INTRODUCTION

In recent years there has been an increase in applications of amniotic tissues in tissue engineering and regenerative medicine, and research to understand the bioactive and signaling components of this tissue type. A rich source of bioactive components for regenerative therapies can be found in amniotic fluid (AF). Many of these bioactive components are present in AF because they are significant contributors to fetal health and development. This includes a diverse set of cell types that have been identified along with a variety of bioactive factors in the fluid itself that can be beneficial in many ways. These factors include proteins, glycosaminoglycans, cytokines, and growth factors that not only facilitate fetal growth and development, but may also participate in wound healing and regeneration of damaged tissue during gestation. This complex medium also contains antimicrobial molecules that protect the fetus in utero from a range of bacterial, fungal, protozoan, and viral infections. Amniotic tissues are an ideal raw material source that is readily available from what would otherwise be clinically wasted tissue. An additional benefit of sourcing this material is that it can be aseptically recovered from a well-screened, healthy patient population. Amniotic tissues present considerably less variability and potential co-morbidities than cadaveric tissues that are used in many regenerative therapies, due to the consistency of donor population age.

THERAPEUTIC AND CLINICAL APPLICATIONS

Amniotic fluid was first considered as a possible therapeutic agent as early as 1935. Early animal studies involved the use of AF concentrate to stimulate the various repair and anti-microbial mechanisms in the peritoneum after trauma. These studies showed that AF concentrate administration into the peritoneal cavity following trauma or surgery presented no unintended negative impacts to patient safety. Studies in rats also showed that the AF concentrate injections stimulated a more robust repair and defense reaction, compared to untreated animals. In 1938, AF concentrate was used in a study to evaluate its efficacy in treating various joint pathologies. This study demonstrated that there were no adverse effects associated with the injections, and that the AF concentrate enhanced the defense and repair mechanisms within the joints, and prevented adhesion formation after closed manipulation of the joint. The AF concentrate also demonstrated a prophylactic effect after arthrotomy.

Today, clinical evidence of therapeutic effects of AF are being investigated. Transected peripheral nerves in rats showed significant reduction in scar tissue at the repair site and significantly faster functional recovery when treated with human amniotic fluid compared to a control treatment using saline. Significant improvement in fiber maturation in the nerves treated with human AF was also reported. Therapeutic effects of AF have also been demonstrated in osteoarthritis. Osteoarthritis occurs when cartilage in a synovial joint begins to deteriorate causing pain and loss of functionality that only increase over time. A press release issued by The American Academy of Pain Medicine in 2015 described interim results of a study conducted by Didier Demesin, M.D. with the University Pain Medicine Center in Somerset, N.J., which showed positive results using an AF allograft for the treatment of knee osteoarthritis. Early analyses of the study outcomes showed that the AF allograft improved pain and functionality and had a lower occurrence of adverse effects compared to other injections in osteoarthritic knees. The AF allograft also demonstrated longer lasting effects and greater overall improvement compared to other injections.

Amniotic fluid has been suggested as a therapeutic agent for use in mitigating chronic discogenic back pain. Chronic discogenic back pain in the lower back is associated with several complications including degenerative osteoporosis, intervertebral disc prolapse, and compression collapse. In a study conducted on 42 patients with lower back pain, an AF injection was evaluated for its efficacy in relieving pain and distress on the affected area compared to steroid injection. The group receiving the steroid injection experienced pain relief, but the group receiving the AF injection scored much better in pain improvement based on the visual analog pain scale (VAS). The group that received the AF injection also scored better on the walking distance (WD) evaluation and the Health Assessment Questionnaire (HAQ). These studies indicate that AF shows promise in various therapeutic and clinical applications. Additional research is required to elucidate the mechanism of action by which AF plays a role in these applications, and several studies have already been published on the subject.

MECHANISM OF ACTION

The mechanism of action of AF in regenerative medicine applications is currently unclear, though efforts are being made to clarify the phenomenon. To date, the focus...
surrounding the application of amniotic tissues in the regenerative medicine space has been centered around the content or concentration of “stem cells”, rather than the bioactive components contained within the processed tissue. Stem cells derived from native AF, referred to as amniotic fluid derived mesenchymal stem cells (AF-MSC), led many to conclude that AF benefits when used as an autologous therapy were attributed solely to the AF-MSC content of the tissue. \(^{10-12}\)

Mesenchymal stem cells (MSCs) are a class of cells that can be readily isolated and expanded in culture, and can be induced to become a variety of mesodermal phenotypes. The term “stem cell” was used to describe this cell type due to in vitro characteristics, and it was thought that once implanted in vivo these cells would migrate to sites of injury where they would then differentiate into regenerating, tissue-producing cells. Historically, the benefits of MSCs were thought to be attributed to their ability to differentiate in vivo, but a paradigm shift has occurred in the way we consider the therapeutic mechanism of action of MSCs.

Over the last several years Professor Arnold Caplan (the “father of the mesenchymal stem cell”) has urged that these cells now be described as “medicinal signaling cells,” instead of “stem cells.” In vivo, infused MSCs do not appear to differentiate or replace tissue; rather, they target a site of injury or inflammation and secrete bioactive factors including growth factors. \(^{13}\) MSCs release many types of growth factors, including epithelial growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF). Mechanistic studies have focused on these critical growth factors to better understand MSC-mediated tissue repair. The benefits attributed to MSCs are now being equally associated with their medicinal signaling mechanisms, in addition to their ability to differentiate in vitro. Extensive clinical trials are being conducted to demonstrate safety and effectiveness of MSC therapies, yet many economic, technical, and regulatory challenges remain for the commercialization of these cell therapies.

Many of the key bioactive signals investigated in MSCs have also been identified in native AF. As more knowledge is gained in the fields of amniotic tissues, MSC functionality, and the synergies between the two, the focus of amniotic tissue has shifted from the MSC content to understanding all of the bioactive components in the tissue, how they work together, and the potential benefits associated with these components.

AMNIOTIC FLUID COMPONENTS

Amniotic fluid contains many complex bioactive and signaling components that are critical to its therapeutic mechanism of action. As the focal point of AF in the regenerative medicine space shifts from the function of MSCs to encompass many of its beneficial components, it is important to explore these components and the contributions they may provide to therapeutic and clinical applications.

Cells

As stated previously, the therapeutic mechanism of action of AF was historically attributed to a small subpopulation of undifferentiated stem cells. Freshly isolated AF samples typically have a total cell concentration on the order of magnitude of \(10^5\) cells/ml \(^{15,16}\). Cell characterization studies have demonstrated about 1% of the initial amniotic cell fraction are CD117\(^+\) which is a common marker used for the selection of AF-MSC. The inherently low population of stem cells in unprocessed AF means minimally manipulated AF products contain a very small sub-section of stem cells and therefore should not be classified as a “stem cell therapy”. In particular MSC therapies typically deliver millions of MSCs per kg of body weight for therapeutic doses, which requires the controlled culture expansion of these undifferentiated cells prior to clinical use. \(^{17}\) Instead, the cells in AF represent a heterogeneous population that includes cell types derived from fetal membranes and from the fetus itself. The majority of cells in the AF are differentiated and include epithelial-like cells and fibroblast-like cells \(^{18}\). These stromal cell fractions (cells that make up certain types of connective tissue) along with their intra-cellular protein and cytokine content are key therapeutic components of AF due to their roles in utero to support fetal development during gestation.

Hyaluronic acid

Hyaluronic acid (HA) is a key component of many of the body’s fluid environments including AF and synovial joints (e.g. knee joints). Hyaluronic acid is a naturally occurring glycosaminoglycan found in AF and provides mechanical cushioning, lubrication, and protection to the fetus inside the womb. It is also thought to be an essential component to fetal wound healing. \(^{19}\) In synovial fluid and joints, HA plays a similarly crucial role. Hyaluronic acid has a unique composition of both solid and fluid characteristics acting as a scaffold for proteoglycan aggregates, extracellular matrix maintenance, and nutrient diffusion. \(^{20,21}\) Native HA also stimulates cell proliferation and differentiation, cell migration, and cell locomotion. \(^{22}\)

Currently, there are synthetic HA viscosupplements available for treatment of osteoarthritis.
Synthetic HA treatments may be generated by extraction and purification of HA from rooster combs or purified from bacterial culture fermentations. Due to differences in production, there is substantial variation in molecular weights of HA in each of these products and the clinical implication of the molecular weight of HA is still inconclusive. Though all of the current synthetic intra-articular HA viscosupplementations show positive impacts on joint pain and functionality, they are associated with certain disadvantages, such as increased immunogenicity and more microbial factor inflammatory properties.

Proteins, Cytokines, Growth Factors

Another essential group of AF components that is important for the growth and development of the fetus are cytokines. “Cytokine” is a general term that is used to encompass a number of molecules such as growth factors, interferon, and interleukins that are multi-functional and have multiple effects on various cell types. While growth factors are characteristically molecules that have positive effects on cell division and proliferation, interferon and interleukins are molecules that are produced during an immune response. Amniotic fluid is rich in growth factors that play a role in cell proliferation, migration, proteoglycan synthesis, and more. Amniotic fluid is also rich in enzyme inhibitors, including tissue inhibitors of metalloproteinases (TIMPs), which inhibit specific matrix metalloproteinases (MMPs). A few key AF cytokines and enzyme inhibitors and their molecular functions in the body are highlighted in Table 1. These can be found in soluble AF protein fractions and many can also be identified within the AF stromal cell fractions.

Non-Therapeutic Components

Amniotic fluid has many components and properties attributed to the positive role the fluid plays in the development of a growing fetus. However, native AF also contains waste and non-essential components including, fetal urine, vernix caseosa, red blood cells, and other wastes. Ideally, these non-therapeutic components should be removed or not selected for during processing of AF for therapeutic applications.

A number of AF products exist on the market for various therapeutic applications. The retention of beneficial components that are critical to the therapeutic mechanism of action of AF depends on how AF is processed and preserved. Today, various methods of processing and preservation are used to create regenerative medicine therapies from AF.

Table 1: Potentially Beneficial Factors and Their Functions Found in Native Amniotic Fluid

<table>
<thead>
<tr>
<th>Factor</th>
<th>Function</th>
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<tbody>
<tr>
<td>TIMP-1</td>
<td>- Inhibits MMPs, some of which may promote inflammation in arthritic joints</td>
</tr>
<tr>
<td></td>
<td>- Inhibits bone matrix degradation, and MMP-13</td>
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<tr>
<td></td>
<td>- Participates in tissue remodeling of extracellular matrix (ECM)</td>
</tr>
<tr>
<td></td>
<td>- Induces growth of chondrocytes, fibroblasts, epithelial cells and endothelial cells</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>- Promotes fibroblast growth</td>
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<tr>
<td></td>
<td>- Inhibits the activity of MMP-2, and MMP-13, which are destructive in diseased joints</td>
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<tr>
<td>EGF</td>
<td>- Stimulates proteoglycan synthesis</td>
</tr>
<tr>
<td></td>
<td>- Stimulates hyaluronan synthesis via production of hyaluronan synthase 2 (HAS2)</td>
</tr>
<tr>
<td></td>
<td>- Participates in re-epithelialization in wounds</td>
</tr>
<tr>
<td>FGF</td>
<td>- Stimulates proteoglycan synthesis</td>
</tr>
<tr>
<td></td>
<td>- Stimulates hyaluronan synthesis via hyaluronan synthases 1 and 2 (HAS1 and HAS2)</td>
</tr>
<tr>
<td></td>
<td>- Stabilizes proteoglycans to reduce proteolysis and the diffusion of proteoglycan from wound sites</td>
</tr>
<tr>
<td></td>
<td>- Stimulates proliferation of various cell types</td>
</tr>
<tr>
<td></td>
<td>- Regulates cell migration and differentiation</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>- Counteracts cartilage degradation by downregulating IL-1 receptor expression</td>
</tr>
<tr>
<td></td>
<td>- Anti-inflammatory</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>- Elevates levels of hyaluronan and HAS2 production and promotes synthesis of type I collagen</td>
</tr>
<tr>
<td></td>
<td>- Anabolic reagent that promotes proliferation and reduces apoptosis of human articular chondrocytes</td>
</tr>
<tr>
<td></td>
<td>- Anti-microbial factor</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>- Required for normal wound repair</td>
</tr>
<tr>
<td></td>
<td>- Stimulates fibroblast proliferation</td>
</tr>
<tr>
<td></td>
<td>- Stimulates fibroblasts to synthesize collagen matrices</td>
</tr>
<tr>
<td></td>
<td>- Stimulates matrix remodeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>- Stimulates angiogenesis</td>
</tr>
<tr>
<td></td>
<td>- Stimulates proliferation of macrovascular endothelial cells</td>
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</table>
AMNIOTIC FLUID PROCESSING FOR THERAPEUTIC APPLICATIONS

Due to the mechanism of action of critical AF components, the ideal processing of AF therapies should be selective to retain the therapeutic cell and protein components and to eliminate the non-therapeutic components. Effective AF therapies should also characterize the components following processing to ensure the desired components are preserved in the final product and remain available throughout the products’ intended shelf-life. Ideally, preservation methods should not introduce undesirable components to the therapy. Current advances in amniotic tissue technologies are now focused on preserving bioactive tissue factors without additives as the key to developing accessible, advanced regenerative therapies.

Many regenerative medicine products are preserved using cryopreservation, requiring the final product to be maintained frozen at very low temperatures (-20°C to -80°C) until use. Cryopreservation of these products allows for a prolonged shelf-life, while maintaining product integrity. Without cryopreservation, these products would have to be used immediately upon preparation, which is not practical given that they are not produced in locations that are readily accessible to the point of care.

Cryopreservation allows product to be stored and delivered to the point of care; however, several disadvantages should be considered. Cryopreservation complicates cold-chain logistics as these products require proper handling and care to ensure they do not thaw in transit. Once thawed, cryopreserved products may only retain integrity for a small amount of time before the components that make them effective begin to degrade.

The use of cryopreservatives to extend shelf-life adds chemical components to these products that not only dilute the active components of the product, but also play no role in the healing processes and may actually have deleterious effects on the healing process. Some AF products are known to contain up to fifty percent (50%) v/v cryopreservation agents. Many AF products purported to have “viable cells” as an active component contain dimethyl sulfoxide (DMSO), a common chemical added to cryopreservatives. DMSO is a permeating cryoprotectant that prevents ice crystal formation, and protects cells from rupture during the freezing process. Despite its protective properties against freezing, DMSO is toxic to viable cells and has negative impacts on cell survivability, proliferation, and induces cell death after thawing. Cryopreservation and thawing methods using DMSO may induce or silence genes that are responsible for cell survival. While cryopreservation using DMSO does protect cellular membrane integrity during the freezing process, it can have detrimental effects on cells once the product is thawed. There have been several reports of complications in cases using DMSO-preserved autologous hematopoietic stem cell transplants isolated from bone marrow. DMSO has dose-dependent adverse effects that occur in some patients but not in others. These symptoms include nausea, vomiting, fever, chills, transient hypertension or hypotension, and anaphylaxis. These symptoms are treated as they appear and usually don’t lead to any long-term effects. Occasionally, more complicated neurologic symptoms can arrive after an infusion of DMSO-preserved stem cell transplants. Isolated cases of severe neurotoxicity, encephalopathy, transient global amnesia, and seizures have been reported in conjunction with use of this preservative in stem cell transplants.

Ideally, accessible AF therapies would be shelf-stable and would not contain any unnecessary additives. Liquid formulations are generally not considered shelf-stable given the susceptibility of proteins to denaturation and aggregation under stresses, such as heating, freezing, changes in pH, and agitation, all of which can result in the loss of biological activity over time. The pharmaceutical and biotechnology industries have used freeze drying (lyophilization) techniques for decades to increase the stability and shelf-life of proteins, cytokines and growth factors. It has been demonstrated that lyophilized MSC extracts can retain equivalent levels of bioactive proteins and exhibit a nature similar to that of intact cells in an osteoinductive application. Tissue engineered constructs (TECs) containing Wharton’s jelly-derived MSCs were devitalized through lyophilization of the tissue constructs and 49 different cytokines and growth factors derived from these MSCs were preserved in the process. The proteins released from these TECs were capable of improving osteogenic behavior and gene expression in bone marrow MSCs. Essentially, these non-viable cells still retained their bioactive contents following preservation via lyophilization. In a similar way, non-viable AF cells can be preserved during a lyophilization process and remain intact to serve as reservoirs that release bioactive molecules capable of signaling. Analysis and characterization of the bioactive signals contained within these preserved AF stromal cells and fluid fractions should be the focus for developers and manufacturers of shelf-stable AF regenerative medicine therapies.

Therapies comprised of human cells carry inherent risks such as communicable disease transmission and generation of an undesirable immunological response in the recipient. The likelihood for occurrence of these risks and appropriate risk mitigation techniques, are dependent on the classification of the tissue therapy. These human cell...
therapies fall under two categories: autologous and allogenic.

- Autologous therapies are produced from a patient’s own cells, which negates the risk of transmitting communicable disease and undesirable immunological responses, but requires the patient to donate their own tissue from which to isolate these cells. While some risks are mitigated with autologous therapies, the collection methods present disadvantages such as lack of economic and regulatory control, and lack of standardization on how the cells are isolated and prepared before being administered to the patient.

- Allogenic cell-based therapies increase the risk of communicable disease transmission as they contain cells isolated from donor tissue intended to be implanted in a different, non-related individual. Dehydrated allogenic products containing non-viable cells can be terminally sterilized by suitable methods, retaining the important bioactive components while effectively reducing the risk of communicable disease transmission from donor to recipient.

Preserved “off-the-shelf” therapies” are ideal as manufacturers can minimize the risk of communicable disease transmission by utilizing terminal sterilization techniques without having to worry about cell viability. Furthermore, allogenic therapies are preferred because they do not require removal of host tissue from the patient to derive therapeutic cells.

CONCLUSIONS

Amniotic fluid is intrinsically a rich tissue source for the development of regenerative medicine products because of its bioactive components that contribute significantly to fetal health and development. The potential of AF as a therapy has been investigated for decades and has shown positive results in studies of various ailments such as osteoarthritis, lower back pain, and peripheral nerve damage. Previously, the benefits of AF were attributed to MSCs present in the fluid; however, the focus has shifted to the idea that multiple constituents of AF are critical to the therapeutic mechanism of action. These bioactive constituents include various cell types, cytokines, growth factors, and enzymes that are multifunctional, and may have applications in a variety of regenerative medicine therapies. Maintaining the integrity of the beneficial components in processed AF depends on the method of preservation. Methods such as cryopreservation allow products to be stored and delivered to the point of care, but utilize unnecessary additives and require proper cold-chain logistics for transport. The prospect of offering allogenic AF as an “off-the-shelf” therapy eliminates a number of challenges facing the field of regenerative medicine. Manufacturing products using preservation techniques such as freeze drying ensures cells, proteins, and cytokines are preserved in these therapies. By eliminating the need for viable cells while ensuring a stable protein and cytokine content, products can be stored on the shelf and be readily available for use at the point of care. These “off-the-shelf” technologies improve consistency, accessibility, and eliminate the need for cold-chain logistics. As current characterization techniques advance it will also be important to take product characterization beyond the cellular level down to the signaling components. Greater attention should be paid to the signaling components of non-viable cells and proteins fractions to offer therapies that provide relief to targeted anatomies.

REFERENCES