Are Amniotic Fluid Products Stem Cell Therapies?

A Study of Amniotic Fluid Preparations for Mesenchymal Stem Cells With Bone Marrow Comparison

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Background: In vivo amniotic fluid is known to contain a population of mesenchymal stem cells (MSCs) and growth factors and has been shown to assist in healing when used as an adjunct in procedures across multiple medical specialties. It is unclear whether amniotic fluid products (AFPs) contain MSCs and, if so, whether the cells remain viable after processing.

Purpose: To determine whether MSCs, growth factors, and hyaluronan are present in commercially available AFPs.

Study Design: Descriptive laboratory study.

Methods: Seven commercial companies that provide amniotic fluid were invited to participate in the study; 3 companies (the manufacturers of PalinGen, FloGraft, and Genesis AFPs) agreed to participate and donated AFPs for analysis. The AFPs were evaluated for the presence of MSCs, various growth factors relevant to orthopaedics (platelet-derived growth factor ββ, vascular endothelial growth factor, interleukin 8, bone morphogenetic protein 2, transforming growth factor β_1), and hyaluronan by enzyme-linked immunosorbent assay and culture of fibroblast colony-forming units. These products were compared with unprocessed amniotic fluid and 2 separate samples of MSCs derived from human bone marrow aspirates. All groups used the same culture medium and expansion techniques. Identical testing and analysis procedures were used for all samples.

Results: MSCs could not be identified in the commercial AFPs or the unprocessed amniotic fluid. MSCs could be cultured from the bone marrow aspirates. Nucleated cells were found in 2 products (PalinGen and FloGraft), but most of these cells were dead. The few living cells did not exhibit established characteristics of MSCs. Growth factors and hyaluronan were present in all groups at varying levels.

Conclusion: The AFPs studied should not be considered ''stem cell'' therapies, and researchers should use caution when evaluating commercial claims that products contain stem cells. Given their growth factor content, however, AFPs may still represent a promising tool for orthopaedic treatment.

Clinical Relevance: Amniotic fluid has been proposed as an allogenic means for introducing MSCs. This study was unable to confirm that commercial AFPs contain MSCs.

Keywords: bone marrow aspirate concentrate; growth factors/healing enhancement; amniotic fluid; stem cell therapy; mesenchymal stem cells

Products derived from amniotic tissue come in various forms such as amniotic fluid, amniotic membrane sheets, and other amniotic particulate derivatives. Mesenchymal stem cells (MSCs) are known to be present in amniotic fluid in vivo,[14,23,28] and amniotic fluid has thus garnered attention as a potential source of these cells. The content of growth factors in amniotic fluid has been well reported in the literature.[4,14,16,23,26,28] The presence of MSCs and growth factors prenatally allows us to theorize about the role of amniotic fluid as an orthobiologic agent due to its anti-inflammatory, proliferative, and reparative therapeutic benefits, which include promotion of neochondrogenesis,[21] peripheral nerve and bone regeneration,[16,20] healing of human skin wounds,[19] and corneal reepithelialization.[7] Some companies assert that their amniotic fluid products (AFPs) can deliver MSCs and growth factors to targeted orthopaedic injuries through an allograft medium.

Riboh et al[24] noted that little research is available on different amnion-derived products for clinical use and stated that equivalency cannot be assumed among different manufacturers. Controversy exists as to whether the

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AFPs on the market actually contain viable MSCs. The proper terminology, role, and formal criteria for the definition of these cells are being debated,[3,5,6,8,18,25] but such discussion is out of the scope of this study. In this study, the term *mesenchymal stem cells* is used to describe the cells in order to remain consistent with previous literature regarding amniotic fluid[14,23,28] and the assertions of manufacturers of amniotic-derived products.

Most investigators who have reported MSCs in amniotic fluid process their samples for cellular analysis immediately after a live birth or an amniocentesis in the second trimester.[4,14,23,28] Although basic science data support that amniotic fluid contains a heterogeneous population of cells including MSCs in vivo,[14,23,28] it is unclear whether these cells are present, viable, or clinically relevant after processing and cryopreservation. This study aims to evaluate 3 commercially available AFPs for the presence of MSCs, growth factors relevant to orthopaedics, and hyaluronan. The cellular and protein content of the preparations was compared with unprocessed cryopreserved amniotic fluid and human bone marrow aspirate.

METHODS

We invited 7 commercial companies to participate in the study; 3 companies agreed to participate and donated AFPs for analysis. The 4 companies that declined participation were Arthrex, MiMedx, Apex Biologics, and AmnioLife (now Alaris Biologic Technologies).

The manufacturers of PalinGen (BioPro), FloGraft (Applied Biologics), and Genesis (Genesis Biologics) each provided three 1-mL vials, corresponding to different production lots. All AFPs were stored at –80°C[15] until the day of experimentation. The manufacturers of PalinGen and FloGraft declared that the provided grafts contained viable MSCs, whereas the manufacturer of Genesis claimed that it was an acellular, particle-free product.

The presence of cells in AFPs was determined by 2 methods. First, immediately after the AFPs were thawed, a small aliquot was diluted 1:1 with trypan blue exclusion dye and inspected under a phase contrast microscope using a hemocytometer (Neubauer chamber). Cells were considered to be dead if the trypan blue dye crossed the cell membrane and was present within the cell, and alive if it did not. Cell viability percentage was calculated by dividing the number of live cells by the number of total cells and multiplying by 100.

Second, 100-μL aliquots of each lot were plated into 6-well plates, with 2 mL of the MSC culture medium per well. The MSC culture medium used MEM-α supplemented with 10% fetal bovine serum. Expansion was performed at 37°C with 5% CO_2 in a humidified incubator for 28 days. Wells were thoroughly inspected weekly for the presence or absence of nucleated cells attached to the sides and bottom of the well. After 28 days, to further determine the presence or absence of these cells, the samples were stained for fibroblast colony-forming units (CFU-Fs). Wells were washed once with phosphate-buffered saline (PBS), fixed with formalin for 15 minutes, rinsed once with PBS, and incubated with Giemsa stain (Gibco) for 30 minutes while gently shaking. Wells were then washed twice with PBS and microscopically evaluated.

Unprocessed amniotic fluid was provided by the Department of Surgery, UC Davis, Sacramento, California. The donor was anonymous, and collection was exempt from institutional review board approval. The unprocessed fluid, obtained from 21 weeks gestational age, was stored at –80°C[15] without any cryopreservation agents. The unprocessed cryopreserved amniotic fluid underwent the same testing and analysis as the 3 commercial AFPs.

Human bone marrow samples (StemExpress) derived from 2 different donors were used as positive controls and served to ensure that our MSC culture expansion technique and medium were conducive to the growth of MSCs and CFU-Fs. MSCs have been defined as being plastic adherent; homogeneous for $CD34^-$, $CD45^-$, $CD73^+$, $CD90^+$, and $CD105^+$; and capable of differentiation into osteoblasts, adipocytes, and chondrocytes.[2,9] MSCs were isolated from the bone marrow based on techniques previously described in the literature.[9] Mononuclear cells were separated from fresh bone marrow by use of a density gradient (Ficoll). Mononuclear cells were then plated into culture flasks and expanded 5 passages through use of the MSC culture medium. For preparation of conditioned media, MSCs were plated into 12-well plates at a density of 10,000 cells/cm^2. The day after plating, the medium was changed to fresh MSC culture medium, and cells were incubated for an additional 24 hours. Supernatants were then stored at –80°C.[15] The bone marrow samples underwent the same testing as the commercial AFPs and unprocessed amniotic fluid.

Detection of Proteins

The growth factors examined were platelet-derived growth factor with 2 β subunits (PDGF-ββ), bone morphogenetic protein 2 (BMP-2), interleukin 8 (IL-8), vascular endothelial growth factor (VEGF), and transforming growth factor β_1 (TGF-β_1) (Table 1). We also tested for hyaluronan, a known element of the extracellular matrix.[13] Commercial enzyme-linked immunosorbent assay kits (R&D Systems) were used following the manufacturer's instructions to

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TABLE 1
Characterization of Biomolecules

Biomolecule	Abbreviation	Function
Platelet-derived growth factor ββ	PDGF-ββ	Cell proliferation, differentiation, migration
Bone morphogenetic protein 2	BMP-2	Induction of osteoblast and chondrocyte differentiation
Vascular endothelial growth factor	VEGF	Cell migration and activation
Hyaluronan	HA	Joint lubrication, source of proteoglycans
Interleukin 8	IL-8	Cell migration, angiogenesis
Transforming growth factor β_1	TGF-β_1	Control of proliferation, differentiation, and apoptosis

detect the presence of the biomolecules in the commercial AFPs, the unprocessed amniotic fluid, and supernatants from bone marrow–derived samples. Total protein concentration was determined with Coomassie brilliant blue (Sigma-Aldrich) staining at 595 nm. To yield sufficient volumes, 3 lots of AFPs per manufacturer were pulled together and tested twice in each plate (each in triplicate), either without further dilution or diluted 1:10.

Statistical Analysis

This was a descriptive laboratory study, and proving statistical significance was not the purpose. As such, statistical analyses were not performed. Additionally, power analysis was not conducted, as the authors were limited to the small number and volume of samples donated by the companies.

RESULTS

MSCs were not identified in the commercial AFPs or the unprocessed cryopreserved amniotic fluid at any time point. MSCs were identified, however, in both bone marrow samples after the 28 days in culture by plastic adherence and growth of CFU-Fs (Figure 1). On initial examination via phase contrast microscopy (immediately after thawing), both PalinGen and FloGraft contained nucleated cells, while Genesis did not (Figure 2A). The nucleated cells, however, did not exhibit MSC characteristics (ie, plastic adherence, CFU-F growth), and most were dead, as noted by an influx of the trypan blue exclusion dye. For the few AFP samples that contained nucleated cells, flow cytometry could not be conducted, as too few cells were available to perform the assays. The overall nucleated cell viability in PalinGen was 50%. Substantial variability across all lots was noted: Lot 1 had 50% viability, lot 2 had 0% viability, and lot 3 had 100% viability. FloGraft had an even lower overall nucleated cell viability, approximately 16%, with an even greater variability across product lots. Genesis, being an acellular product, did not have any cells present in any of the lots provided. In addition to containing cells, PalinGen and FloGraft contained small (<100 μm) tissue fragments. The unprocessed cryopreserved amniotic fluid did not contain any live cells.

Genesis had a total protein concentration most similar to unprocessed amniotic fluid in solution, whereas PalinGen and FloGraft had significantly lower levels (Figure 2B). Among the growth factors measured, detectable levels

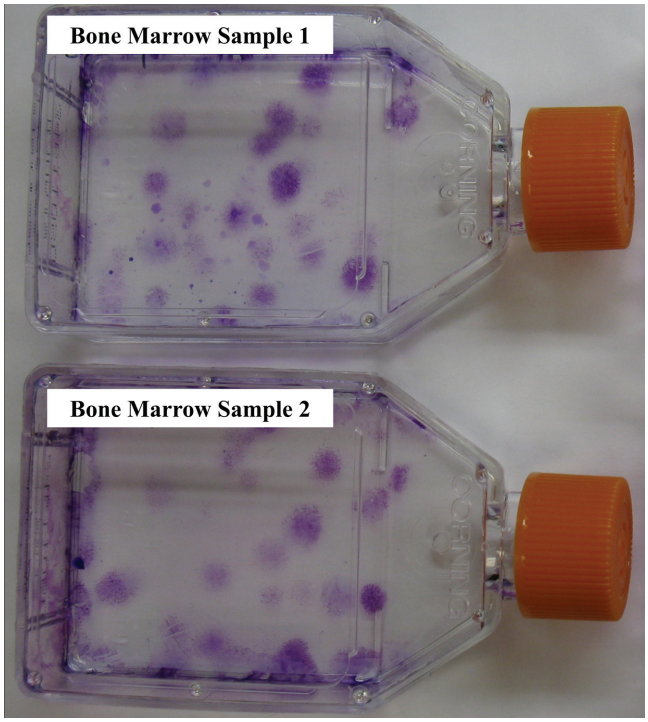

Figure 1. Bone marrow samples showing growth of fibroblast colony-forming units (CFU-Fs). These samples served as examples that the culture and expansion techniques were appropriate for the growth of CFU-Fs. No CFU-Fs could be cultured from any amniotic fluid samples by use of the same techniques.

of PDGF-ββ, hyaluronan, BMP-2, and TGF-β_1 were found in PalinGen (Figure 3). PalinGen did not contain VEGF or IL-8. FloGraft contained hyaluronan, BMP-2, and low levels of IL-8 but did not contain PDGF-ββ, TGF-β_1, or VEGF. Genesis contained hyaluronan, BMP-2, and IL-8 but did not contain PDGF-ββ, VEGF, or TGF-β_1. All measured factors were detected in the unprocessed amniotic fluid, except for VEGF. In the bone marrow–derived controls, all factors except for PDGF-ββ were detected. Of note, hyaluronan levels in PalinGen, unprocessed amniotic fluid, and MSC supernatants were above saturation levels (ie, ≥100 pg/mL). Measurements of the individual proteins and hyaluronan revealed that PalinGen is the closest to regular amniotic fluid overall, although no IL-8 could be detected. In addition, we found that the individual protein

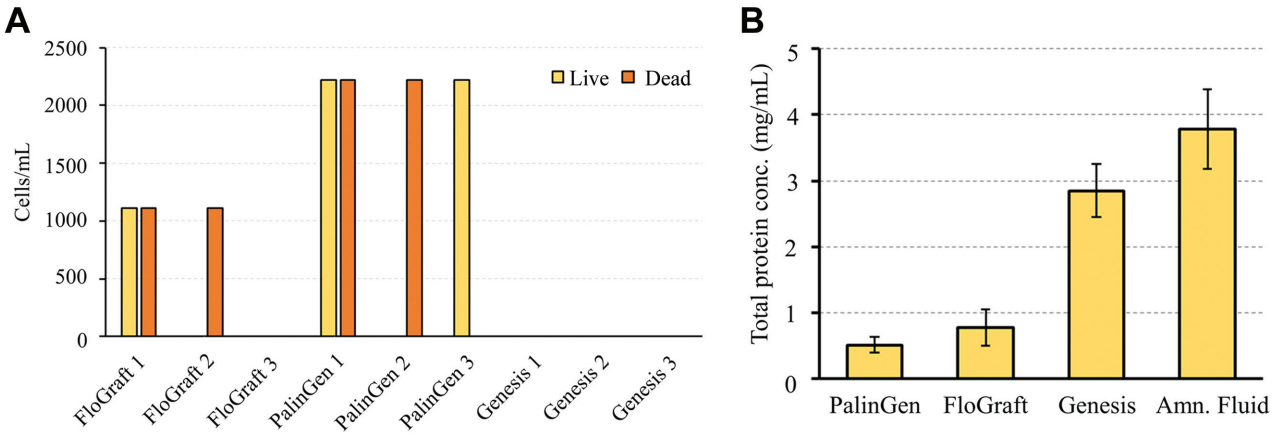

Figure 2. (A) Total cell count (cells/mL) for the amniotic fluid products. Each company provided 3 samples corresponding to different production lots for analysis. (B) Total protein concentration (mg/mL) for the amniotic fluid products and unprocessed amniotic fluid. Data is represented as mean ± standard deviation.

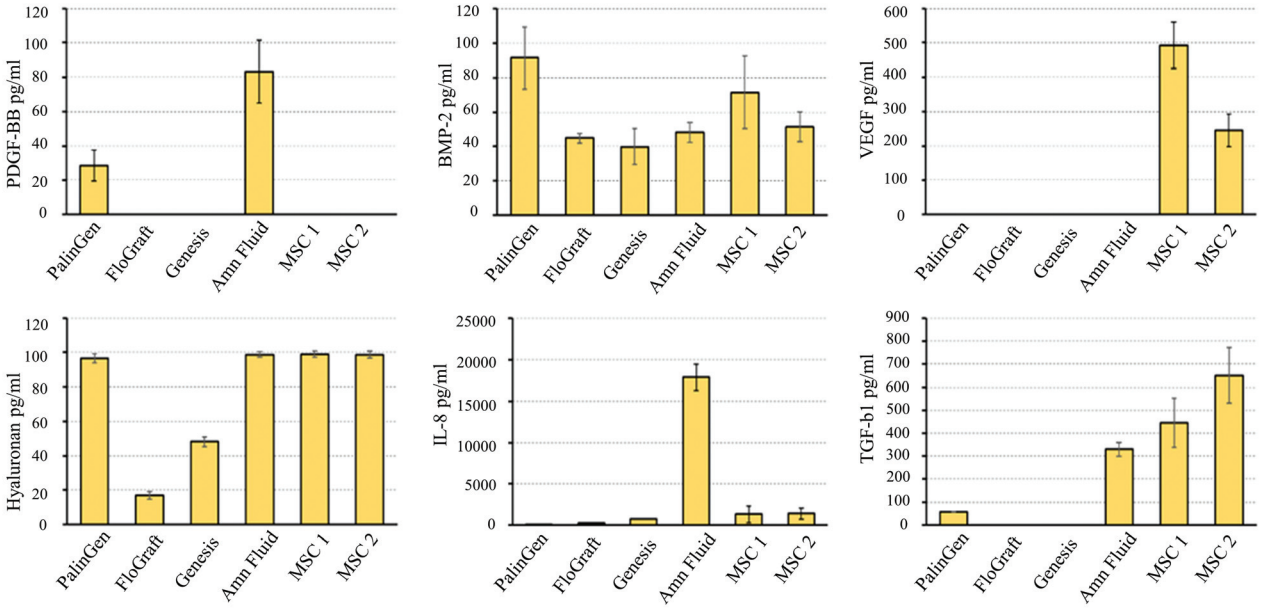

Figure 3. Individual measurements of the growth factors and hyaluronan (pg/mL) for the amniotic fluid products, unprocessed amniotic fluid, and mesenchymal stem cell (MSC) samples derived from human bone marrow aspirates. PDGF-ββ, platelet-derived growth factor with two β subunits; BMP-2, bone morphogenetic protein 2; IL-8, interleukin 8; VEGF, vascular endothelial growth factor; TGF-β_1, transforming growth factor β_1. Data is represented as mean ± standard deviation.

levels of FloGraft and Genesis were very similar to each other.

DISCUSSION

This study was unable to identify any cells in the AFPs or in the unprocessed amniotic fluid that expressed MSC characteristics. MSCs could be cultured in the bone marrow aspirates through use of the same protocols, demonstrating that the culture medium and expansion techniques used were appropriate for growing MSCs. The products did contain hyaluronan and varying levels of PDGF-ββ, TGF-β_1, BMP-2, IL-8, and VEGF.

While studies have shown that MSCs are present in amniotic fluid during the second trimester and immediately after birth,[14,23,28] controversy exists as to whether these MSCs are present in sufficient quantities for therapeutic applications and whether they remain viable after commercial processing and cryopreservation.[10,15] Cryopreservation, in isolation, is known to cause cell death due to crystal formation and cell dehydration. This was

exemplified by the present study in which the unprocessed amniotic fluid did not retain any cells after being frozen without any cryopreservation agents. The potential harm of cryopreservation and processing on cells was also illustrated in this study by the low viability of nucleated cells found in the cellular AFPs. Although processing resulted in retention of some living cells, the question remains as to whether cells died strictly due to the cryopreservation or whether the processing methods played any role. In addition, it should be determined whether the inability to culture MSCs is a result of the processing protocols or whether insufficient numbers of MSCs are present in amniotic fluid in the first place. Research should be advanced on specific processing methods designed to preserve, isolate, and eventually culture MSCs from amniotic fluid.

Clinically, Hernigou et al[11] suggested a dose-dependent relationship between the number of MSCs and healing. In repairs augmented with bone marrow concentrate, patients with an MSC concentration that exceeded 2500/mL had greater instances of healing; when the MSC concentration did not surpass 1500/mL, there were more cases of nonhealing. Patients who received a greater quantity of MSCs (>30,000 cells) also experienced healing of the rotator cuff footprint quicker than those with lower cell counts.[11] These findings further highlight that MSCs not only need to be present but also may need to be present in sufficient quantities to yield a clinically significant result. The results of this study suggest that even if the nucleated cells found in the AFPs were in fact MSCs, they were either dead or in such low quantity that they were ultimately insignificant from a clinical perspective.

Each commercial product contained either 3 or 4 of the 6 measured biomolecules at varying levels. Hyaluronan and 4 of the growth factors were detected in the unprocessed amniotic fluid and at higher concentrations than in the commercial products except in one instance (BMP-2; Figure 2). These bioactive factors play a key role in the inflammatory and healing response commonly seen in many orthopaedic injuries. Upon injury, PDGF-ββ stimulates the influx of inflammatory factors and fibroblasts and accelerates extracellular matrix deposition and collagen formation.[22] TGF-β_1 is an important mediator of tissue repair and is released by platelets during acute responses to injury. An influx of TGF-β_1 to a wound is critical for macrophage and fibroblast chemotaxis to the injury site.[17] In response to inflammatory stimuli, monocytes secrete IL-8, which induces neutrophil chemotaxis and increases VEGF expression.[12] VEGF is a potent stimulator of the angiogenic cascade. It regulates endothelial migration and proliferation, induces vascular permeability for increased nutrient delivery, and promotes epithelization and collagen deposition.[1] BMP-2 induces chondrogenic and osteogenic differentiation and is vital in regulating cell interactions. The role of BMP-2 in cartilage restoration has been theorized but has yet to be fully elucidated.[27] Hyaluronan is a primary component of the extracellular matrix and essential to lubrication in synovial fluid.[13]

Although this study was unable to identify MSCs, AFPs remain an interesting orthobiologic option due to the volume of these various cytokines and growth factors that are involved in the immunomodulatory response. Future analyses should be conducted to conclude whether a dose-dependent relationship exists between the proteins studied in this analysis and clinical outcomes. Additional research should be focused on discovering the true potential of amniotic fluid and where it fits in the regenerative medicine spectrum of orthopaedics and sports medicine.

Study Limitations

The authors are aware of limitations of this study. Each company provided three 1-mL samples for use in all assays. Analysis with larger AFP volumes may have allowed for better quantification of the various biomolecules. The precision of our method in determining cell viability was also suboptimal, as the low cell counts in each lot may not have met hemocytometer detection limits. Additionally, the low cell volumes did not allow for flow cytometry analysis and further characterization of the few living cells. The unprocessed amniotic fluid sample was frozen at –80°C without cryopreservation agents. It is theoretically possible that nucleated cells in the unprocessed amniotic fluid that died as a result of the freezing would have survived if cryopreservation agents had been used.

CONCLUSION

Our study was unable to identify any MSCs in 3 commercially available AFPs or in unprocessed, fresh-frozen amniotic fluid. Using identical methods and medium, we were able to grow MSCs in culture from bone marrow samples. Although these AFPs should not currently be categorized as “stem cell” treatments, they may still represent a promising tool for orthopaedic treatments given the presence of cytokines and growth factors. Caution should be exercised when examining commercial AFPs claiming concentrations of MSCs for clinical use.

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