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FULL LENGTH PAPER

Collection and characterization of amniotic fluid from scheduled C-section deliveries

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Abstract Amniotic fluid (AF) possesses anti-inflammatory, anti-microbial and regenerative properties that make it attractive for use in clinical applications. The goals of this study were to assess the feasibility of collecting AF from full-term pregnancies and to evaluate non-cellular and cellular properties of AF for clinical applications. Donor informed consent and medical histories were obtained from pregnant women scheduled for C-sections and infectious disease testing was performed the day of collection. AFs were evaluated for total volume, fluid chemistries, total protein, and hyaluronic acid (HA) levels. AF was also assessed with quantitative antibody arrays, cellular content and for an ability to support angiogenesis. Thirty-six pregnant women consented and passed donor screening to give birth tissue. AF was successfully collected from 17 individuals. Median AF volumes were 70 mL (range 10–815 mL; n = 17). Fluid chemistries were similar, but some differences were noted in HA levels and cytokine profiles. Cytokine arrays revealed that an average of 304 ± 20 of 400 proteins tested were present in AF with a majority of cytokines associated with host defense. AF supported angiogenesis. Epithelioid cells were the major cell type in AF with only a minor population of lymphoid cells. Cultures revealed a highly proliferative population of adherent cells capable of producing therapeutic doses of mesenchymal stromal cells (MSCs). These findings showed that significant volumes of AF were routinely collected from full-term births. AF contained a number of bioactive proteins and only a rare population of MSCs. Variations noted in components present in different AFs, warrant further investigations to determine their relevance for specific clinical applications.

Keywords Amniotic fluid · C-section · Cytokines · Growth factors

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Background

Early after conception and until the mother's water breaks for the delivery of their infant, the fetus is bathed in amniotic fluid (AF). AF functions is a supportive cushion to the fetus and provides a protective environment. AF is a rich source of nutrients, cytokines and growth factors that are

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required for fetal development and maturation (Underwood et al. 2005). AF also contains stem cells with the potential to differentiate along multiple cell lineages (In' t Anker et al. 2003; Prusa et al. 2003; Bottai et al. 2012). The protective and regenerative properties of AF are achieved via the exchange of water and solutes with surrounding tissues. This is accomplished via the utilization of different pathways during the course of a pregnancy that likely contribute to changes in the composition of the AF with gestational age (Underwood et al. 2005).

Among some of the first evidence that AF has protective biological properties is a report describing that concentrates of AF inhibit the development of peritonitis (Johnson et al. 1936). This is followed by a report by Shimberg and co-workers that AF accelerates defense-repair mechanisms within damaged joints (Johnson et al. 1936; Shimberg 1938). Since these early publications, more sophisticated evaluations have revealed the presence of antimicrobial, immunomodulatory, and growth-promoting activities in AF (Underwood et al. 2005). Reports of antimicrobial activity in AF differ (Ismail et al. 1989) among investigators. Some studies show that AF is inhibitory, while others show no effect against the same microorganisms. Another report provides evidence that AF with low antimicrobial activity is associated with a high incidence of an infectious syndrome in pregnant women (Ojo et al. 1986). Components with antimicrobial, antiviral and anti-fungal activity that are present in AF include lysozyme, peroxidase, transferrin, β-lysin, immunoglobulins and zinc-peptide complexes (Ismail et al. 1989). Immunomodulatory properties of AF are evident from studies showing that enteral feeding of AF suppresses the pro-inflammatory responses in preterm pigs with necrotizing enterocolitis (Siggers et al. 2013). While growth promoting activities of AF are supported by animal studies and by in vitro culture studies showing that AF can enhance neochondrogenesis (Ozgenel et al. 2004), regenerate peripheral nerves (Ozgenel and Filiz 2003) and bone (Karacal et al. 2005), accelerate reepithelialization in corneas (Castro-Combs et al. 2008), and promote healing of human skin wounds (Nyman et al. 2013). Some of the factors that are found in AF that may contribute to these activities include inflammatory mediators that include, but are not limited to TNF-α, IL-6, IL8, and IL-10 (Weissenbacher et al. 2012), trophic factors that include EGF, IGF-1, FGF, HGF and TGF-α (Merimee et al. 1984; Watanabe 1990; Lang and Searle 1994; Kurauchi et al. 1995; Hirai et al. 2002), and HA, an important factor in promoting re-epithelialization in human skin wounds (Nyman et al. 2013).

Based on the hypothesis that nutrients, cytokines and growth factors contained in the non-cellular fraction of AF are useful for reparative and regenerative treatments in patients, the purpose of this study was to address three issues. The first was to determine the feasibility of consenting and screening volunteer donors for the routine collection of AF from full-term pregnant women that were scheduled for caesarean-section (C-sections) and for processing the AF for clinical applications. The second aim was to develop a processing method that resulted in a cell-free AF preparation suitable for clinical applications. The third goal was to gain a better understanding about components of AF procured from full-term pregnancies.

Materials and methods

Donor consent, screening and infectious disease testing

AF was procured from volunteer donors according to protocols approved by the Institutional Review Board at the University of Utah. To participate in the study, women were required to be 18 years or older with an uncomplicated singleton pregnancy. Informed consent was obtained from women who were scheduled to undergo a C-section. As part of the donor selection process, medical and social history screening was performed using a self-administered questionnaire designed to ask broad leading questions to determine the health of the potential donor and to minimize any communicable risks to the recipient. Donors were selected based on medical and social history responses that met the Standards of the American Association of Tissue Banks and Title 21, Code of Federal Regulations, Part 1271 as issued under the authority of Section 361 of the United States Public Health Service Act (Dock et al. 2012, CFR-Title21 2015). Maternal blood samples were collected prior to delivery and infectious disease testing was performed for HBsAg (Hepatitis B surface antigen), HBcAb (Hepatitis B core antibody), HCV (Hepatitis C antibody screen and HCV nucleic acid test), HIV I/II-Ab (Human

Immunodeficiency Virus Types 1 and 2 antibody and HIV nucleic acid test), Syphilis (RPR test), and CMV (antibody screen).

Amniotic fluid collection

Human AF was collected by the staff of the Obstetrical and Gynecological department at the University of Utah hospital. A physician executed abdominal fenistil incision was performed through the abdominal and uterine muscles without cutting into the amnion membrane. Using a sterile soft suction catheter connected to a sterile MediVac Suction Container (Cardinal Health, Waukegan, IL), a blunt end insertion with a catheter was made into the amnion membrane and the AF was aseptically suctioned into a MediVac Container. The container was labelled, wrapped in frozen Insul-ice mats (Fisher Scientific, Hanover Park, IL) and placed in a temperature monitored shipper that is validated for transport between 2 and 8 °C to the Cell Therapy and Regenerative Medicine (CTRM) facility at the University of Utah. Upon arrival at the facility, the product was immediately placed in a refrigerator at 2–8 °C until processing occurred.

Processing

The MediVac container with AF was aseptically placed in a biological safety cabinet and the AF was transferred via aseptic techniques into sterile centrifuge tubes. The total volume and gross appearance of the AF were recorded and samples were removed for sterility testing, cell counts and other relevant testing. The AF was centrifuged at $1400 \times g$ for 20 min at 4 °C. Once centrifugation was complete, the supernatant was expressed into a new transfer pack and the remaining cell pellet was characterized and cultured as described below. The supernatant from the AF was processed using a proprietary filtration technology to sterilize and eliminate cellular debris from the final product.

Cell counts, cell cultures, and flow cytometry

Cell pellets were re-suspended in Dulbecco's phosphate-buffered saline (Life Technologies Corporation, Grand Island, NY) and passed through a 40 μM sterile cell strainer (BD Biosciences, Durham, NC) to remove non-cellular material/aggregates. The filtered material was centrifuged at $400 \times g$ for 10 min and the resulting isolate was assessed by microscopic evaluation. Manual cell counts and viabilities were performed using trypan blue (Sigma-Adrich, St. Louis, MO).

Cell pellets were isolated from freshly collected AF and were plated on tissue culture-treated plastic at 500 cells/cm^2 in Prime XV AFSC® (Amniotic Fluid Stem Cell) expansion medium (Irvine Scientific, Santa Ana, CA). This typically generated focal adherent populations of cells. The focal outgrowths were trypsinized, re-established as a monolayer, and passaged multiple times.

After the final passage, the harvested cells were immunophenotyped using fluorescence-conjugated mouse anti-human monoclonal antibodies: CD90-fluorescein isothiocyanate (CD90-FITC), CD73-allophycocyanin (APC), CD166-phycoerythrin (PE), CD14-PE, CD34-APC, CD45-peridinin-chlorophyll protein, HLA-DR-FITC and HLA-ABC-FITC. Appropriate isotype controls were set up in parallel (antibodies are from BD Biosciences, San Jose, CA). The cells were incubated for 30–60 min at 4 °C in the dark and were washed in phosphate-buffered saline containing 0.5 % bovine serum albumin. A minimum of 10,000 events were collected using a Cyflow Space, Sysmex-Partec flow cytometer (Sysmex; Lincolnshire, IL). Data acquisition and analysis was performed using FlowMax software.

Tri-lineage differentiation

Adipogenic and osteogenic induction was performed using a modification of the manufacturer's instructions (STEMPRO® Adipogenesis and Osteogenesis Differentiation Kits; Gibco Life Technologies). Briefly, adherent cells were removed from culture by adding 0.05 mL/cm^2 of 0.05 % trypsin-ethylenediamine tetraacetic acid. The cells were centrifuged and resuspended in Prime XV AFSC and the cells were seeded at 10,500 cells/cm^2 for adipogenic and osteogenic differentiation in six well plates. Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2. After a couple of days in culture, the medium was replaced with either adipocyte differentiation medium or osteocyte differentiation medium and the cells were cultured for an additional 14–15 days. Differentiation medium was changed every 2–3 days. Staining was initiated by fixing cells with 10 % formalin and the cells were made permeable with

isopropanol. Adipocytes were stained by applying oil red O for 5 min and osteocytes by adding 2 % alizarin red S for 20 min and counterstaining with hematoxylin for 1 min. Before visualization on an inverted light microscope, the wells were washed with deionized water.

Chondrocyte differentiation was also performed using the manufacturer's instructions (STEMPRO® Chondrogenesis Differentiation Kit). MSCs were harvested and cells were resuspended with an appropriate volume of pre-warmed MesenPRO RS medium at a concentration of 1.6×10^7 viable cell/mL. Micromass cultures were generated by seeding 5 μL of the cell solution into each well of a 6-well plate, incubating the cells for 2 h and then adding chondrogenesis differentiation medium to the culture vessels. Differentiation medium was changed every 2–3 days and after 21 days of culture, cells were fixed with 10 % formalin and stained with 1 % Alcian Blue.

Chemistry and sterility testing

Chemistry assessments for sodium, potassium, chloride, urea nitrogen, creatinine, and HA were performed by ARUP Laboratories (Salt Lake City, UT). Total protein levels were determined using a Pierce™ BCA Protein Assay Kit (Life Technologies, Grand Island, NY). Pre-processing microbiologic testing was performed for aerobic, anaerobic and fungal microorganisms using the BACTEC system (Becton–Dickinson, Sparks, MD). BACTEC Plus Aerobic/F, Plus Anaerobic/F, and a Myco F/Lytic culture bottles were each inoculated with 1 mL of AF. The bottles were sent to ARUP Laboratories for a 5 day culture and microorganism detection. Post-processing or final product 14-day sterility testing was performed using United States Pharmacopeia <71> guidelines. This was accomplished by sending aliquots of AF to LABS, Inc (Centennial, CO) for testing.

Endothelial tube formation

Endothelial tube formation assays were performed using an in vitro angiogenesis kit according to the manufacturer's instructions (Life Technologies, Grand Island, NY). Briefly, human umbilical vein endothelial cells (HUVECs) were established at 2×10^5 cells per T-75 flask using 200PRF medium containing a low serum growth supplement [i.e. 2 % (v/v) FBS and bFGF (3 ng/mL)]. Medium was changed every other day until cultures were approximately 80 % confluent. HUVEC cells were trypsinized and washed with non-supplemented 200PRF medium.

HUVEC cells were then suspended with one of three selected medium to achieve a plating concentration of 4.0×10^4 cells/cm^2. The three different medium included LSGS-supplemented medium 200PRF (positive inducer control), LSGS-supplemented medium containing 30 μM suramin (positive inhibitor control) or AF. After suspending HUVECs (8000 viable cells/cm^2) in each of the different selected medium, cells were seeded in 24-well plates pre-coated with Geltrix™. To pre-coat plates, Geltrex™ (50–100 μL) was added to each well of a 24-well plate and incubated for 30 min at 37 °C to allow the gel to solidify. The HUVECs were incubated at 37 °C, 5 % CO_2 for 3 h. Each well was scored from a 0–4+ for tubule formation relative to the amount of tube formation in the positive control.

Protein arrays

AF (1 mL) from three maternal collections were sent to RayBiotech for quantitative screening using the Quantibody ® Human Cytokine Antibody Array 9000 (RayBiotech, In., Norcross, GA). This array set consists of nine glass chips that simultaneously and quantitatively measure the concentration of 400 human cytokines. Controls and serial dilutions of cytokine standards were prepared according to the manufacturer's instructions. Chips were blocked with 100 μL of Sample Diluent at room temperature for 30 min. After decanting the diluent from each chip, cytokine standards, controls and test samples were added to chip wells and were incubated at room temperature for 1–2 h. Each chip was washed three times and then incubated for 1 h at room temperature in the dark with a Cy3 equivalent dye-streptavidin conjugate. The dye was decanted and chips were washed five times with a 1× wash buffer at room temperature, dried and imaged using a laser scanner equipped with a Cy3 wavelength. Quantitative data analysis was performed using the Quantibody® Q-Analyzer software (RayBiotech, Inc.). Positive controls in each array were used for normalization. Classification of proteins according to biological function was obtained by surveying the Human Protein Reference

Database (http://www.hprd.org/index_html), Cytokines and Cells Online Pathfinder Encyclopedia (COPE) http://www.copewithcytokines.de/), GeneCards® (http://www.genecards.org/), and the biomedical literature in PubMed (http://www.ncbi.nlm.nih.gov/pubmed). Cluster Analysis was performed using the free software program from The R Project for Statistical Computing.

Epidermal growth factor (EGF) Elisa

Quantitative sandwich enzyme-linked immunosorbent assays (Elisa) were conducted according to the manufacturer's instructions (R&D, Systems, Inc, Minneapolis, MN). Briefly, duplicate standards and duplicate test samples of AF were tested in different wells of a microplate pre-coated with monoclonal antibody specific for EGF. After incubating the plate at room temperature for 2 h, unbound substance was washed away and an enzyme-linked polyclonal antibody specific for EGF was added to the wells. The plate was incubated at room temperature for 1 h. After washing, substrate solution was added to each well, incubated for 20 min in the dark followed by the addition of a stop solution. The optical density of each well was read at 540 nm using a microplate reader and the concentrations of the samples were determined using the standard curve. Criteria for intra- and inter-assay variations were a coefficient of variation of $\leq$10 %.

Statistical analysis

Data are presented as mean $\pm$ standard deviation from the mean and in some cases a median is provided. A student *t* test was used to determine differences or similarities between populations. *P* values <0.05 were designated as significant.

Results

Amniotic fluid (AF) collection

Donors were required to sign a consent form and were asked to complete a medical/social history questionnaire for eligibility determination as well as donate blood samples for infectious disease testing. Donor eligibility rules as outlined by the FDA in 21 CFR1271 Subpart C were followed to establish selection criteria for AF donations (CFR-Title21 2015). All donor screening and testing was performed to ensure that the donor was healthy and to rule out risk factors and clinical evidence of infection due to relevant communicable disease agents. To participate in the study, women were required to be 18 years or older with an uncomplicated singleton pregnancy and were scheduled for a C-section. Human AF was collected by the Obstetrical and Gynecological department and was transported to the CTRM facility at the University of Utah for processing and storage.

From October 10, 2013 to December 31, 2014, a total of 62 pregnant women out of a possible 159 potential donors were approached for donation of birth tissue. Thirty-six of 62 individuals consented to donate their birth tissue and also passed the donor history questionnaire. AF was successfully collected from 17 donors and there was one failed attempt at a collection. Due to staffing issues, no attempt was made to collect AF from the other 18 donors. AF was immediately transported to the CTRM facility and was processed within 24 h of collection. Upon arrival of the AF at the CTRM facility and prior to processing, each AF collection was evaluated for total volume and appearance. AF collections with meconium and/or excessive blood contamination were not processed.

Pre-processing characterization of amniotic fluid

The average total volume of AF collected was 152 $\pm$ 230 mL (n = 17; mean $\pm$ SD) with a median collection volume of 70 mL and a range of 10–815 mL. When visibly contaminated with blood, the color of AF went from red to pink. When no blood contamination was evident, the color of the AF ranged from colorless to dark yellow. Turbidity scores of 1+ to 4+ were assigned to each AF, where zero equals no turbidity and 4+ equals a flocculent appearance. A majority of pre-processed AF collections scored a 4+. No samples received turbidity scores of 2+ or 0.

Randomly selected AFs were evaluated for fluid chemistries. Before processing the AF, average total protein levels were 3.3 $\pm$ 0.2 mg/mL with a median value of 3.3 mg/mL (n = 3) Average electrolyte, sodium, potassium, chloride, carbon dioxide, urea nitrogen, creatinine, and glucose levels are shown in Table 1 (n = 6). HA levels in AF averaged 311 $\pm$ 75 ng/mL with a median of 313 ng/mL and a range of 198–416 ng/mL (n = 8).

Table 1 Pre-processing amniotic fluid chemistries

Parameters	Mean ± SD	Median
Sodium (mmol/L)	122 ± 7	125
Potassium (mmol/L)	4.2 ± 0.4	4.3
Chloride (mmol/L)	98 ± 5	101
Urea nitrogen (mg/dL)	18 ± 5	17
Creatinine (mg/dL)	2.2 ± 0.4	2.1
Glucose (mg/dL)	10 ± 8	8
Calcium (mg/dL)	6.4 ± 0.8	6.3

SD standard deviation; n = 6

Post-processing characterization of amniotic fluid supernatant

AF was processed by first centrifuging and then filtering the fluid to remove particulates (i.e. lanugo, vernix and cells). After centrifugation, the supernatant was removed from the cell pellet and the supernatant was sequentially filtered to obtain a sterile filtered fluid with volume recoveries of 71 ± 23 % (n = 11). Turbidity scores of the AF decreased from an average pre-filtration score of 2.6+ to a post-filtration score of 1+. Post-processing recoveries for HA were 99.8 ± 4.2 %. Post processing recovery levels for sodium, potassium, chloride, carbon dioxide, anion gap, urea nitrogen, creatinine and glucose were all >90 % (Fig. 1). Overall, processing of AF resulted in an average decrease in total protein levels from 3.3 ± 0.2 to 2.8 ± 0.3 mg/mL (mean ± SD; n = 3) with a recovery of 84.4 ± 12.8 % of the total protein. EGF pre-filtration levels for AF averaged 204.2 ± 80.6 ng/mL and after filtration EGF levels averaged 203.9 ± 100.7 ng/mL for overall recovery levels of 95.2 ± 16.4 %, Post-processing sterility test results for aerobic, anaerobic and fungal microorganisms were negative for six of six randomly selected AFs.

The angiogenic activity of AF was measured using an in vitro endothelial tube formation assay. Three different AFs were randomly selected for testing to determine whether pre- and post-filtered AF supported endothelial tube formation. Representative figures of endothelial tube formation for negative and positive controls as well as for pre- and post-filtered AF are shown in Fig. 2. The results showed that endothelial tube formation was supported independent of whether AF was filtered or not. Also, endothelial tube formation was observed to be similar for each of three different AFs that were tested.

Cytokine profile of AF

Cytokine antibody arrays were used to simultaneously identify and quantitate protein levels for 400 human cytokines from three randomly selected AF donors.

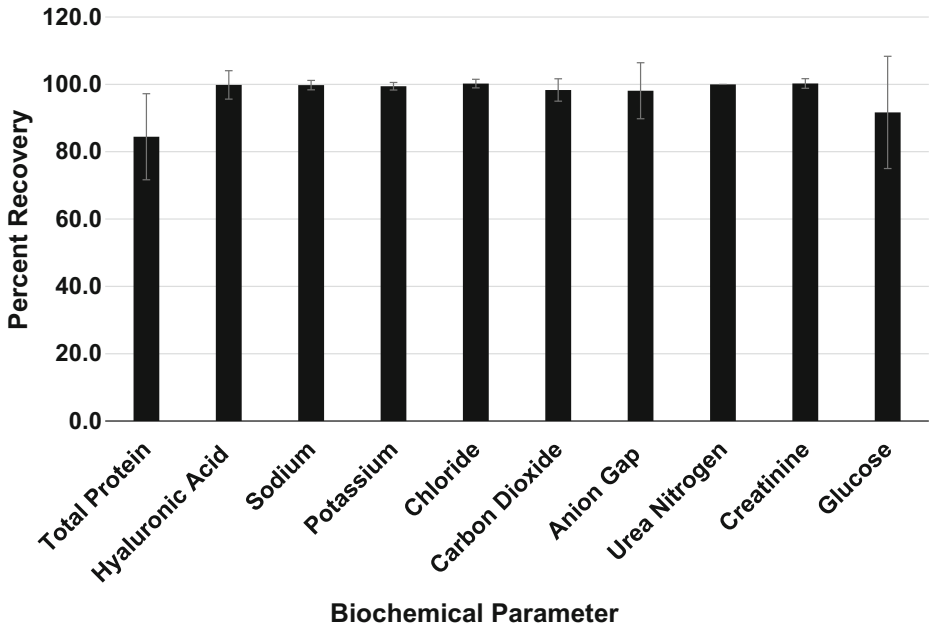
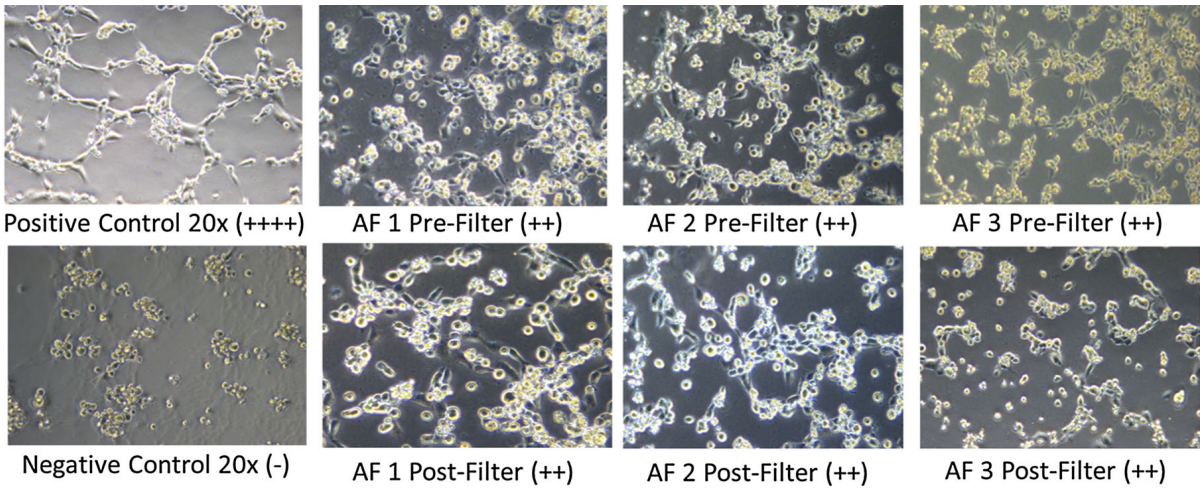


Fig. 1 Post-processing recoveries. Mean values for the indicated parameters were determined for pre- and post-processed AF as described under "Materials and methods" section. Percent recoveries of each parameter indicated in the graph were determined by dividing post-processed AF mean values by pre-processed AF mean values and multiplying by 100. *Error bars* represent standard deviation

A



B

		Qualitative Functional Assessment	
Lot Identification		2-hour	4-hour
AF Sample 1	Pre-Filter	+	++
	Post Filtration	+	++
AF Sample 2	Pre-Filter	+	++
	Post Filtration	+	++
AF Sample 3	Pre-Filter	+	++
	Post Filtration	+	++
POSITIVE INDUCTION		+++	++++
INDUCTION INHIBITION		-	-

AF= Amniotic Fluid

Fig. 2 Endothelial tube formation. **a** Representative images of HUVECs suspended with pre-filtered AF or post-filtered AF from three different sources of AF (magnification ×20). **b** Each well was qualitatively scored according to the number of tubes formed. 4+ = maximum tube formation; 2+ = intermediate tube formation and negative = no tube formation. HUVEC = umbilical vein endothelial cell; AF1, 2, and 3 = amniotic fluids from donor #1, #2 and #3 respectively

Prior to performing the arrays, each of three lots of AF underwent a sterile filtration process. Protein arrays were performed in replicates of four for each of three lots. Using a mean cut-off of 8 ± 8 pg/mL the total number of proteins present in each of the three lots was 318, 313 and 282 (see supplemental data). This resulted in an average of 304 ± 20 cytokines being detected out of 400 cytokines tested or 72 % of the cytokines that were tested showed a positive signal in AF.

Each protein with a positive signal was assigned a biological function based on annotated information obtained from Entrez Gene, GeneCards, UniProtKB/Swiss-Prot, Gene Wiki, and the Human Protein Reference databases. Cytokines were assigned to 12 different functional categories. There were also a handful of proteins that were designated as having poorly described functional activities (i.e. unknown) and some proteins that were designated as miscellaneous because they did not meet the criteria for one of the twelve defined functional categories (i.e.) (Fig. 3a).

A majority of cytokines (i.e. 39 %) were categorized as participating in host defense (Fig. 3a) The host defense proteins were further sub-categorized and found to have known functions associated with the inflammatory response, innate immunity or as an antimicrobial (Fig. 3b). The next biggest categories of

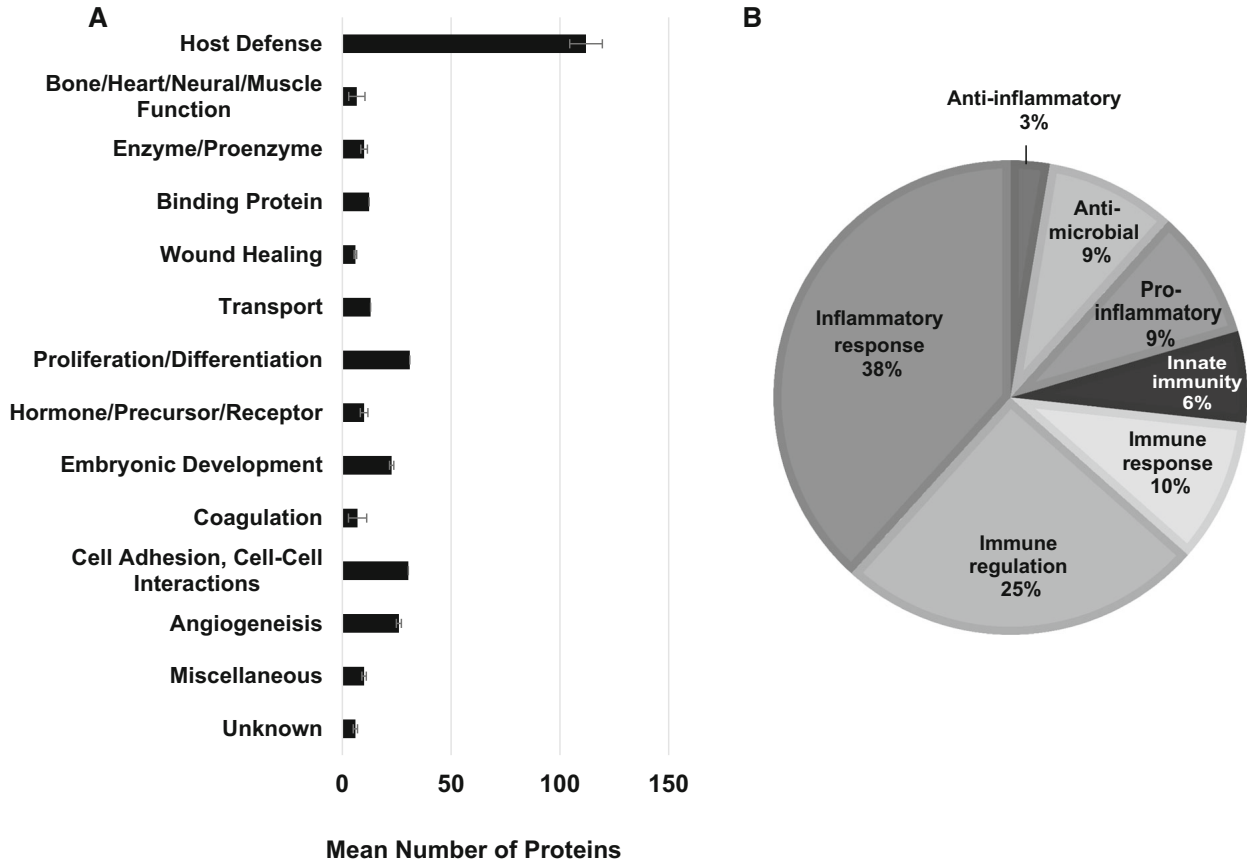


Fig. 3 Classification of proteins that were identified in AF according to biological function. An average total of 304 ± 20 proteins were positively identified in AF by Quantibody ® Human Cytokine Antibody Arrays 9000. **a** Biological functions were assigned based on information queried from Entrez Gene, GeneCards, UniProtKB/Swiss-Prot, Gene Wiki, and the Human Protein Reference databases. Each of the proteins were matched to 12 defined biologically functional groups. Proteins with defined functional activities that did not meet any of the 12 functional categories were designated as miscellaneous proteins and proteins with no known functional activity were designated as unknown. **b** Proteins classified as having involvement in host defense were further sub-categorized as proteins with inflammatory activity, anti-microbial activity, involvement in the immune response, and immune regulation

similar functioning proteins were for proteins involved in cell adhesion, proliferation and angiogeneisis (Fig. 3a). Proteins that were classified as having poorly defined functional activities included serum amyloid A (SAA), cathepsin S (CTSS), Interleukin-36 alpha (IL-36a), mycobacterial porin (MSPa), interferon-alpha/beta receptor beta chain (IFNAR2), and G protein-coupled receptor associated sorting protein (GASP2). Proteins assigned to the miscellaneous category included proteins involved in Ca++ regulation, apopotosis, cell migration, lysosomal sorting and ectodermal organ morphogenesis. More specifically this included the proteins: Procalcitonin, two death proteins (HTRA2 and TRAIL-R4), ectodysplasin A (EDA-A2), a protease inhibitor, Cystatin C, a nucleosome assembly protein, NAP-2, protein-coupled receptor associated sorting protein (GASP-1), stromal derived factor-1a, (SDF-1a), carbohydrate antigen 19-9 (CA19-9) and agouti-related protein (AgRP).

Approximately 90 % of the proteins that were detected in AF were present in all 3 AF samples. The remaining 10 % of proteins were found in either one or two of the AF samples, but not in all three lots. Variances in expression levels for proteins present in all three collections were identified using the criteria that a $\geq$1.5-fold increase or $\leq$0.65-fold decrease in signal intensity between protein levels was a measurable and significant difference among analytes. Using these criteria, no measurable differences in expression

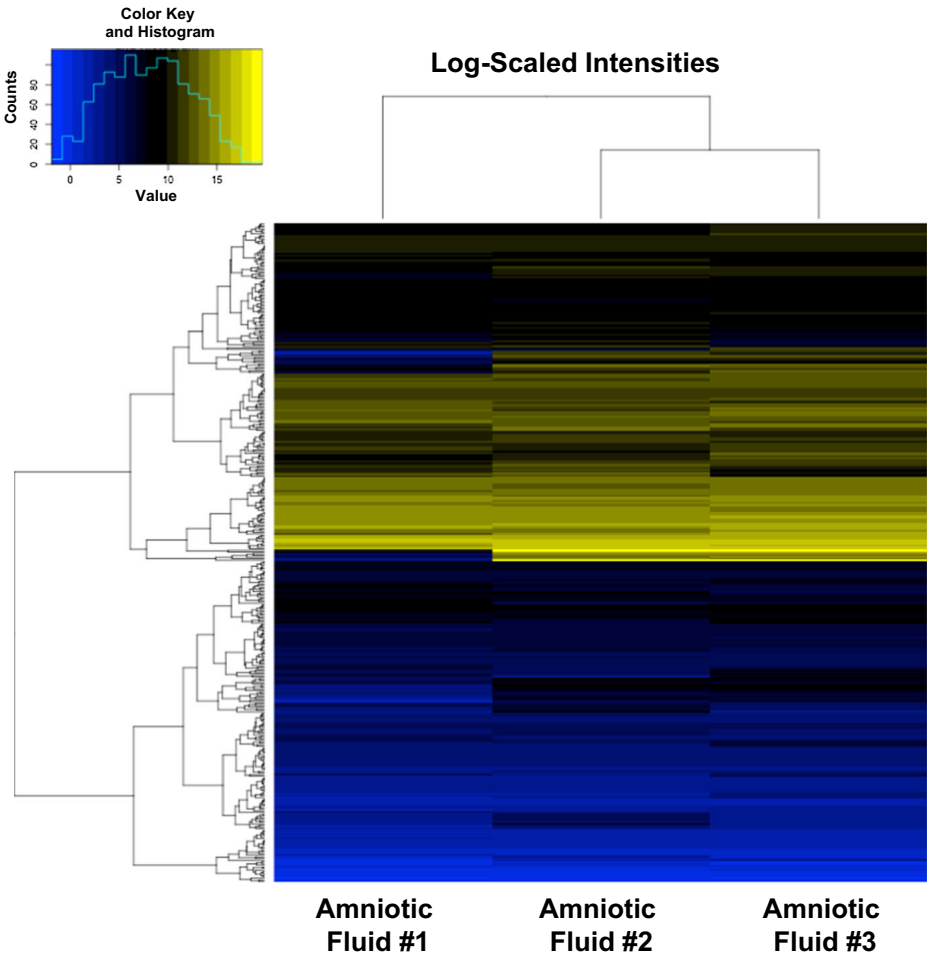


Fig. 4 Hierarchical cluster analysis heat map. Cluster analysis was performed after removing proteins that showed no detectable signal from all three AF samples. Low expression protein values are represented as *blue*, high expression protein values are designated as *yellow*, and intermediate values are *black*. Dendrograms for clustering of AFs indicate that AF#2 and AF#3 are more alike one another than AF#1 is to AF#2 or AF#3

levels were observed for 53.5 ± 4.2 % of the cytokines, whereas, 47.5 ± 2.8 % of the cytokines were noted to have measurable differences in expression levels. Differences in protein expression levels among three lots of AF were best illustrated by the proteins, periostin and PDGFRα, which were the two most highly expressed proteins in two of the three AF tested. Periostin levels in these two AF collections were 6.3 and 1.1 μg/mL, but in the third AF was only 3.7 pg/mL. PDGFRα levels were 2.5 and 5.7 μg/mL in two of the AF collections, but were undetectable in the third fluid. An overall manual inspection of the data suggested that two of the AFs were more alike to one another than the third AF. This observation was confirmed using a statistical and graphical program to perform hierarchical cluster analysis. After removing proteins that had no signal, cluster analysis of the data confirmed that samples #2 and #3 were more alike one another than to sample #1 (Fig. 4).

Characterization of the AF cell pellet

Microscopic examination of cell pellets obtained after centrifugation of AF from four randomly selected donors revealed that a majority of cells were epithelioid cells with the bulk of these cells being non-viable (Fig. 5b). Among the non-viable epithelioid cells was a minor population of lymphoid cells that were present at an average concentration of 6045 ± 780 cells per mL of fluid (n = 4). When cell pellets from AF were plated using an adherent cell culturing strategy, an average of 39 ± 17 adherent focal colonies were formed at approximately 2–3 weeks of culture. The focal colonies exhibited extensive proliferation potential with subsequent passages (Fig. 5d). AF#1, AF#2, AF#3 and AF#4 underwent 6, 3, 3 and 5 passages, respectively. The immunophenotype of the expanded cells expressed cell surface antigens associated with MSCs, (Krampera et al. 2013; Table 2) and cells

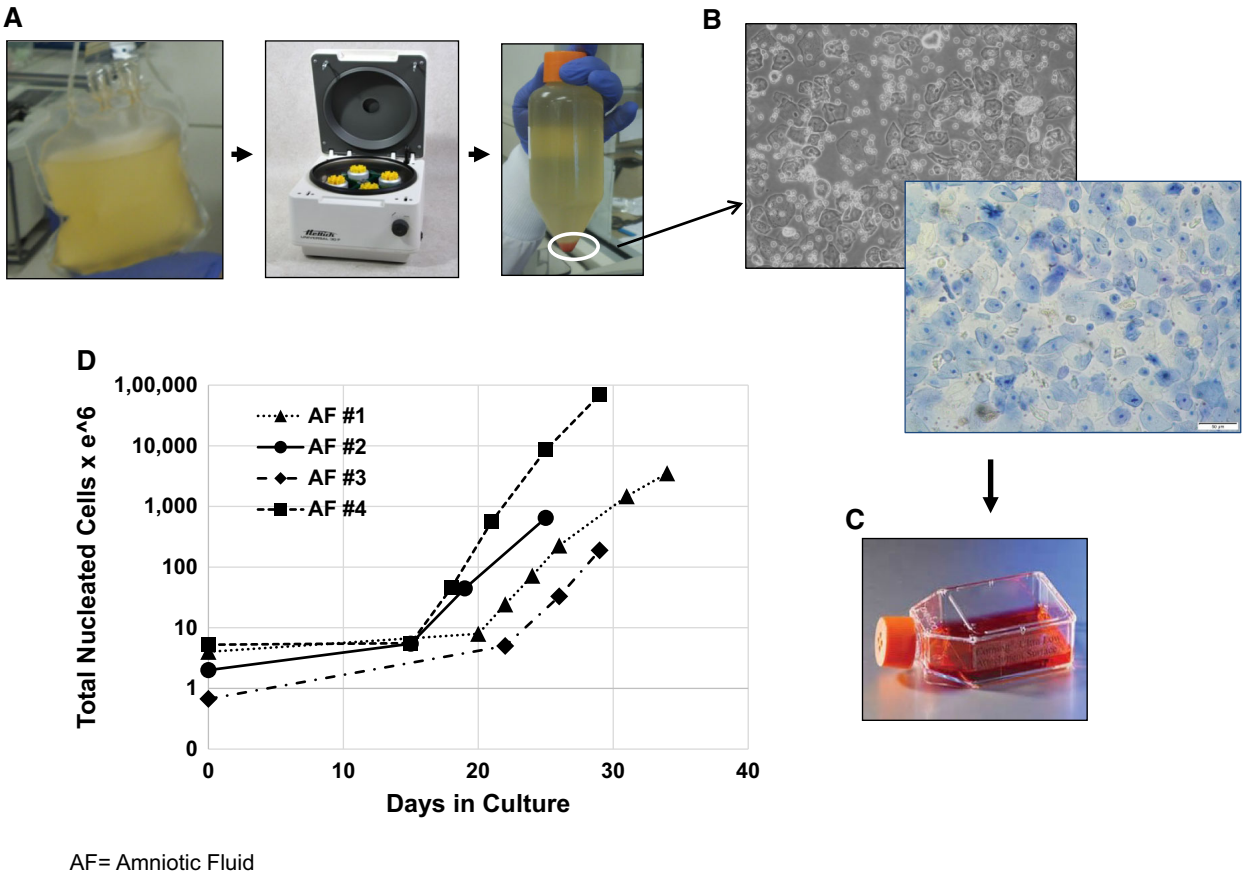


Fig. 5 A highly proliferative population of adherent cells in amniotic fluid. **a** Cell pellets were isolated and examined from AF after transferring fluid to 250 mL conical tubes and centrifuging them. **b** Supernatant was removed and the cell pellet was examined microscopically. The *upper micrograph* is a phase contrast image and the *lower micrograph* shows that a majority of cells are non-viable epithelioid cells by trypan blue staining. **c** The cell pellets from AF were plated using an adherent cell culturing strategy. **d** The graph shows the proliferative response of adherent cells over multiple passages for the defined time periods. Each symbol except for the time zero point represents when a passage was performed for a given culture. For example, AF#1 was passaged on day 20, 22, 23, 26, 31, and 34. AF#2 was passaged on day 15, 19, and 25. AF#3 was passaged on day 21, 26, and 29 and AF#4 was passaged on day 15, 18, 21, 25 and 29

differentiated into adipocytes, osteocytes and chondrocytes (data not shown).

Discussion

This study demonstrates that it is feasible to routinely collect AF at scheduled C-sections from full-term pregnancies. Over half of pregnant women who are asked to donate birth tissue responded positively by giving consent and by completing a health history questionnaire. This is a response rate that is better than what we expected, which may have been impacted by an awareness of public campaigns that promote donations of umbilical cord blood for hematopoietic stem cell transplantation. Although further investigation is required to confirm reasons for individual participation, the generous donations provided by these women are greatly appreciated and will foster collections of AF for banking purposes.

A high variability in collection volumes occurred with AF volumes ranging from 10 to 815 mL. Our observation that the most successful collections were linked to the skills of one particular physician, suggest that variations in collection volumes may be partly due to the inexperience of the obstetrical staff. Especially, when considering that they were using a newly designed device for collecting AF during a

Table 2 Culture-derived adherent cells from amniotic fluid display a mesenchymal stromal cell phenotype

Antigen	Percent positive			
	AF#1	AF#2	AF#3	AF#4
CD90+	94.7	98.1	75.2	98.8
CD73+	98.4	99.6	98.6	99.5
CD166+	100.0	92.3	98.6	99.6
CD14+	ND	0.0	0.22	ND
CD34+	0.0	0.0	0.23	0.7
CD45+	0.0	0.0	0.40	ND
HLA-DR+	0.0	0.0	0.29	0.67
HLA-ABC+	100.0	99.2	ND	99.6

AF amniotic fluid

C-section. Thus, the implementation of a training program is likely to promote an optimization of collection practices that will have a positive impact on future C-section collections.

In several in vitro and in vivo studies it has been shown that sterile filtered AF can support human corneal re-epithelialization (Castro-Combs et al. 2008) as well as the wound healing process (Nyman et al. 2013; Bazrafshan et al. 2014). Given that these studies used sterile filtered AF and that the filtration process of AF eliminates cellular elements, hair, lanugo, and vernix from the fluid, we hypothesize that it is the soluble components of AF that are responsible for corneal re-epithelialization and for wound healing processes. Consequently, one of the aims of this study was to examine the biochemical properties of pre- and post-filtered AF. Our results show that electrolytes, creatinine, urea nitrogen, glucose and total protein levels are similar among donors. However, HA levels did vary among full-term pregnancy donors (median 313 ng/mL; range 198–416). Also, the HA values that we found at full-term pregnancies are lower than HA values (i.e. 1 μg/mL) reported at 30 weeks of gestation (Dahl et al. 1983). The sterile filtration process used in this study did result in a retention of HA, electrolytes, creatinine, urea nitrogen, and glucose levels, but total protein levels decreased by ~26 % in the AFs. The decline in protein levels during the filtration process may be attributable to the removal of proteins embedded in vernix (Akinbi et al. 2004) as it is caught in the filters. Exactly which proteins are the most affected by the filtration process are not yet known, but it is currently an area of investigation in our laboratory. Of note, is the result that despite the decrease in protein levels is that EGF levels remained unaffected by filtration.

Protein arrays of post-filtered AFs show that the fluid remains rich in cytokines with antimicrobial, immunomodulatory, and growth-promoting activities. Given that AF functions as a supportive cushion to the fetus and provides a protective environment, it is interesting that a majority of proteins fell into the category of host defense. The host defense proteins identified by our study add to an arsenal of bioactive molecules that are already known to be present in AF (i.e. lysozyme, peroxidase, transferrin, β-lysin, immunoglobulins and zinc-peptide complexes) (Ismail et al. 1989) to combat an invasion of microorganisms during the gestational period.

Proteins for angiogenesis are identified with the protein arrays and we also show that AF is angiogenic as illustrated by the endothelial tube formation assay. In light of evidence that the epithelial side of amnion membrane (i.e. the side of the membrane in association with AF) inhibits angiogenesis (Niknejad et al. 2013), and that AF has angiogenic potential, this raises the question of why AF supports the process of angiogenesis and the epithelial side of amnion does not. We speculate, that because AF is ingested and inhaled by the fetus and the amnion membrane is not, that angiogenic properties of the fluid might assist with the formation of blood vessels in the gastrointestinal and respiratory systems.

Even though there is donor variability in both cytokine levels and composition, the arrays show that a large majority of the same cytokines are present in AFs collected from different donors. The cytokine arrays show that the profiles of two of the three AFs are more similar to one another than to a third fluid (Fig. 4). An examination of maternal donor charts to determine whether there is any obvious differences between the two similar samples and the third sample, did not reveal any significant maternal donor variables that might account for protein profile differences/similarities based on factors such as the gestational age of the fetus, the reason for C-section, or maternal history. Given the complexity and changes in the regulatory pathways utilized during pregnancy that define the composition of AF (Underwood et al. 2005), it is not surprising that donor variability is observed among AF collections.

We also report that a majority of nucleated cells found in AF are non-viable squamous epithelial cells. Among these cells is a minor population of cells with a high proliferative potential that can be isolated and expanded using an adherent cell culture strategy. The resulting cells display an MSC immunophenotype and have tri-lineage potential. This is consistent with observations that a highly proliferative population (In 't Anker et al. 2003) of a rare population of stromal cells is present in AF (Zia et al. 2013; Ekblad et al. 2015). Due to a rarity of viable precursor MSCs in AF, it is unlikely in a clinical setting that non-expanded cells from small volumes of AF (i.e. 1–2 mL) will contribute significantly to reparative and regenerative processes.

In conclusion, it is feasible to routinely collect, process and bank large volumes of AF procured from full-term pregnancies during scheduled C-sections. The cellular content of full-term gestational age AF consists predominately of non-viable epithelial cells. AF is rich in cytokines and growth factors and these factors are likely to contribute to the effects that AF have on promoting the healing process (Shimberg 1938; Colombo et al. 1993; Ozgenel et al. 2001, 2004; Castro-Combs et al. 2008; Ghaderi et al. 2011; Nyman et al. 2013; Feizi et al. 2014). AF from full-term pregnancies also contain HA. However, not surprisingly there are differences in HA levels and the cytokine profiles of AFs obtained from different donors. These differences in constituent levels do not preclude the use of AF for clinical applications, but emphasizes the importance of fully characterizing AF and defining release criteria of AF for specific clinical applications. Although the experience presented here is still rather limited, the results are encouraging and indicate that further effort is required to fully characterize AF from full-term pregnancies to maximize the benefit of using this valuable fluid in a clinical setting. Until specific parameters are identified to predict the efficacy of using AF for a specific application(s), minimum guidelines that we have established for a good quality sample of AF are that there is no visible evidence of maternal blood contamination, maternal samples shall test negative for infectious disease agents (i.e. human immunodeficiency virus, hepatitis B virus, Hepatitis C Virus, Syphilis) and there is no detection of any bacterial and fungal contamination based on USP <71> guidelines. We also currently have acceptance criteria for endotoxin levels (i.e. <5.0 EU/mL), HA levels (i.e. >150 ng/mL) and total protein levels (i.e. 0.16–10 mg/mL).

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Conflict of interest JP, PJ and JAR have no conflicts of interest.

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