, cytokine, chemokine, exosomes and micro RNA concentrations as well.

2. Background: Placental tissue and its components

Placental tissue has proven to be a very powerful, versatile and safe allograft therapy in a wide range of pathologies treated in the regenerative medicine arena (1). The human amnion is a single layer of epithelial cells separating the amniotic cavity from the vascularized chorion. The Amniotic Membrane (AM) is the innermost layer of the placenta and consists of a thin epithelial layer, a thick basement membrane, and an avascular stroma. It contains collagen types III, IV, V, and VII and fibronectin and laminin.(40,41,42) It also contains fibroblasts and growth factors, cytokines, exosomes, alpha2macroglobulin, miRNA and a wide spectrum of growth factors at high concentrations. AM has been shown to have unique properties including the ability to suppress pain, fibrosis, and bacteria and to promote wound healing (43,44,45,46,47).

The AM contains 2 cell types of different embryologic origin; specifically, amnion epithelial cells, derived from the embryonic ectoderm, and amnion mesenchymal cells, derived from embryonic mesoderm (48). The recommendation of the International Society for Cellular Therapy has been that mesenchymal cells derived from amnion be referred to as *amniotic membrane-human mesenchymal stromal cells* (AM-hMSCs) (40).

Early in gestation amniocytes start off flattened and produce the amniotic fluid; however, as pregnancy progresses, they become cuboidal and have increasing numbers of microvilli on their apical surface. Tortuous intercellular channels exist between the tight junctions of amniocytes. Vascular endothelial growth factor (VEGF) in the fetal membranes appears to be a mediator of this process. VEGF promotes blood vessel development within the amnion and influences the permeability of the micro-vessels, which perfuse the fetal and placental surfaces (48). In a study, Moshiri and Oryan (45) demonstrated the effectiveness of FGF (Fibroblast growth factor) in restoring the morphologic and biomechanical properties of injured tendon in rabbits (50,51). The innate immune system is the first line of defense against pathogens and includes anatomic and physiologic barriers, enzymes and antimicrobial peptides, phagocytosis, and release of proinflammatory mediators by neutrophils and macrophages. Many of the substances that constitute the innate immune system have been identified in AM and have been shown to have significant antimicrobial properties, including defensins (human neutrophil defensins lactoferrin, lysozyme, bactericidal/permeability-increasing protein, (2,3) calprotectin, secretory leukocyte protease inhibitor, RIS-1/psoriasin (expression in epithelial skin cells indicates their selective role in innate immunity and in inflammatory skin diseases including acne), and a Cathelicidin (Cathelicidin-related antimicrobial peptides are a family of polypeptides, found in lysosomes of macrophages and polymorphonuclear leukocytes (PMNs) and keratinocytes)(15). These potent antimicrobials have shown broad-spectrum activity against bacteria, fungi, protozoa, and viruses. Perhaps the most important of these are the defensins (human neutrophil defensins 1-3), which are found in in AM. Furthermore, lactoferrin is a glycoprotein with 2 binding sites for ferric ions. Lactoferrin is likely secreted by neutrophils and amniotic cells. Lactoferrin has both bacteriostatic activity, owing to the sequestration of iron which is then unavailable for microbial growth, and bactericidal activity, by binding to bacterial outer membranes and triggering release of the lipopolysaccharide lactoferricin. Lactoferricin shows antimicrobial effects against viruses, protozoa, and fungi (53).

There are contributing factors that appear to minimize scarring, such as hyaluronic acid and the presence of hyaluronic acid-stimulating factor. In a study of the effect of AM on proteases important to wound healing, human AM was shown to enhance collagenase activity but to inhibit activation of hyaluronidase, elastase, and cathepsin (54,55).

The amnion has been used as a physiologic wound dressing and as a graft for skin wound coverage (44,45,46,47). Human AM has proved to be a versatile temporary biologic dressing in studies involving hundreds of patients during the past century. The first reported use of fetal membranes was in skin transplantation in the early 1900s (59,60). AM was also used on burned and ulcerated skin surfaces, and clinicians reported a lack of infection, a marked decrease in pain, and an increased rate of re-epithelialization of the traumatized skin surfaces. Others have demonstrated the use of AM as a biologic dressing for open wounds, including burns and chronic ulceration of the legs (61). In traditional medicine, the first reported use was by Davis (59) in 1910 at Johns Hopkins Hospital for burns and ocular wounds in 550 cases. In 1914, Sabella (60) reported similar positive findings. Numerous reports were published during the 1940s and 1950s, until the 1970s when the human immunodeficiency virus/acquired immunodeficiency syndrome became epidemic, and its source was unclear (61). AM was no longer favorable as a treatment choice until 1995 when Kim and Tseng (60) presented their findings. Subsequently, published research has been increasing.

The wounds treated with AM responded to a protocol that allowed coverage of tissues as diverse as exposed bowel, pleura, pericardium, blood vessels, tendon, nerve, and bone. Wounds unresponsive to standard therapeutic measures have also responded to application of AM, and human AM dressings have become a useful adjunct in the care of complicated wounds (63,64).

Reports on the immunogenicity of human amniotic epithelial cells after transplantation into human volunteers have also been published (65-69). Amnion, consisting of a monolayer of epithelium on a basement membrane with an underlying collagen matrix containing a few fibroblasts (which, in theory, would express HLAs, although the epithelium itself lacks them), has been transplanted into subcutaneous pouches in normal human volunteers. None of the volunteers showed clinical signs of rejection (graft-host reaction), and amniotic epithelial cells were demonstrated by biopsy up to 7 weeks after implantation.

HLA antibodies were not detected in serum samples, and no in vitro lymphocyte reaction to the amniotic cells was found in 2 out of 100 volunteers. These results suggest that acute immune rejection does not occur after allotransplantation of human amniotic epithelial cells.

In 1979, Trelford and Trelford-Sauder (42) found that AM transplantation promoted epithelial healing, reduced inflammation, increased comfort, and decreased the severity of insufficient vascularization. In 2002, Ucakhan et al (68) did not find any infectious, inflammatory, or toxic reactions related to AM transplantation. Amnion surface epithelial cells do not express HLA-A, -B, -C, or -DR or b2-microglobulin (71-72).

Ucakhan et al (68) evaluated safety and efficacy of non-preserved AM transplantation with or without limbal autograft transplant in acute and chronic eye injuries. In the transplantation of human organs, whether skin, kidney, liver, or other tissue, the major problem has been rejection of the grafted tissue owing to the host immune response. Despite this risk, amnion has been used successfully as a skin graft without concern for tissue typing and matching of the donor to the host (69). This unique attribute (the lack of immunogenicity) has been described in numerous clinical studies and scientific journals and has led to the characterization of the placental organ as *immune privileged*. Thus, granulized AM and AF (gAM-AF) has been considered by many to be ideal for use in all patients, including the most immunocompromised, such as post-transplant and human immunodeficiency virus-positive patients, and others with compromised immune systems, who could be adversely affected by human tissue transplantation or infection. The unique biologic structure of amniotic tissue, coupled with the low risk of an adverse host immune response, makes gAM-AF ideal for an in vivo wound covering.

Experimental and clinical studies have demonstrated that AM transplantation promotes re-epithelialization, decreases inflammation and fibrosis, and modulates angiogenesis (72). Several growth factors produced by AM are involved in these processes, including TGF-b and basic FGF (73). Additionally, recent published reports have reported that specific soluble factors secreted by human amniotic epithelial cells into AF might be effective in ameliorating liver fibrosis, COPD, and chronic kidney conditions. Extracellular vesicles (EVs) are secreted nanosized (40-100 nm) membrane vesicles that may act as a novel cell-cell communicator. BioStem Life Sciences will address this more specifically in additional reports to be published.

One needs to also consider the Hyaluronic Acid component of AF (amniotic fluid). A study by Lockington (98) demonstrates that AM is able to remove reactive oxygen species (ROS) from its environment. Demonstrating total antioxidant capacity in AM provides evidence for its use as a free radical scavenger. An increased awareness of the role of free radicals in corneal disease may lead to treatment strategies utilizing antioxidant agents derived from HA or AM.

The AM anti-inflammatory action may be mediated in part by interleukin-10 **(IL-10)**, of which we detect significant amounts in AM extracts. IL-10 is known to suppress or counteract the actions of pro-inflammatory cytokines such as IL-611 and tumor necrosis factor-alpha (TNF-a) (49). IL-10 also suppresses amniotic cell production of IL-8,13 which is a pro-inflammatory chemokine attracting the migration of neutrophils. TCF-13 superfamily provides the protein substrates for production of inhibin and activin. Activin promotes the production of prostaglandin **PGE2**. (51,52) A low dose of activin stimulates, but a high dose of activin inhibits, the production of IL-6, IL-8, and PGE2 by the AM. (53) No such effect is noted in the chorion or decidua. TNF-a is significantly inhibited by activin in the chorion and decidua's (53). The AM contains various protease inhibitors, including anti-trypsin inhibitor (54), which may exert an anti-inflammatory effect (55). Future studies are needed to determine whether IL-10, activin, protease inhibitors, and/or a combination of them are responsible for the anti-inflammatory action of AM when it is transplanted to the ocular surface.

The AM contains IL-1 receptor antagonist **(IL-1)** and helps transport it to the amniotic fluid.19 IL-lRA is a potent inhibitor of lL-1, and thus will suppress the inflammation mediated by IL-1. Data has shown that limbal epithelial cells cultured on the AM stromal matrix downregulate the expression and production of IL-1 but upregulate the expression and production of IL-lRA, resulting in a higher ratio of IL-lRNIL-1 (56). Such an effect withstands the challenge of lipopolysaccharide (56). These findings support the concept that the AM exerts its anti-inflammatory action by suppressing the signaling pathway via IL-1.

As a review of cytokines, chemokines are proteins found in amniotic membrane and fluid allografts. Cytokines are a broad and loose category of small proteins (~5–20 kDa) that are important in cell signaling. They are released by cells and affect the behavior of other cells, and sometimes the releasing cell itself. Some cytokines enhance or inhibit the action of other cytokines in complex ways. Cytokines include chemokines, interferons, interleukins, lymphokines, tumor necrosis factors, but generally not hormones or growth factors.

Chemokines are a family of small cytokines or signaling proteins secreted by cells. Their name is derived from their ability to induce directed chemotaxis in nearby responsive cells; they are chemotactic cytokines.

Growth factor cytokine	Function
AR	Amphiregulin (AR) (Colorectum cell-derived growth factor) (CRDGF)
BDNF	Brain-derived neurotrophic factor (BDNF) (Abrineurin)
bFGF	Fibroblast growth factor 2 (FGF-2) (Basic fibroblast growth factor) (bFGF) (Heparin-binding growth factor 2) (HBGF-2)
BMP-4	Bone morphogenetic protein 4 (BMP-4) (Bone morphogenetic protein 2B) (BMP-2B)
BMP-5	Bone morphogenetic protein 5 (BMP-5)
BMP-7	Bone morphogenetic protein 7 (BMP-7) (Osteogenic protein 1) (OP-1) (Eptotermin alfa)
b-NGF	Beta-nerve growth factor (Beta-NGF)
EGF	Pro-epidermal growth factor (EGF) [Cleaved into: Epidermal growth factor (Urogastrone)]
EGF R	Epidermal growth factor receptor (EC 2.7.10.1) (Proto-oncogene c-ErbB-1) (Receptor tyrosine-protein kinase erbB-1)
EG-VEGF	Prokineticin-1 (Endocrine-gland-derived vascular endothelial growth factor) (EG-VEGF) (Mambakine)
FGF-4	Fibroblast growth factor 4 (FGF-4) (Heparin secretory-transforming protein 1) (HST) (HST-1) (HSTF-1) (Heparin-binding growth factor 4) (HBGF-4) (Transforming protein KS3)
FGF-7	Fibroblast growth factor 7 (FGF-7) (Heparin-binding growth factor 7) (HBGF-7) (Keratinocyte growth factor)
GDF-15	Growth/differentiation factor 15 (GDF-15) (Macrophage inhibitory cytokine 1) (MIC-1) (NSAID-activated gene 1 protein) (NAG-1) (NSAID-regulated gene 1 protein) (NRG-1) (Placental TGF-beta) (Placental bone morphogenetic protein) (Prostate differentiation factor)
GDNF	Glial cell line-derived neurotrophic factor (hGDNF) (Astrocyte-derived trophic factor) (ATF)
GH	Somatotropin (Growth hormone) (GH) (GH-N) (Growth hormone 1) (Pituitary growth hormone)
HB-EGF	Proheparin-binding EGF-like growth factor [Cleaved into: Heparin-binding EGF-like growth factor (HB-EGF) (HBEGF) (Diphtheria toxin receptor) (DT-R)]
HGF	Hepatocyte growth factor (Hepatopoietin-A) (Scatter factor) (SF) [Cleaved into: Hepatocyte growth factor alpha chain; Hepatocyte growth factor beta chain]
IGFBP-1	Insulin-like growth factor-binding protein 1 (IBP-1) (IGF-binding protein 1) (IGFBP-1) (Placental protein 12) (PP12)
IGFBP-2	Insulin-like growth factor-binding protein 2 (IBP-2) (IGF-binding protein 2) (IGFBP-2)
IGFBP-3	Insulin-like growth factor-binding protein 3 (IBP-3) (IGF-binding protein 3) (IGFBP-3)
IGFBP-4	Insulin-like growth factor-binding protein 4 (IBP-4) (IGF-binding protein 4) (IGFBP-4)
IGFBP-6	Insulin-like growth factor-binding protein 6 (IBP-6) (IGF-binding protein 6) (IGFBP-6)
IGF-1	Insulin-like growth factor I (IGF-I) (Mechano growth factor) (MGF) (Somatomedin-C)
Insulin	Insulin [Cleaved into: Insulin B chain; Insulin A chain]
MCSF R	Macrophage colony-stimulating factor 1 receptor (CSF-1 receptor) (CSF-1-R) (CSF-1R) (M-CSF-R)

	(EC 2.7.10.1) (Proto-oncogene c-Fms) (CD antigen CD115)
NGF R	Tumor necrosis factor receptor superfamily member 16 (Gp80-LNGFR) (Low-affinity nerve growth factor receptor) (NGF receptor) (p75 ICD) (CD antigen CD271)
NT-3	Neurotrophin-3 (NT-3) (HDNF) (Nerve growth factor 2) (NGF-2) (Neurotrophic factor)
NT-4	Neurotrophin-4 (NT-4) (Neurotrophin-5) (NT-5) (Neutrophic factor 4)
OPG	Tumor necrosis factor receptor superfamily member 11B (Osteoclastogenesis inhibitory factor) (Osteoprotegerin)
PDGF-AA	Platelet-derived growth factor subunit A (PDGF subunit A) (PDGF-1) (Platelet-derived growth factor A chain) (Platelet-derived growth factor alpha polypeptide)
PIGF	Placenta growth factor (PlGF)
SCF	Kit ligand (Mast cell growth factor) (MGF) (Stem cell factor) (SCF) (c-Kit ligand) [Cleaved into: Soluble KIT ligand (sKITLG)]
SCF R	Mast/stem cell growth factor receptor Kit (SCFR) (EC 2.7.10.1) (Piebald trait protein) (PBT) (CD antigen CD117)
TGFa	Protransforming growth factor alpha [Cleaved into: Transforming growth factor alpha (TGF-alpha) (EGF-like TGF) (ETGF) (TGF type 1)]

AF also contains carbohydrates, proteins and peptides, lipids, lactate, pyruvate, electrolytes, enzymes, and hormones. The concentration of epidermal growth factor in amniotic fluid is fourfold greater than in maternal serum AF also contains transforming growth factor (TGF)- TGF-b1, and fibroblast growth factor (FGF).
Heil et al (56) demonstrated that patients with occlusive vascular disease developed a prominent collateral vascular network below the occlusive site through spontaneous arteriogenesis (the remodeling of existing arterio-arteriolar anastomoses to developed functional arteries) and angiogenesis (new capillary growth induced by hypoxic conditions) after being injected.

Amniotic fluid represents an abundant source of multipotent stem cells, referred as broadly multipotent given their differentiation potential and expression of pluripotency-relate. These amniotic fluid-derived stem cells (AFSCs) have a phenotype similar to bone marrow-derived (multi-potent) mesenchymal stem cells (BMSCs) AFSCs are often referred to as "broadly multipotent" given the expression of factors involved in the maintenance of pluripotency in embryonic stem cells (ESCs), such as OCT4,NANOG, andSSEA-4 (2,3). AFSCs have also been differentiated into cell types of all three germ layers, including adipogenic, osteogenic, myogenic, hepatic, neuronal, and endothelial lineages. (4)(1)(5)(6) In humans, AFSC genes being involved in the undifferentiated state of cells have received major attention given that AFSCs are thought to represent an intermediate stage between pluripotent stem cells and lineage-restricted adult stem cells and ultimately can reacquire pluripotency through reprogramming more easily. (1)

Human amniotic fluid stem cells (hAFS) have shown a distinct secretory profile and significant regenerative potential in several preclinical models of disease. hAFS actively release extracellular vesicles (EV) endowed with significant paracrine potential and regenerative effect. hAFS secreted EV ranged from 50 up to 1,000 nm in size. In vitro analysis defined their role as biological mediators of regenerative, paracrine effects while their modulatory role in decreasing skeletal muscle inflammation in vivo was demonstrated. (7–9) Mounting evidence has shown that transplanted stem cells can release trophic signals that influence the microenvironment. This has led to a significant shift in paradigms, from exploring the stem cell genome to analyzing the stem cell "secretome,"

the latter of which includes the entirely of growth factors and chemoattractant molecules produced by stem cells. In this scenario, growing interest has focused on the characterization of stem cell-

secreted extracellular vesicles (EV). EV are membrane-bound cellular components enriched with soluble, bioactive factors (proteins, lipids, etc.) and RNA (mainly regulatory microRNA — miRNA). They elicit a wide range of effects while mediating intercellular transfer of information

on the responder cell, consequently modulating its function. EV

are secreted as micro-sized (micro-vesicles: 100–1,000 nm) and

nanosized (exosomes: 30–150 nm) particles, acting as key

biological effectors of paracrine signaling. (10) Microvesicles are

released as shedding vesicles by direct budding of the plasma

membrane, while exosomes are produced in endosomal

multivesicular compartments (multivesicular body, MVB) and secreted as the MVB fuses with the plasma membrane. (11) Recent studies

have shown that the beneficial effects observed following stem

cell transplantation in several preclinical models of experimental

ischemic disease and injury could be mediated by stem cell-EV.

These include the activation of antiapoptotic and pro-survival path-

ways eliciting angiogenic, anti-inflammatory, and antifibrotic

responses, and the stimulation of resident endogenous

progenitors, overall enhancing organ function. (12) In particular, many

studies have reported the potential efficacy of EV from adult

mesenchymal stem cells (MSC) in providing cardioprotection against

acute myocardial infarction (MI) (13–15) in enhancing wound heal-

ing, (16) counteracting graft-versus-host-disease (17) reducing

renal injury, (18) mediating liver regeneration (19) and stimulating

neural plasticity following stroke (20) Since cell-free delivery of

bioactive cargos by EV recapitulates the same beneficial

responses of stem cell transplantation, they offer remarkable

benefits over conventional cell therapy as immunologically

unresponsive agents (21) (22)

Because of their fetal, but non-embryonic origin, hAFS overcome many ethical concerns and can be easily obtained upon the expression of the stem marker c-KIT from eligible cesarean delivery (23)
c-KIT1hAFS exert remarkable cardioprotective paracrine effects reducing the infarct size in a rat acute model of MI by approximately 14% (19) possibly via the secretion of the cardioactive peptide thymosin beta 4, which, in turn, can reactivate epicardial progenitor cells to give rise de novo to fully mature cardiomyocytes [20]. Moreover, when injected in an established model of necrotizing enterocolitis, rodent c-KIT1AFS

have been shown to improve survival and clinical status, while

maintaining the gut structure and function via paracrine modulation of resident stromal cells expressing cyclooxygenase 2 (COX2)

(24) Similar remarkable results have been also obtained in a

model of skeletal muscle atrophy carrying a homozygous deletion

of the exon 7 within the survival motor neuron (Smn) gene,

specifically directed to new forming skeletal muscle fibers (HSA-Cre, Smn f7/f7 mice), where mouse c-KIT1AFS confirmed their

regenerative potential by providing significant improvement in

muscle strength and in the survival rate of the affected animals,

together with the replenishment of the depleted skeletal muscle

niche. (25)

MicroRNA Profiling of the hAFS-EV

Balbi et al provide an excellent review of the micro RNA profile in amniotic fluid cellular components. (20) hAFS-EV Normo and hAFS-EV Hypo showed to contain small non-coding RNAs and miRNAs (20–40 nucleotides). In particular, hAFS-EV Hypo showed significant enrichment over the hAFS-EVNormo of the following miRNAs (Fig. 6B): miR-223 (***p<.001,1.3-fold), miR-146a (**p<.01, 1.6-fold), miR-let7c (***p<.001,twofold), miR-21 (***p<.001, twofold), miR-126 (****p<.0001,2.5-fold), miR-146b (****p<.0001, almost threefold), miR-199a-3p (**p<.01, almost threefold), miR-210 (****p<.0001, 3.6-fold). (20)

Structural analyses revealed that hAFS actively release immunologically inert EV, as similarly indicated for other MSC-EV (26), which are heterogeneous in size. These include nanosized exosomal particles identified by the expression of TSG101, ALIX, CD81,

CD9, AnnV, and CD63 [5, 34] (11) (27) (28), along with cell specific markers such as CD105. Similar to other stem cells (29), hAFS are likely to originate from a hypoxic niche within the amniotic fluid, and they demonstrated remarkable antiapoptotic effects when transplanted in an ischemia/reperfusion injury rat model (30)
Even if the AM contains the aforementioned anti-inflammatory mediators, its anti-inflammatory action may require a dose contact with its stromal matrix. Clearance of granulocytes by pushing them rapid apoptosis is an effective anti-inflammatory strategy. Recently, Ueta etal (31) also reported that human AM can suppress alloreactive responses and downregulate production of Thl and Th2 cytokines in mouse lymphocytes in vitro. This finding suggests that the AM may also suppress acquired immunity.

3. Methods

A.Ray BioTech method: One standard glass slide is spotted with 16 wells of identical cytokine antibody arrays. Each antibody is arrayed in quadruplicate. Detection Method Fluorescence: with laser scanner: Cy3 equivalent dye. It combines the advantages of the high detection sensitivity & specificity of ELISA and the high throughput of arrays. Like a traditional sandwich-based ELISA, it uses a pair of cytokine-specific antibodies for detection. A capture antibody is first bound to the glass surface. After incubation with the sample, the target cytokine is trapped on the solid surface. A second biotin-labeled detection antibody is then added, which can recognize a different epitope of the target cytokine. The cytokine-antibody-biotin complex can then be visualized through the addition of the streptavidin-labeled Cy3 equivalent dye using a laser scanner. Unlike the traditional ELISA, Quantibody (Ray Biotech) products use array format. By arraying multiple cytokine-specific capture antibodies onto a glass support, quantitative, multiplex detection of cytokines in one experiment is made possible.

For cytokine quantification, the array-specific cytokine standards, whose concentration has been predetermined, are provided to generate a standard curve for each cytokine. By comparing signals from unknown samples to the standard curve, the cytokine concentration in the samples will be determined.

B. DaVinci laboratory method: RHEO product was shipped on dry ice and maintained at -80°C until ready for testing. Each vial was carefully thawed using an active thaw technique in order to rapidly thaw the vial. An aliquot of Rheo product was taken and stained using acridine orange (AO) and propidium iodide (PI) followed by quantification using the Nexcelom Cellometer Auto 2000. Half the vials of cells were then pooled in order to run a flow cytometry for specific cell markers FITC Anti Human S100A4 Antibody for fibroblast, APC Mouse Anti Human CD90 Antibody a marker indicative of mesenchymal stem cells (MSCs) and Mouse Anti Human Cytokeratin7 FITC Antibody for keratinocytes using the BD Accuri C6 Flow Cytometer. The reminder of the vials were processed for proteins in order to run ELISA for vascular endothelial growth factor (VEGF) a known growth factor which increases vasculature, interleukin-1 receptor antagonist (IL-1ra) a known protein which regulates the pro-inflammatory response, beta fibroblast growth factor (B-FGF) a known growth factor that is a strong mitogen and plays a role in cartilage repair, and platelet derived growth factor beta beta (PDGF-BB) a known growth factor that plays a role in cell growth and division besides a significant role in blood vessel formation.

BioStem Life Sciences had quantification analysis completed on 41 proteins, cellular components for our Rheo (cryopreserved amniotic fluid, ECM, Wharton's jelly) utilizing the Ray Biotech Quantibody system and Davinci Labs.

4. Results

Target Cytokine – Growth Factor	Fluid-ECM-Whartons Median Reported Value *picograms per milliliter (pg/mL)*
AR	35.6
BDNF	11.4
bFGF	340.6
BMP-4	0.9
BMP-5	408.7
BMP-7	281.3
b-NGF	0.4
EGF	50.5
EGF R	4,491.9
EG-VEGF	1,300.0
FGF-4	98.0
FGF-7	44.2
GDF-15	1,522.3
GDNF	4.8
GH	67.1
HB-EGF	13.4
HGF	2,315.5
IGFBP-1	3,970.3
IGFBP-2	1,843.3
IGFBP-3	46,676.0
IGFBP-4	15,236.8
IGFBP-6	44,255.6
IGF-1	8.2
Insulin	133.0
MCSF R	1,807.3
NGF R	6.8
NT-3	12.1
NT-4	442.0
OPG	1,031.7
PDGF-AA	216.9
PIGF	57.9
SCF	16.5
SCF R	142.2
TGFa	81.4
TGFb1	4,271.6
TGFb3	84.5
VEGF	10.7
VEGF R2	1,931.2
VEGF R3	52.6
VEGF-D	3.0

For flow cytometry all vials were pooled and total pooled count for analysis was 4.07E+05 cells/ml with a viability of 12.8% because of time lapse of 2 hours and processing of cells for the test being performed. We assessed cell markers indicative of fibroblast, MSCs and keratinocytes, S100-A4, CD90 and Cytokeratin 7 respectively (Figure 2). Each corresponding IgG isotype control is directly below the respected marker (Figure 2). There were 82% of the cells positive for fibroblast cell marker S100-A4, 23.9% positive for MSCs marker CD90 and 0.3% of the cells positive for keratinocyte marker cytokeratin 7. In many instances there could be overlap of cell markers (i.e. cells positive for both S100-A4 and CD90).

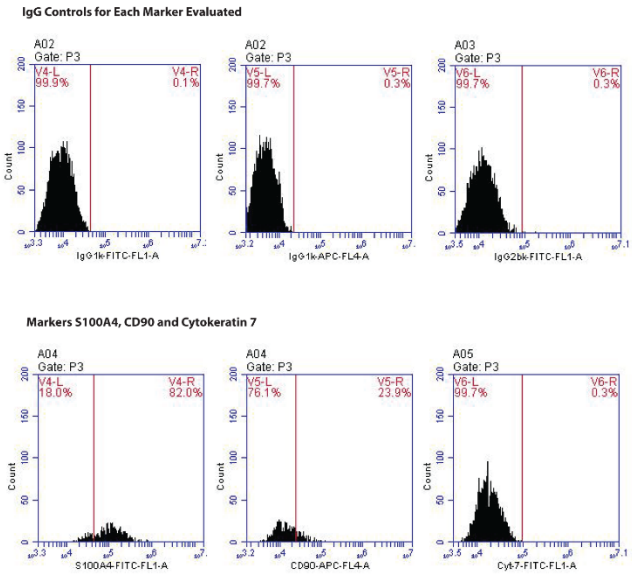

Figure 2: Flow cytometry of BPSRs regenerative medicine product. There were 82% of the cells positive for fibroblast cell marker S100-A4, while 23.9% of the cells were positive for MSCs marker CD90 and 0.3% of the cells were positive for keratinocyte marker cytokeratin 7.

5. Discussion *X*

This review and study indicate that our ANUsureTech proprietary cryopreserved amniotic fluid, Wharton's jelly, ECM (RHEO) allograft process is successful. With minimal manipulation of the allograft components, we retain functional bioactive proteins (cytokines, chemokines, growth factors, robust cellular components). The RHEO allografts' bioactive proteins, combined with the presence of the extracellular matrix, lamin, and fibronectin, present a functional allograft that is effective in a wide array of applications. The applications range from topical wound healing to rapid response for use in orthopedic pathologies, organ system pathologies, pain / anti-inflammatory control, scar management by following the inflammatory chemotactic pathways and providing components, immunomodulation, and scaffolding for healthy tissue generation and repair of injured tissue..

The anti-inflammatory action appears to require contact with the various growth factor/ cytokines in suspension. The mechanism by which RHEO exerts its anti-inflammatory action plays a role in modulating balance M1 and M2 macrophages. Several authors have reported that human amniotic ECM can suppress alloreactive responses suggesting that the amniotic ECM may also suppress acquired immunity.

The high efficiency of WJ-MSC recovery, the minimal ethical concerns associated with its acquirement and use, low immunogenicity, and the fact that they are from healthy, young donors make them an ideal source of MSC for autologous and allogeneic applications.(32) The amniotic fluid has been identified as an untapped source of cells with broad potential, which possess immunomodulatory properties and do not have the ethical and legal limitations

of embryonic stem cells. CD117(c-Kit)1cells selected from amniotic fluid have been shown to differentiate into cell lineages representing all three embryonic germ layers without generating tumors, making them ideal candidates for regenerative medicine applications. Moreover, their ability to engraft in injured organs and modulate immune and repair responses of host tissues,

suggest that transplantation of such cells may be useful for the treatment of various degenerative and inflammatory disease. (22)

The amniotic fluid provides the signaling required for these cells to maintain their undifferentiated status, which would be consistent with a stem cell niche role for the amniotic fluid.

Fetal stem cells retain capacity for proliferation and differentiation greater than that of their adult counterparts, however data reported so far do not describe spontaneous teratoma formation (33). Indeed, Guillot's group have tested the potential of AFSC to form teratoma extensively but proved they could only achieve it. Stultz et al (34) noted that hAF MSC's actually safer than postnatal cells because the MSC in prolonged culture have been found to exhibit defects in genetic stability and differentiation capacity.

In Figure 3 below based on work in multiple centers have demonstrated success, additional multiple authors have cited success. The usefulness to a large pool of patients where injured tissue can be stimulated to repair and regenerate with allogeneic graft. The therapeutic effect appears to be related to the paracrine and immunomodulating effects. Demonstrated T-cell proliferation being inhibited. (24,35)

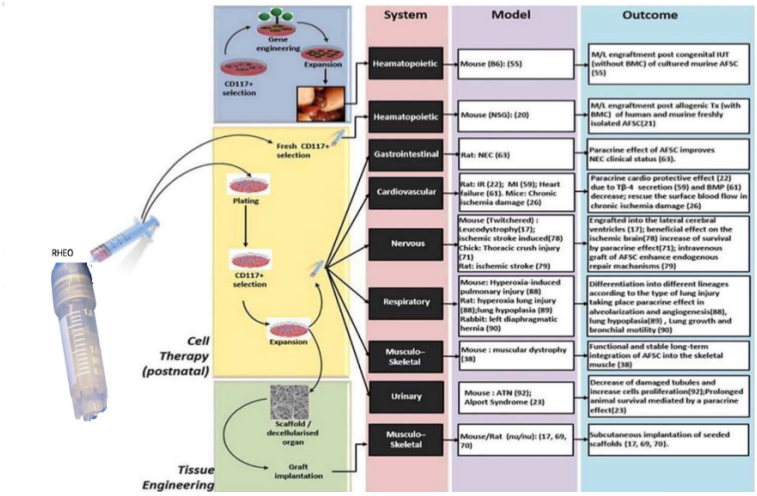

Figure.3 Summary of key supporting evidence from animal models for potential clinical applications of freshly isolated and expanded AFSC.(24,35,36) figure from stem cells (37)

The anti-fibrosis anti-scarring capacity of the ECM present in RHEO is due to the presence of the anti-inflammatory cytokine, IL-10, which can inhibit the production of IL-6 (3,11). Diminished IL-6 production contributes to fetal wound repair without scarring (4,31). The phenomenon of fetal wounds healing without scar was confirmed in a study by Liechty. Three different TGF-beta (1, 2, 3) are the most potent cytokine, promoting myofibroblast differentiation by upregulating expression of alpha-SMA, integrin alpha5beta1, and EDA containing fibronectin **Fn-46** in a number of cell types, including fibroblasts. These factors contribute to healthy tissue healing to form fully functional, flexible tissue, with strong anatomic layering. TGF-beta also upregulates the expression of such matrix components as collagens and proteoglycans, downregulates proteinase and matrix metalloproteinases (MMPs), and upregulates their inhibitors.

The presence of neurotrophic factors that control the growth and targeting of sensory and autonomic nerves to the peripheral tissues (6) are present: nerve growth factor **(NGF)**, brain-derived neurotrophic factor **(BDNF)**,

and neurotrophin-3 **(NT-3)**. The fact that the AM contains these neurotrophic factors suggests that it has a significant role in the development of the fetal nervous system, enabling scarless wound healing. The presence of NGF makes it extremely effective in treating corneal eye lesions (7, 8).

It is the combination of each of these powerful cytokines, chemokines and ECM present in our Amniogen Amnion/Chorion allografts that amplify the healing potential of the recipients.

6. Conclusion

In this review we demonstrate our evidence that regenerative therapeutics requires a combination of components to drive the healing regenerative process using healthy processed placental tissues. It is clear that the proprietary ANUsureTech processing techniques provide enhanced levels of growth factors, miRNA, exosomes, and cellular components to our allografts. The growth factors, cytokines, and ECM all need to work together to provide a safe and efficacious pathway to healing traumatized, injured tissue.

The data above represents characterization of RHEO allograft product done by an independent lab. It characterizes the product by assessing what the end user will get in terms of cells (viability, quantity and cell type) and proteins/growth factors. The product does contain stem cells and many growth factors/proteins such as VEGF, B-FGF, IL-1ra and PDGF-BB. These growth factors play a role in growth, cartilage repair, reducing the inflammatory response and creating new vasculature which increases blood flow.

BioStem Life Sciences, is on a mission to increase the understanding and awareness of the plethora of proteins and extra-cellular matrix interactions in our placental tissue allografts.

As we develop additional assays of cellular components, exosomes, miRNA, various secretome vesicles, and Alpha 2 macroglobulin in our minimally manipulated placental allografts, we will bring this information to our clinical studies in multiple pathologies. BioStem Life Sciences demonstrates that placental tissue allografts processed appropriately bring the whole symphonic orchestra to play in regenerative healing of injured, traumatized and/ or diseased tissue.

ACKNOWLEDGEMENTS

Growth factor assays performed by Ray Biotech. Flow Cytometry by Davinci Labs.

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