Mechanism of Action for Amniotic Fluid

Amniotic fluid naturally contains the necessary "ingredients" for developing an extracellular matrix that can repair damaged tissue. Amniotic fluid contains a number of components that are imperative in the development of this foundational extracellular matrix, such as collagen, which forms fibrils that provide structure for tissues like ligaments, tendons, and skin. In addition to collagen, cytokines, chemokines, and hyaluronan in amniotic fluid work together within the matrix to regulate inflammation, maximize communication, and initiate cell regrowth within the tissue. An Amniotic Fluid Injection is an injectable scaffold that utilizes a naturally formed mixture of bioactive molecules and solidifiable precursors found in pure amniotic fluid. By injecting Amniotic Fluid into defected joints or soft tissues, a new 3D structure of regenerated healthy tissue is created. This entire process generally takes 3-6 weeks.

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The following pages contain the previously listed references and additional supporting studies.

Technology Insight: adult mesenchymal stem cells for osteoarthritis therapy

Ulrich Nöth, Andre F Steinert and Rocky S Tuan*

SUMMARY

Despite the high prevalence and morbidity of osteoarthritis (OA), an effective treatment for this disease is currently lacking. Restoration of the diseased articular cartilage in patients with OA is, therefore, a challenge of considerable appeal to researchers and clinicians. Techniques that cause multipotent adult mesenchymal stem cells (MSCs) to differentiate into cells of the chondrogenic lineage have led to a variety of experimental strategies to investigate whether MSCs instead of chondrocytes can be used for the regeneration and maintenance of articular cartilage. MSC-based strategies should provide practical advantages for the patient with OA. These strategies include use of MSCs as progenitor cells to engineer cartilage implants that can be used to repair chondral and osteochondral lesions, or as trophic producers of bioactive factors to initiate endogenous regenerative activities in the OA joint. Targeted gene therapy might further enhance these activities of MSCs. Delivery of MSCs might be attained by direct intra-articular injection or by graft of engineered constructs derived from cell-seeded scaffolds; this latter approach could provide a three-dimensional construct with mechanical properties that are congruous with the weight-bearing function of the joint. Promising experimental and clinical data are beginning to emerge in support of the use of MSCs for regenerative applications.

KEYWORDS articular cartilage, biomaterial scaffold, gene delivery, osteoarthritis, mesenchymal stem cell

REVIEW CRITERIA

We searched for full-text, English-language articles in the PubMed database up to December 2007 using the term “stem cells” in combination with “osteoarthritis”, “articular cartilage”, “biomaterial scaffold” and “intra-articular injection”, as well as the combination of “stem cells and cartilage and gene therapy”. We also searched the reference lists of identified articles for additional published reports.

U Nöth is an Assistant Professor of Orthopedic Surgery and Head of the Division of Tissue Engineering at the Orthopedic Center for Musculoskeletal Research, König-Ludwig-Haus, Julius-Maximilians-University, Würzburg, Germany, where AF Steinert is a Senior Resident and Head of the Division of Gene Therapy. RS Tuan is Chief of the Cartilage Biology and Orthopedics Branch at the National Institute of Arthritis, Musculoskeletal and Skin Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA.

Correspondence
*Cartilage Biology and Orthopedics Branch, National Institute of Arthritis, Musculoskeletal and Skin Diseases, National Institutes of Health, Building 50, Room 1523, 50 South Drive MSC 8022, Bethesda, MD 20892-8022, USA
tuanr@mail.nih.gov

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INTRODUCTION

Osteoarthritis (OA), the most common form of joint disease, is characterized by degeneration of the articular cartilage and, ultimately, joint destruction.[1] Currently, OA is a major cause of disability in the elderly; the prevalence of this disease is expected to increase dramatically over the next 20 years with an increasingly aged population.[2] The burden of OA is exacerbated by the inadequacies of current therapies. Nonpharmacologic and pharmacologic treatments are used for early and moderately early cases of OA, but protection of articular cartilage has so far not been convincingly shown.[3,4] Surgical intervention is often indicated when the symptoms cannot be controlled and the disease progresses.[5] Whether arthroscopic lavage and/or debridement can provide symptomatic relief is unclear.[6] Methods for the repair of articular cartilage lesions include the transplantation of osteochondral grafts, microfracturing, and autologous chondrocyte implantation, with or without the assistance of a scaffold matrix to deliver the cells;[7–12] however, all of these techniques are limited to the repair of focal lesions.[13] Consequently, patients with OA are currently excluded from these treatments. In the case of joint malalignment,[14] osteotomy can provide pain relief for several years, until the new weight-bearing articular cartilage erodes, but this tactic merely buys time until a total knee replacement becomes necessary. The challenge for researchers to develop disease-modifying OA treatments is, therefore, of paramount importance.

Adult mesenchymal stem cells (MSCs), which have the ability to differentiate into cells of the chondrogenic lineage, have emerged as a candidate cell type with great potential for cell-based articular cartilage repair technologies. MSCs can be isolated from a variety of adult tissues, readily culture-expanded without losing their multilineage differentiation potential, and have been induced to undergo chondrogenic differentiation *in vitro* and *in vivo*.[15–17] Unlike chondrocytes,

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the use of MSCs is not hindered by the limited availability of healthy articular cartilage or an intrinsic tendency of the cells to lose their phenotype during expansion. The use of MSCs also obviates the need for a cartilage biopsy and, thereby, avoids morbidity caused by damage to the donor-site articular surface.

In this Review, we will discuss current MSC-based strategies for the treatment of OA. We first address the etiopathophysiology of OA and the mechanisms responsible for breakdown of the cartilage extracellular matrix. We then discuss the potential of MSCs for articular cartilage repair in patients with OA, with particular respect to the chondrogenic differentiation potential of MSCs, and review the currently used experimental strategies (intra-articular injection, matrix-guided technologies, and gene therapy). An example of the repair of articular cartilage defects by use of a hydrogel seeded with MSCs is presented, to highlight the current strategies, limitations and perspectives of using MSCs to treat OA.

ETIOPATHOPHYSIOLOGY OF OA

The late stage at which OA is diagnosed, difficulties in studying the disease in humans, and inadequacies in animal models of OA account for (or contribute to) the poor understanding of this disease. Much research into the pathophysiology of OA has focused on the loss of articular cartilage, caused by mechanical and oxidative stresses, aging or apoptotic chondrocytes.[18] Articular chondrocytes within diseased cartilage synthesize and secrete proteolytic enzymes, such as matrix metalloproteinases and aggrecanases, which degrade the cartilaginous matrix. The proinflammatory cytokine interleukin 1 (IL-1) is the most powerful inducer of these enzymes and of other mediators of OA in articular chondrocytes. The induction of these factors leads to matrix depletion through a combination of accelerated breakdown and reduced synthesis.[18] Other proinflammatory cytokines, such as tumor necrosis factor, are also involved in cartilage breakdown and, together with biomechanical factors implicated in OA etiopathophysiology,[19,20] contribute to induction of the disease. Despite the considerable efforts put into development of inhibitors of these molecules for use in treating OA, clinical success with respect to the prevention of further cartilage matrix breakdown or cartilage restoration in OA remains elusive.[21,22]

POTENTIAL OF MESENCHYMAL STEM CELLS TO AID CARTILAGE RESTORATION

Some of the various OA pathologies might be obviated by the application of cell-based treatments. MSCs are multilineage progenitors that can be stimulated to differentiate along specific pathways, including chondrogenesis.[15] In contrast to mature chondrocytes, which must be surgically harvested from a limited supply of non-weight-bearing articular cartilage, MSCs can be readily harvested from bone marrow or other tissues of mesenchymal origin, and will maintain their multilineage potential even with extended passage, which enables their considerable expansion in culture.[16,17] MSCs are commonly isolated by adherence to cell-culture plastic or by density-gradient fractionation and, therefore, represent a heterogeneous population of cells.[16,17] Although no definitive marker(s) for MSCs has been identified, an immunophenotype that is positive for STRO-1, CD73, CD146, CD105, CD106, and CD166, and negative for CD11b, CD45, CD34, CD31 and CD117 has been shown to be the most reliable means of characterizing the MSC population.[16,17]

For the purpose of cartilage regeneration, extensive analyses of microenvironments that promote chondrogenesis in MSCs *in vitro* have been performed. Conditioning the culture medium with growth factors such as fibroblast growth factor 2 or transforming growth factor β during monolayer expansion enhances positive selection for chondroprogenitor cells.[23] The development of effective methods to maintain an articular cartilage phenotype without hypertrophy, ossification or fibrinogenesis, and a delivery system to localize the cells within a lesion without compromising their chondrogenic differentiation or the integrity of the repair tissue[13] are additional requirements for the use of MSCs in articular cartilage regeneration.

EXTENDING THE APPLICATION OF MESENCHYMAL STEM CELLS TO OA CARTILAGE

Despite the promising features of MSCs and their potential to reverse some of the pathology associated with OA, cartilage defects that arise from an underlying disease process (such as occurs in OA) are distinct from focal cartilage lesions that result from acute injury or osteochondrosis dissecans, and this difference must be taken into consideration. Specifically, acute cartilage injury and osteochondrosis dissecans often

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occur in an otherwise healthy joint; the patient might be young, and the focal defect will probably require localized treatment. By contrast, patients with OA are likely to be elderly, and often the entire articulating surface will require treatment. Repair of lesions might provide symptomatic relief and delay the progression of OA symptoms, but without effective treatment of the underlying disease, any improvement is likely to be short-lived.

Some researchers have suggested that tissue damage in progressive, degenerative, joint diseases might be related to the depletion or functional alteration of MSC populations.[24] Of importance, when considering the potential application of MSCs in OA treatment, researchers should ascertain whether MSCs obtained from the patient with OA differ functionally from those of healthy individuals, in terms of their chondrogenic capacity and longevity. The proliferative, chondrogenic and adipogenic capacities of MSCs obtained from patients with OA are reportedly reduced.[25] Perhaps the altered activity status of these MSCs is related to their exposure to elevated levels of proinflammatory cytokines and/or anti-inflammatory drugs. Whether susceptibility to OA might result from reduced mobilization or proliferation of MSCs remains to be ascertained.[24] Another factor associated with OA is advanced age; several studies have described an age-dependent reduction in the number of progenitor cells isolated from human bone marrow,[26,27] although others could not find any such inverse relationship between age and MSC numbers.[25,28] Also, an age-dependent decline in the differentiation capability of MSCs has been reported by several investigators.[25,27–29] In this context, however, researchers and clinicians should note that sufficient numbers of MSCs with adequate chondrogenic differentiation potential can be isolated from patients with OA, irrespective of their age or the etiology of their disease.[23,30,31] These results, therefore, suggest that the therapeutic use of MSCs for the regeneration of cartilage in patients with OA is feasible.

DELIVERY MODES FOR MESENCHYMAL STEM CELLS

A crucial requirement for MSC-based OA therapy is the delivery of the cells to the defect site. Direct intra-articular injection might be possible in early stages of the disease when the defect is restricted to the cartilage layer, whereas a scaffold or matrix of some kind would be required to support the MCSs in cases where the subchondral bone is exposed over large areas.

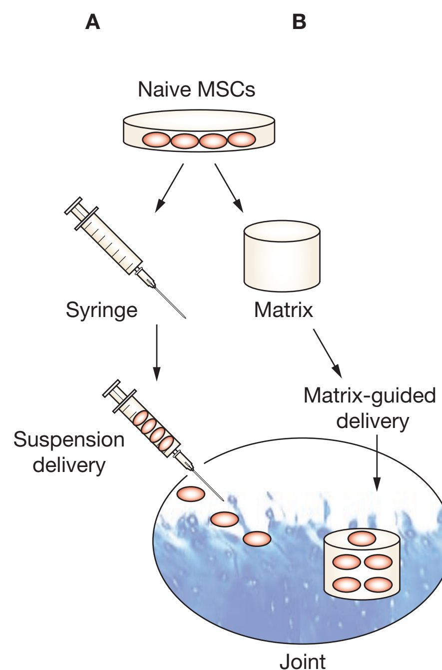

Figure 1 Delivery of MSCs to diseased cartilage in patients with osteoarthritis. (**A**) Direct intra-articular injection of naive MSCs. After harvest from an appropriate source, MSCs can be delivered in suspension to the joint space, where they encounter all intra-articular tissues. (**B**) Matrix-guided application of naive MSCs. Restoration of the deep cartilage defects that occur in osteoarthritis might require MSCs to be seeded into a biodegradable scaffold, which enables their controlled, local application to damaged areas of cartilage. Abbreviation: MSC, mesenchymal stem cell.

Direct intra-articular injection of MSCs

Direct intra-articular injection of MSCs is, technically, the simplest approach to their use in OA therapy (Figure 1A). Following injection, MSCs would be distributed throughout the joint space, and would interact with any available receptive cells and surfaces. The highly cellular synovium lines all the internal surfaces of the joint space, except for the cartilage and meniscus, so it is likely to be a primary tissue for MSC interaction.

Direct intra-articular injection of MSCs has only been carried out a few times. In one study, autologous MSCs in a dilute solution of sodium hyaluronan (hyaluronic acid) were directly injected into the knee joints of goats, in which

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OA had been induced by a total medial meniscectomy and resection of the anterior cruciate ligament.[32] Joints exposed to MSCs showed evidence of marked regeneration of the medial meniscus, and implanted cells were detected in the newly formed tissue. Articular cartilage degeneration, osteophytic remodeling, and subchondral sclerosis were also reduced in the treated joints. There was no evidence of repair of the ligament in any of the joints.[32] Whether the changes observed in MSC-treated joints resulted from direct tissue repair by the transplanted cells or from their interaction with host synovial fibroblasts at the site of injury is still unclear.

In another study, a freshly created, partial-thickness cartilage defect in the knee joints of mini-pigs was also treated by direct intra-articular injection of MSCs suspended in hyaluronic acid.[33] The cell-treated group of animals showed improved cartilage healing compared with the control group. The authors postulated that hyaluronic acid might facilitate the migration and adherence of MSCs or MSC-like cells—probably derived from the synovium—to the defect, which might explain the occurrence of partial healing at 6 weeks in animals that were treated with hyaluronic acid alone. The repair tissue in animals treated with hyaluronic acid alone was of inferior quality, however (possibly because an insufficient number of endogenous MSCs were recruited to the injury site), and was shown to deteriorate further by 12 weeks.

The exact mechanisms that guide homing of implanted or mobilized MSCs are not known, but it is clear that these cells secrete a broad spectrum of bioactive molecules that have immunoregulatory[34,35] and/or regenerative activities.[36] Bioactive factors secreted by MSCs have been shown to inhibit tissue scarring, suppress apoptosis, stimulate angiogenesis, and enhance mitosis of tissue-intrinsic stem or progenitor cells. The complex, multifaceted effects that result from the secretory activity of MSCs have been referred to as 'trophic activity'. Of note, the trophic activity of MSCs is distinct from their capacity to differentiate.[37]

Matrix-guided application of MSCs

Compared with direct intra-articular injection, MSC application to eroded cartilage surfaces via a scaffold offers more control (Figure 1B). Seeding MSCs into a scaffold, such as a biodegradable template, for proliferation and matrix production offers the advantage of providing an accessible, easy-to-manipulate, self-renewing source of progenitor cells (which would otherwise be of limited availability). The ideal scaffold should be biocompatible and biodegradable upon tissue healing, highly porous so as to permit cell penetration and tissue impregnation, sufficiently permeable to allow nutrient delivery and gas exchange, and adaptable to the mechanical environment. Also, the scaffold should have a surface that is conducive to cell attachment and migration, and permits appropriate extracellular matrix formation and the transmission of signaling molecules.[13,17,38,39] Various biomaterials have been utilized as vehicles to deliver MSCs for articular cartilage repair. However, few—if any—of the currently available scaffolds fulfill all of the requirements described above,[40] and further developments in biomaterial design are clearly needed to achieve optimal neocartilage formation with the use of cell–scaffold constructs.

Synthetic scaffolds

Synthetic scaffolds can be designed to offer optimal fiber diameter, pore size, degradation time and reproducibility in production. Many synthetic scaffolds commonly used in cartilage repair are fabricated using α-hydroxy polyesters, including polyglycolic acid, poly-L-lactic acid, the copolymer poly-DL-lactic-co-glycolic acid, and poly-ε-caprolactone.[41–43] The topography and material properties of these scaffolds are important in their ability to support MSC differentiation—for example, a nanofibrous scaffold of biodegradable polymers has demonstrated enhanced support of MSC proliferative and multilineage differentiative activities.[39,42]

Natural scaffolds

Native biomaterials, including collagen type I, hyaluronan, chitosan and alginate,[44,45] present a more natural microenvironment for MSCs than synthetic scaffolds do. Collagen type I hydrogels have several advantages: these matrices are biodegradable, can be metabolized by MSCs via the action of endogenous collagenases, elicit minimal, if any, inflammation, and surround the MSCs in three dimensions. The material properties of collagen hydrogels are similar to those of hyaline cartilage. Collagen gels can also be adapted as desired to most defect shapes. Compared with meshes or fleeces, in which cell seeding is often limited to superficial regions of the scaffold

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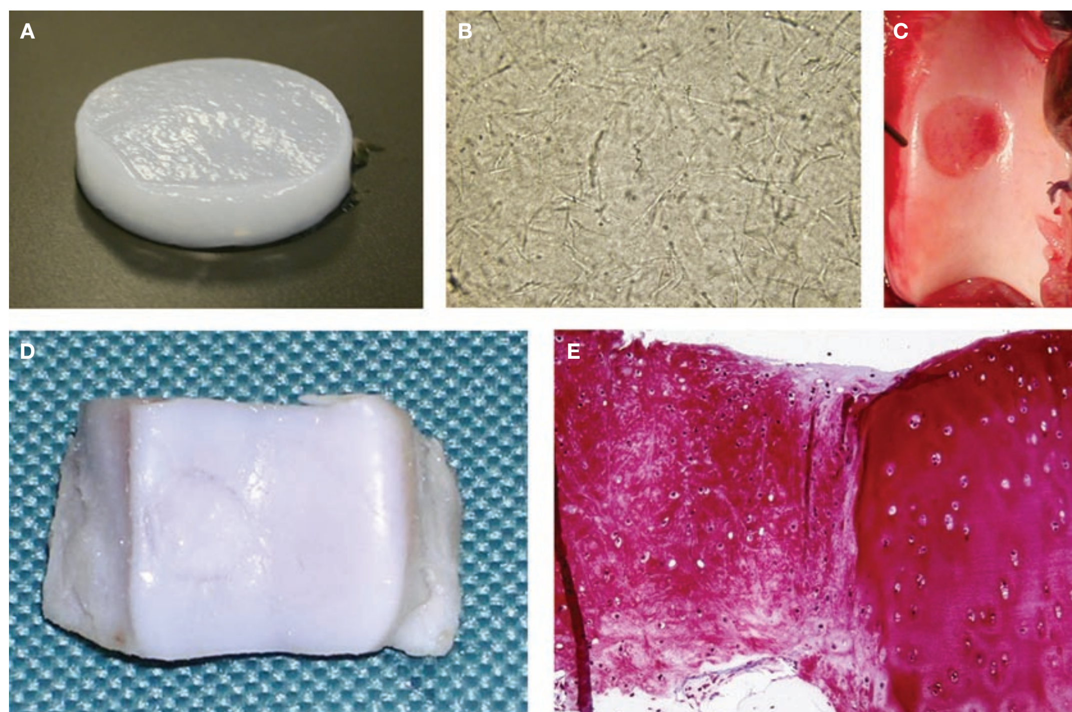

Figure 2 MSCs embedded in a collagen type I hydrogel can be used for tissue engineering of cartilage (U Nöth, unpublished data). (**A**) The collagen type I hydrogel used for matrix-based MSC transplantation was fabricated from rat-tail collagen (Arthro Kinetics, Esslingen, Germany). The implant (3 mm high and 7 mm wide) was seeded with MSCs and used to treat a cartilage defect in the trochlea of the mini-pig. (**B**) Magnified view of the MSC-containing collagen type I hydrogel, after 10 days of culture *in vitro* with Dulbecco's Modified Eagle's Medium plus 10% serum. The seeded cells are homogeneously distributed within the gel and show a fibroblast-like phenotype (×20 magnification). (**C**) Isolated chondral defect of the trochlea in a 6-month-old mini-pig. (**D**) Macroscopic appearance of the chondral defect 6 months after treatment with autologous MSCs seeded in a collagen type I hydrogel. (**E**) Immunohistochemical staining of the cartilage graft shows a cartilaginous, collagen type II-rich extracellular matrix, which contains chondrocytes that differentiated from MSCs. Bonding of the implanted gel to the host cartilage tissue was evident (×40 magnification). Abbreviation: MSC, mesenchymal stem cell.

material, hydrogels permit a more even distribution of seeded MSCs, which promotes homogeneous production of extracellular matrix.[46] Matrix-based implantation of autologous chondrocytes uses a collagen type I hydrogel for cell delivery.[47] Similarly, collagen hydrogel seeded with MSCs and implanted in mini-pig knee joints showed a homogeneous cell and extracellular matrix distribution 6 months after implantation (Figure 2).

Clinical studies of MSC implantation in collagen hydrogels

The first results for use of transplanted MSCs seeded within collagen type I hydrogels to repair isolated, full-thickness, cartilage defects in humans were reported by Wakitani *et al.*[48] Two patients with a patellar defect were treated with collagen gels containing MSCs, which were covered with a periosteal flap. Fibrocartilaginous filling of the defects was found after 1 year, and both patients showed significantly improved clinical outcomes in their respective follow-ups after 1, 4, and 5 years. The same group[49] has also used this protocol to treat another patient with a full-thickness cartilage defect in the weight-bearing area of the medial femoral condyle. The patient's clinical symptoms had improved significantly 1 year after surgery. Histologically, the defect was filled with a hyaline-like type of cartilage tissue that stained positively with safranin O, which indicated that the transplanted MSCs had differentiated into chondrocytes.

These pilot studies have been performed on isolated or focal articular cartilage defects in an otherwise healthy joint. The loss of joint

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Table 1 Vectors used for *ex vivo* intra-articular gene delivery.

Vector	Efficiency of transgene expression	Duration of transgene expression	Features	DNA capacity (kb)	Host range
Nonviral	Weak	Transient	Inflammatory Used in many clinical trials of RA	>20	Broad
Adenovirus	High	Transient	Inflammatory Approved for use in clinical trials	8–28	Broad
AAV	Moderate	Transient	Cause no known disease in humans Used in clinical trials of RA	4	Broad
HSV	High	Transient	Cytotoxic	40	Broad
Retrovirus	Moderate	Stable	Risk of insertional mutagenesis Used in clinical trials of RA	8	Dividing cells
Lentivirus	High	Stable	Risk of insertional mutagenesis Safety concerns	8	Broad
Spumavirus	Moderate	Stable	Cause no known disease in humans	>8	Broad

Abbreviations: AAV, adeno-associated virus; HSV, herpes simplex virus; kb, kilobases; RA, rheumatoid arthritis.

homeostasis in OA creates a very different microenvironment, which will influence MSC engraftment and tissue differentiation. The potential outcome of matrix-based cell transplantation in an OA joint is still unclear.[45] Generally, cartilage lesions in OA are usually large, unconfined, and affect more than one location—opposed (or 'kissing') lesions are common. In the knee joint, kissing lesions are regularly seen, and are frequently accompanied by a varus or valgus deformity or patella maltracking. The direct contact between opposed matrices bearing the transplanted cells creates a high probability that implanted matrices will be rapidly worn down as a result of joint articulation. Consequently, we must point out that current biological and technological developments do not indicate sufficient retention of cell-loaded scaffolds in OA lesions.

MESENCHYMAL STEM CELLS AS VEHICLES FOR GENE DELIVERY

MSCs seem to be receptive to transduction with various viral vectors, including adenovirus, adeno-associated virus, retrovirus, herpes simplex virus, lentivirus and spumavirus (also termed foamyvirus) (Table 1), so it is conceivable that some of the aforementioned limitations of current OA therapies might be overcome by adaptation of MSC-based gene-transfer technologies.[50] This approach will involve isolation of MSCs, *ex vivo* genetic modification of the MSCs, and transplantation of the modified cells into the diseased joint.

Generally, *ex vivo* gene-delivery approaches are more invasive, expensive and time-consuming than *in vivo* approaches (in which therapeutic vectors are applied directly into the body), but they do permit control of the transduced cells and safety testing before reimplantation.[51] In particular, use of MSCs should allow the development of techniques for delivering genes that encode proteins that might reverse some of the major pathologies of OA (Table 2).[13,51] Analogous to the delivery approaches described above for native MSCs (Figures 1A and 1B), genetically modified MSCs can be delivered to joints either as a cell suspension to counteract the inflammatory and matrix degradation processes, or via matrix-based strategies to induce formation of neocartilage tissue (Figure 3).

Delivery by cell suspension

Following delivery of cell suspensions, the aim is for transduced MSCs to release therapeutic proteins that interact with all available tissues, including cartilage. Considerable progress has been made towards defining the parameters that prolong intra-articular transgene expression, an approach that was originally developed for the treatment of rheumatoid arthritis (RA).[52] Current research suggests that immunologically compatible vector systems allow sustained intra-articular transgene expression.[53] In a phase I clinical study, IL-1 receptor antagonist complementary DNA was successfully retrovirally delivered by an *ex vivo* strategy to the metacarpophalangeal joints of individuals with

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Table 2 Classes of gene products used to augment MSC-based therapy for OA.

Potential therapeutic targets	Gene product class	Examples
Chondrocyte induction and protection		
Chondrogenic differentiation	Anabolic growth factors Signal-transduction molecules Transcription factors	TGF-β, BMP, Wnt Smad4, Smad5 SOX, brachyury
Osteogenic inhibition	Osteogenic inhibitors Inhibitors of chondrocyte terminal differentiation Signal-transduction molecules	Noggin, chordin PTHrP, IHH, SHH, DHH Smad6, Smad7, mLAP-1
Apoptosis inhibition	Caspase inhibitors Agents that block FasL Inhibitors of NO-induced apoptosis TNF, TRAIL inhibition	Bcl-2, Bcl-XL Anti-FasL antibodies Akt, PI3K NFκB
Senescence inhibition	Inhibitors of telomere erosion Free-radical antagonists	hTERT NO antagonists, SOD
Cartilage matrix induction and protection		
Cartilage matrix synthesis	Anabolic growth factors Extracellular matrix components Enzymes for glycosaminoglycan synthesis	TGF-β, BMPs, IGF-I Collagen type II GlcAT-1
Inhibition of inflammation	Cytokine antagonists Proteinase inhibitors Anti-inflammatory cytokines Enzymes that inhibit IL-1	IL-1Ra, sIL-1R, sTNFR, anti-TNF antibodies TIMP1, TIMP2 IL-4, IL-10, IL-11, IL-13 GFAT

Abbreviations: Akt, protein kinase B; Bcl-2, B-cell chronic lymphocytic leukemia and/or lymphoma 2; Bcl-XL, B-cell chronic lymphocytic leukemia and/or lymphoma apoptosis regulator; BMP, bone morphogenetic protein; DHH, Desert hedgehog; FasL, Fas ligand or CD178; GFAT, glutamine fructose 6 phosphate amidotransferase; GlcAT-1 glucuronosyltransferase I; hTERT, human telomerase reverse transcriptase; IGF-I, insulin-like growth factor I; IHH, Indian hedgehog; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; mLAP-1, murine latency-associated peptide 1; MSC, mesenchymal stem cell; NF-κB, nuclear factor κB; NO, nitric oxide; OA, osteoarthritis; PI3K, phosphatidylinositol 3 kinase; PTHrP, parathyroid-hormone-related protein; sIL-1R, soluble IL-1 receptor; sTNFR, soluble TNF receptor; TGF-β, transforming growth factor β; SHH, Sonic hedgehog; Smad, mothers against decapentaplegic homolog 1; SOD, superoxide dismutase; SOX, sex-determining region Y-box-containing proteins; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; Wnt, wingless-type mouse mammary tumor virus integration site family member.

RA.[54] This study shows that genes can indeed be delivered safely to human joints, and highlights the clinical utility of *ex vivo* gene transfer as a treatment for arthritis.[55] Data are beginning to emerge on the potential of such an approach for treating OA; encouraging results have been reported for IL-1 receptor antagonist adenovirally delivered to the joints of horses with experimental OA.[56] Furthermore, insulin-like growth factor 'administered' by intra-articular delivery partially reversed matrix degradation in OA.[51,57,58] Other cell types were initially investigated, but MSCs have the potential to be at least as beneficial when used in *ex vivo* approaches.[13,16,59]

A growing body of literature indicates that many of the pleiotropic gene products considered necessary for cartilage repair and regeneration are compatible with intra-articular delivery in suspension. However, delivery of transforming growth factor β1 or bone morphogenetic protein 2 to the synovium resulted in severe swelling, fibrosis, and osteophyte formation within joints.[60,61] Candidate complementary DNAs for synovial gene transfer should, therefore, be carefully chosen, safety-tested and validated (Table 2).

Delivery within a matrix

The above-mentioned anti-inflammatory treatments for RA and OA are, in principle, useful for preventing disease progression, but might not be able to restore damaged cartilage. An alternative strategy uses genetically modified MSCs in matrix-guided approaches to cartilage regeneration.[59,62] MSCs are first stimulated to undergo chondrogenic differentiation, stabilized as chondrocytes, then introduced on a matrix to the defect site, with the aim of establishing a cartilage phenotype without progression to hypertrophy or dedifferentiation.[13] A number of *in vitro* systems that use various transgenes (Table 2) demonstrate that MSCs can undergo chondrogenesis efficiently in defined, three-dimensional, serum-free, culture conditions.[44] Data indicating that delivery and expression

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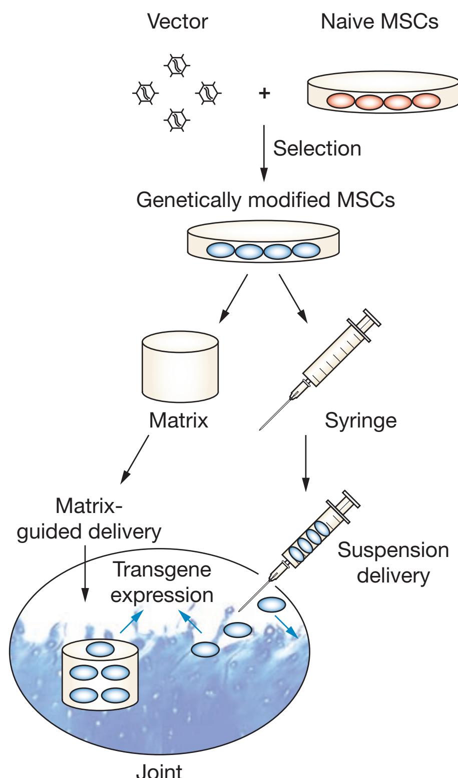

Figure 3 MSCs can be used as vehicles for *ex vivo* gene delivery. Cell-based approaches to osteoarthritis therapy might be augmented by use of genetically modified MSCs, which would involve gene transduction of culture-expanded MSCs. Successfully transduced cells would be isolated and applied to the joint space either as a cell suspension, or seeded within a biological matrix that can be implanted in a cartilage defect. Depending on which delivery approach is chosen, ubiquitous or local transgene expression is induced by the genetically modified MSCs, and the gene products could beneficially influence osteoarthritis pathology. Abbreviation: MSC, mesenchymal stem cell.

of certain genes might bias the repair response towards the synthesis of normal articular cartilage *in vivo* are beginning to emerge.[59] As already mentioned, however, this approach has been used mainly to treat focal cartilage defects. Future studies will show whether such technology will be suitable for repairing large areas of eroded cartilage, as occurs in advanced OA.[63]

CONCLUSIONS

OA is associated with the loss of homeostasis in joint tissues, particularly in the articular cartilage and the underlying bone. An insufficient repair response in articular cartilage, which results from a reduction in cell number and the loss of phenotypic stability, is a major contributor to disease progression. Further investigation will determine whether the titers of existing MSCs—both locally and throughout the body—as well as the quality of these cells might be important in the rate and extent of the repair of the damaged tissue.

The delivery of an appropriate MSC population is currently being investigated in the search for new therapeutic approaches to treat OA. The principal attraction of MSCs lies in their proliferative and chondrodifferentiation abilities, since articular chondrocytes are in limited supply. Understanding the biological activities and mechanisms of action of MSCs is crucial for a rational approach to their clinical application; specifically, conditions must be optimized to maintain MSC-derived chondrocytes in a stable, hyaline, chondrocyte-like state, without hypertrophy. Although MSC-based approaches might be developed and adapted for the treatment of both localized cartilage lesions and diseased or degenerate cartilage, as in OA, these states should be recognized as different entities.

Although direct intra-articular injection of cells is considered a technically simple approach to treatment of advanced OA, whether this approach can elicit beneficial effects (such as minimizing further cartilage damage) in human OA joints remains to be seen—and, if so, to what extent and under which conditions. The engineering design of matrix and scaffold material for cell-based articular cartilage repair has taken substantial strides, but the ideal scaffold material is still being sought, particularly for OA joints. Defects such as kissing lesions necessitate the design and engineering of new biomaterials that can be seeded with cells and can withstand significant mechanical loads. The use of MSCs in combination with bioactive substrates, natural or synthetic, also has significant clinical potential and is likely to be important in future, MSC-based, cartilage-repair technologies. In this context, MSCs might also offer promise in the future as vehicles for therapeutic gene delivery. In the long term, we hope that MSC-based technologies will permit the engineering of cartilage not only for repair of focal lesions but also as a treatment option for OA joints, to realize the ultimate goal of a fully biological prosthesis.

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KEY POINTS

- Osteoarthritis (OA), the most common joint disease, is characterized by degeneration of the articular cartilage that ultimately leads to joint destruction
- Current treatment strategies for OA are inadequate
- Delivery of an appropriate mesenchymal stem cell (MSC) population is currently being investigated in the search for new therapies for OA
- MSCs could be used as trophic producers of bioactive factors to initiate endogenous regenerative activities in the OA joint; their activities might be further enhanced via targeted gene therapy
- Delivery of MSCs might be achieved either by direct intra-articular injection or by implantation of engineered constructs derived from MSC-seeded scaffolds
- In the long term, MSC-based technologies could permit the engineering and repair of cartilage as a treatment option for OA joints

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Potential use of the human amniotic membrane as a scaffold in human articular cartilage repair

Silvia Díaz-Prado · Mª Esther Rendal-Vázquez · Emma Muiños-López · Tamara Hermida-Gómez · Margarita Rodríguez-Cabarcos · Isaac Fuentes-Boquete · Francisco J. de Toro · Francisco J. Blanco

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Abstract The human amniotic membrane (HAM) is an abundant and readily obtained tissue that may be an important source of scaffold for transplanted chondrocytes in cartilage regeneration in vivo. To evaluate the potential use of cryopreserved HAMs as a support system for human chondrocytes in human articular cartilage repair. Chondrocytes were isolated from human articular cartilage, cultured and grown on the chorionic basement membrane side of HAMs. HAMs with chondrocytes were then used in 44 in vitro human osteoarthritis cartilage repair trials. Repair was evaluated at 4, 8 and 16 weeks by histological analysis. Chondrocytes cultured on the HAM revealed that cells grew on the chorionic basement membrane layer, but not on the epithelial side. Chondrocytes grown on the chorionic side of the HAM express type II collagen but not type I, indicating that after being in culture for 3–4 weeks they had not de-differentiated into fibroblasts. In vitro repair experiments showed formation on OA cartilage of new tissue expressing type II collagen. Integration of the new tissue with OA cartilage was excellent. The results indicate that cryopreserved HAMs can be used to support chondrocyte proliferation for transplantation therapy to repair OA cartilage.

Keywords Amniotic membrane · Chondrocytes · Cartilage · Osteoarthritis · Cell therapy

All authors were involved in the research presented and drafted and approved the final manuscript.

S. Díaz-Prado · I. Fuentes-Boquete · F. J. de Toro
Department of Medicine, INIBIC-University of A Coruña, A Coruña, Spain

S. Díaz-Prado · I. Fuentes-Boquete · F. J. de Toro · F. J. Blanco
CIBER-BBN-Cellular Therapy Area, Barcelona, Spain

M. E. Rendal-Vázquez · M. Rodríguez-Cabarcos
Tissue Bank, INIBIC-Hospital Universitario A Coruña, A Coruña, Spain

E. Muiños-López · T. Hermida-Gómez · F. J. Blanco
Rheumatology Division, INIBIC-Hospital Universitario A Coruña, A Coruña, Spain

F. J. Blanco (✉)
Osteoarticular and Aging Research Laboratory, Hospital Universitario A Coruña, C/As Xubias S/N, 15.006 A Coruña, Spain
e-mail: francisco.blanco.garcia@sergas.es; fblagar@canalejo.org

Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by deterioration in the integrity of hyaline cartilage and subcondral bone (Ishiguro et al. 2002). OA is the most common articular pathology and the most frequent cause of disability. Genetic, metabolic and physical factors interact in the pathogenesis of OA producing cartilage damage. The incidence of OA is directly related to age and is

expected to increase along with the median age of the population (Brooks 2002).

The capacity of articular cartilage to repair is very limited (Steinert et al. 2007; Mankin 1982), largely due to its avascular nature. Currently, there are no effective pharmaceutical treatments for OA, although some medications slow its progression (Brandt and Mazzuca 2006; Steinert et al. 2007). There are also no surgical approaches to treat OA; however, surgery is an important tool for the repair of cartilage injuries, which if left untreated may result in secondary OA.

To date, most efforts made to repair an articular cartilage injury are intended to overcome the limitations of this tissue for healing by introducing new cells with chondrogenic capacity (Koga et al. 2008) and facilitating access to the vascular system. Current treatments generate a fibrocartilaginous tissue that is different from hyaline articular cartilage. To avoid the need for prosthetic replacement, different cell treatments have been developed with the aim of forming a repair tissue with structural, biochemical, and functional characteristics equivalent to those of natural articular cartilage.

Cell therapy is a new clinical approach for the repair of damaged tissues. Cell therapy using mesenchymal stem cells (Koga et al. 2008) or differentiated chondrocytes (autologous chondrocyte implantation, ACI) is one therapeutic option for the repair of focal lesions of articular cartilage, which is most successful in young people producing repair tissue of high quality (Brittberg et al. 1994; Minas and Chiu 2000). Aging diminishes the cell density of cartilage and the ability of chondrocytes to proliferate and form cartilage in vivo (Froger-Gaillard et al. 1989).

ACI has several technical limitations, among which are the effects of gravity causing the chondrocytes to sink to the dependent side of the defect, resulting in an unequal distribution of cells (Jin et al. 2007) that hampers the homogenous regeneration of the cartilage. To overcome some of the limitations of ACI, cell delivery supports can be used for cell transplantation. The transplantation of chondrocytes seeded on natural and synthetic scaffolds has been used for cartilage tissue engineering (Kuo et al. 2006). Scaffolds must readily integrate with host tissues and provide an excellent environment for cell growth and differentiation. Scaffolds must also provide a stable temporary structure while cells seeded within the biodegradable matrix synthesize a new and natural tissue. A number of scaffolds have been developed and investigated, in vitro and in vivo, for potential use in tissue engineering. The human amniotic membrane (HAM) is considered to be an important potential source for scaffolding material (Niknejad et al. 2008) and has begun to be appreciated for its usefulness in the field of regenerative medicine (Toda et al. 2007).

HAMs develop from extra-embryonic tissue and consist of both a fetal component (the chorionic plate) and a maternal component (the decidua) that are comprised of an epithelial monolayer, a thick basement membrane and an avascular stroma (Niknejad et al. 2008; Jin et al. 2007). The amnion is a fetal membrane attached to the chorionic membrane. Both the amnion and chorion form the amniotic sac filled with amniotic fluid, providing and protecting the fetal environment. The outer layer, the chorion, consists of trophoblastic chorionic and mesenchymal tissues. The inner layer, the amnion, consists of a single layer of ectodermally-derived epithelium uniformly arranged on the basement membrane, which is one of the thickest membranes found in any human tissue, and a collagen-rich mesenchymal layer (Wilshaw et al. 2006). This mesenchymal layer can be subdivided into the compact layer forming the main fibrous skeleton of the HAM, the fibroblast layer and an intermediate layer, which is also called the spongy layer or *zona spongiosa* (Niknejad et al. 2008). The amnion is a thin (up to 2 mm), elastic, translucent and semi-permeable membrane, which adheres firmly to an exposed surface. These properties enable surgeons to apply the graft on various tissue surfaces without need for suturing or application of secondary dressings. Immediately after grafting, the process of biodegradation begins and the membrane self-dissolves over a period of time from days to 3–4 weeks depending on the characteristics of the wound, the presence or absence of co-existing pathogens, the polarization of the applied graft and the type of graft applied.

The HAM possesses clinical considerable advantages to make it potentially attractive as a biomaterial. It is anti-microbial, anti-fibrosis, anti-angiogenic, anti-tumorigenic and has acceptable mechanical properties. It also reduces pain and inflammation, inhibits scarring, enhances wound healing and

epithelialization, and acts as an anatomical and vapor barrier. All these characteristics are not shared by other natural or synthetic polymers, highlighting the clinical advantages of amniotic membrane as a scaffold compared to other biocompatible products. Also, amnios shows little or no inmunogenicity and the immune response against the graft, if there is, is slight and ineffective, so it does not represent transplantation risks. On the contrary, chorion shows high immunogenicity and for this reason it is not used as biomaterial for transplantation purposes. Importantly, HAMs are inexpensive and easily obtained with an availability that is virtually limitless, negating the need for mass tissue banking (Toda et al. 2007; Niknejad et al. 2008; Hennerbichler et al. 2007; Wilshaw et al. 2006). The extracellular matrix (ECM) components of the HAM include collagens (types I, III, IV, V and VI), fibronectin, nidogen, laminin, proteoglycans and hyaluronan, as well as growth factors (Niknejad et al. 2008; Rinastiti et al. 2006; Jin et al. 2007). The HAM, therefore, has abundant natural cartilage components, which are important in the regulation and maintenance of normal chondrocyte metabolism (Jin et al. 2007); this suggests that the HAM is an excellent candidate for use as native scaffold for cartilage tissue engineering (Niknejad et al. 2008).

The aim of this study was to evaluate the potential usefulness of cryopreserved HAMs as human chondrocyte graft support for human articular cartilage repair. For this purpose, we developed an in vitro model to evaluate the capacity of human chondrocytes to grow on a HAM and repair human articular cartilage lesions.

Materials and methods

Harvest and preparation of HAMs

Human placentas were obtained from selected Cesarean-sectioned mothers in Hospital Materno Infantil-Teresa Herrera from La Coruña, Spain. All mothers gave written informed consent prior to collection. This study was approved by the Ethics Committee of Clinical Investigation of Galicia (Spain). Under stringent sterile conditions harvested placentas were placed in 199 medium (Invitrogen S.A., Spain) with antibiotics: cotrimoxazol 50 μg/ml (*Soltrim*®, Almirall-Prodesfarma S.A., Spain), vancomycin 50 μg/ml (*Vancomicina Hospira*®, Laboratorio Hospira S.L., Spain), amykacin 50 μg/ml (*Amikacina Normon*®, Laboratorios Normon S.A., Spain) and B amphotericin 5 μg/ml (*Fungizona*®, Bristol-Myers Squibb S.L., Spain). The HAM was carefully separated from the chorion of the placenta and the chorion was discarded. The amnion was then washed 3–5 times with 0.9% NaCl solution to remove blood and mucus. The HAM was then incubated in 199 medium with antibiotic solution: metronidazol 50 μg/ml (*Metronidazol G.E.S.*, G.E.S. Genéricos *Españoles Laboratorio S.A., Spain*), vancomycin 50 μg/ml, amykacin 50 μg/ml and B amphotericin 5 μg/ml for 6–20 h at 4°C and cryopreserved. In some cases, the HAMs were pretreated with 1% trypsin–EDTA (Sigma–Aldrich Química S.A., Spain) for 30 min, as previously described (Ma et al. 2006), to remove epithelial cells and to facilitate chondrocyte penetration into the porous structure of the denuded HAM.

Cryopreservation and thawing of HAMs

The HAM was cut in 6 × 6 cm patches and placed on a supportive sterile nitrocellulose filter in 20 ml of medium without antibiotics but with a cryoprotectant, 10% dimethyl sulfoxide (DMSO). Each patch of HAM was cryopreserved following a protocol of controlled freezing using a CM 2000 (Carburos Metálicos, Spain). Freezing rates were −1°C/min to a temperature of −40°C, −2°C/min to −60°C, and −5°C/min to −150°C. All HAMs were stored in the gas phase of liquid nitrogen at −150°C. Thawing was carried out for 5 min at room temperature followed by 37°C until thawing was complete (Fig. 1a–c). To reduce cell damage due to osmotic changes, the DMSO was removed by sequential washing and progressive dilution with 0.9% NaCl at 4°C.

Harvest of human cartilage and isolation of articular chondrocytes

Femoral heads were provided by the Autopsy Service and Orthopaedic Department at Hospital Universitario A Coruña, Spain. Samples comprised 25 donors (15 male and 10 female) with a mean age of 67, 24 years and a range from 25 to 85 years. All samples came from knee donors (13 were diagnosed of osteoarthritis and 12 were healthy). The population

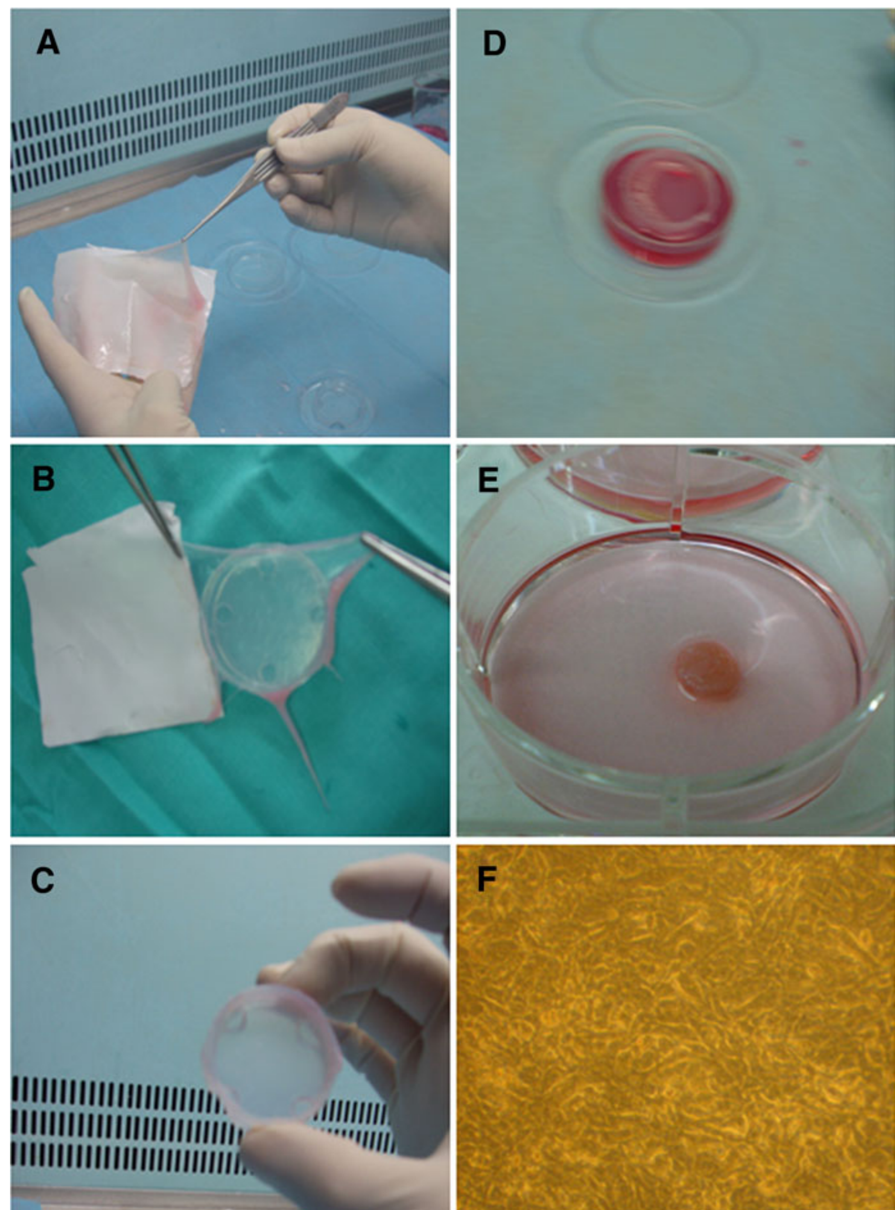

Fig. 1 Illustrations of the developed methodology for articular cartilage repair. A cryopreserved human amniotic membrane (HAM) was thawed (**a**) and placed over ring-shaped support (**b**, **c**) that was then placed in a petri dish containing growth medium. Human articular chondrocytes were seeded (5×10^5) on the HAM (**d**). After chondrocyte proliferation the HAM with chondrocyes was used for in vitro cartilage repair (**e**). Human chondrocytes grown on the chorionic basement membrane layer of the HAM (10X) (**f**)

of patients included 17 living donors and 8 deceased donors. To obtain chondrocytes, articular cartilage full-thickness slices were used. To develop the in vitro cartilage repair model, 6 mm diameter discs of articular cartilage were used.

Cartilage slices were aseptically removed from femoral heads, sliced full thickness (excluding the mineralized cartilage and subchondral bone), and washed in Dulbecco's modified Eagle's medium (*DMEM*, Sigma–Aldrich Química S.A., Spain) as previously described (Blanco et al. 1998; Rendal-Vázquez et al. 2001). Briefly, slices were minced with a scalpel and transferred into a digestion buffer containing DMEM + Glutamax (Sigma–Aldrich Química S.A., Spain), 1% L-glutamine (Sigma–Aldrich Química S.A., Spain), ciprofluoxacin 10 µg/ml (*Ciprofluoxacina*, Laboratorios Vita S.A., Spain), penicillin 100 UI/ml (Invitrogen S.A., Spain) streptomycin 100 µg/ml (Invitrogen S.A., Spain), insulin 100 UI/ml (*Actrapid*®, Novo Nordisk Pharma S.A., Spain), deoxyribonuclease I (25,000 UI/l) (Sigma–Aldrich Química S.A., Spain), and 1%

trypsin–EDTA. The cartilage tissues were then incubated on a shaker at 37°C for 5–10 min until digestion was complete. The supernatant (without chondrocytes) was discarded and the trypsinized cartilage was subjected to a second digestion buffer containing DMEM + Glutamax,
1% L-glutamine, ciprofluoxacin 10 μg/ml, penicillin (100 UI/ml), streptomycin (100 μg/ml), insulin (100 UI/ml), deoxyribonuclease I (25,000 UI/l) and 2 mg/ml clostridial collagenase (Type IV) (Invitrogen S.A., Spain), incubated at 37°C overnight and washed 3 times before being used for culture or cryopreservation. Fresh or thawed primary chondrocytes were grown directly on the basement layer of HAMs prepared as described above.

Chondrocyte proliferation studies on HAMs

For human chondrocyte growth on the chorionic basement layer of the HAM, a suspension containing 5×10^5 primary chondrocytes was deposited on the central part of the amniotic membranes (6×6 cm^2). These chondrocytes on the HAM membrane were grown in DMEM + Glutamax medium containing 20% foetal bovine serum (*FBS*, Invitrogen S.A., Spain), ciprofloxacin 10 μg/ml, penicillin (150 UI/ml), streptomycin (50 mg/ml), insulin (100 UI/ml), deoxyribonuclease I (25,000 UI/l) for 3–4 weeks in a humidified 5% CO_2 atmosphere at 37°C until they reached a confluency of 80–90%. At this time, the membranes were employed to develop an in vitro model for articular cartilage repair (Fig. 1d, f). The number of assays performed in chondrocyte proliferation studies was $n = 59$.

Development of an in vitro model for articular cartilage repair

Each 6×6 cm patch of HAM was cut into three fragments of 0.7×0.7 cm and placed on the superficial surface of three different 6 mm OA cartilage discs such that the basement layer of the HAM, on which the chondrocytes were grown, was in direct contact with the superficial surface of the cartilage. The three cartilage discs layered with the chondrocyte-cultured HAM were placed in six-well culture plates (Costar®, USA) (Fig. 1e). In each well, 2 ml of culture medium containing DMEM supplemented with penicillin (100 UI/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum was placed. The culture plates were incubated in humidified 5% CO_2 atmosphere at 37°C. The culture medium was replaced twice weekly. All procedures were performed under stringent sterile conditions. After 4 weeks, the first cartilage disk was retrieved from the culture plate, fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. The same procedure was followed at 8 and 16 weeks with the second and third discs. The resulting blocks were cut into 4 μm-thick sections using a microtome that were mounted on poly-L-lysine-coated glass slides for histological and immunohistochemical analyses. The number of in vitro models for articular cartilage repair developed was $n = 44$. Also, one group of controls, with amniotic membrane but without chondrocytes, were included ($n = 4$).

Histological and immunohistochemical analyses

For general histological analyses, 4 μm-thick paraffin sections were deparaffinized in xylol, rehydrated in a graded series of ethanol, and stained with hematoxylin and eosin (H–E), Masson's trichrome or Safranin O staining for proteoglycans.

Paraffin sections (4 μm-thick), which had been deparaffinized and hydrated as described above, were incubated with monoclonal antibodies to detect the presence of collagen types I (Abcam, Spain) and II (BioNova Científica, Spain), Ki-67 (Novocastra, UK), integrin β-1 subunit (Abcam, Spain) and glycosaminoglycans: chondroitin-4-sulphate (ICN Biomedicals Inc, Spain), chondroitin-6-sulphate (ICN Biomedicals Inc, Spain) and keratan sulphate (Seikagu America Inc, Rockville, MD). To facilitate the exposure of epitopes, sections stained for collagens were pretreated with hyaluronidase (Sigma–Aldrich Química S.A., Spain), and those stained for glycosaminoglycans were pretreated with chondroitinase ABC (Sigma–Aldrich Química S.A., Spain). The peroxidase/DAB ChemMateTM DAKO EnVisionTM detection kit (Dako Citomation, USA) was used to determine antigen-antibody interaction. Negative staining controls were achieved by omitting the primary monoclonal antibody or the secondary detector antibody. Samples were visualized using an optical microscope.

Results

Selection of the appropriate side of the HAM

As a first approach to the study of the potential usefulness of the HAM as a support for the cultivation of human chondrocytes, we cultured chondrocytes on the HAM. In this study, the chorion and amnion were carefully separated from the human placenta to assess only the amnion as a human chondrocyte delivery support for human articular cartilage repair. To determine which side of the amnion would be the most appropriate, we first tested the growth of human chondrocytes on both the epithelial side, the single monolayer of epithelial cells from the extra-embryonic ectoderm ($n = 9$), and the chorionic thick basement membrane side ($n = 8$). The amnion also consists of a delicate avascular mesenchymal layer, the extra-embryonic remainder of the mesoderm, located under the thick basement membrane. Preparations that are shown in this study do not include this mesenchymal layer.

Primary human chondrocytes were grown on the chorionic and epithelial sides of the HAM. When chondrocytes reached confluency at approximately 3–4 weeks (Fig. 1f), they were stained with H–E and Masson's trichrome. As shown in Figs. 2 and 3, the distribution of chondrocytes on both the chorionic and epithelial sides of the HAM showed a characteristic monolayer pattern of cell growth. However, on the epithelial side the chondrocyte monolayer was not attached to the extra-embryonic ectoderm. Despite the fact that the epithelial cells of the extra-embryonic ectoderm of HAM showed good preservation, the cultured chondrocytes were not in contact with the epithelial cells, indicating a possible competition between these cell types. As a result of this incompatibility, we observed apparent cell death of both chondrocytes and epithelial cells and, in fact, epithelial cells from the HAM caused the release of chondrocytes cultured upon it. In several areas the layer of cultured chondrocytes is lifted, detached and fragmentized. This cellular competition produced eosinophilia and massive necrosis in the epithelium in addition to the detachment of the chondrocyte monolayer cultured upon it.

Human chondrocytes cultured on HAMs maintain their phenotype

Histological techniques demonstrated that type II but not type I collagen was expressed by the human chondrocytes cultured on the basement membrane layer of the HAM ($n = 10$) (Fig. 4). This confirms that human chondrocytes grown on HAMs for 3–4 weeks until confluent did not de-differentiate into fibroblasts or another cell type, but maintained the characteristic phenotype of human chondrocytes.

The effect of trypsin on chondrocyte growth on HAMs

To remove epithelial cells from HAMs and facilitate chondrocyte penetration into the porous structure of the denuded HAM, we treated HAMs with various trypsin concentrations ($n = 7$). As a result of trypsin treatment we observed a massive necrosis of epithelial cells from the extra-embryonic ectoderm of HAMs. Chondrocytes cultured on trypsin-treated HAMs had disintegrated cytoplasmic membranes, and many of the chondrocytes were necrotic. Chondrocytes were found in very few areas, sometimes appearing in clusters, and they were separated from the basement membrane. We did, however, observe areas with well-conserved chondrocyte monolayers attached to the basement membrane of the generally fragmented HAM (Fig. 5). For this reason we only performed 3 in vitro repair models using trypsin-treated HAMs.

In summary, these results demonstrate that chondrocytes grew when cultured on the chorionic basement membrane layer of the HAM, but did not proliferate well when grown on the epithelial side. For this reason the articular cartilage repair experiments were carried out using the chorionic basement membrane layer of the HAM.

In vitro cartilage regeneration

The surface of OA articular cartilage is irregular with numerous fissures and small cavities distributed along the edge of the lesion. The HAM with chondrocytes grown on the cartilage biopsy provided a more regular surface with the new tissue able to fill the fissures and cavities of the OA cartilage. The newly-formed tissue

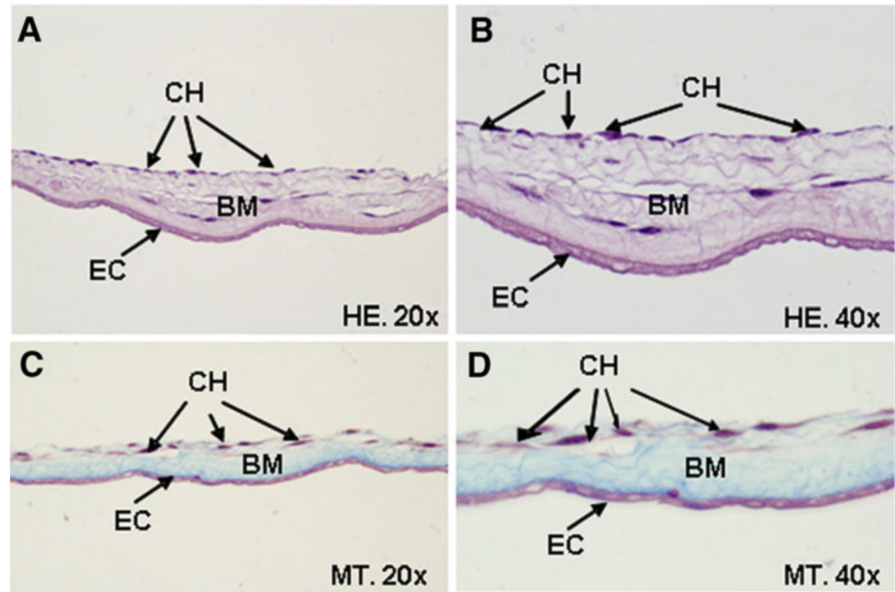

Fig. 2 Distribution of human articular chondrocytes cultured on the basement membrane of human amniotic membranes (HAMs). H–E (hematoxylin–eosin) (**a**, **b**) and M–T (Masson's Trichrome) (**c**, **d**) staining. CH, monolayer of cultured cells (chondrocytes) on the thick basement membrane of the HAM; *BM* basement membrane; *EC* epithelial cells from the extra-embryonic ectoderm

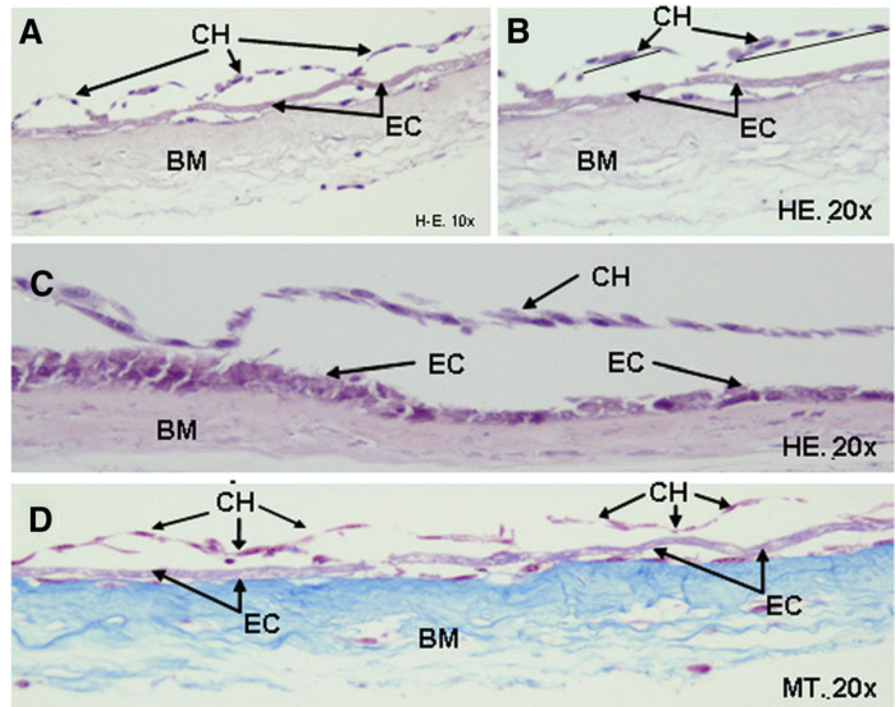

Fig. 3 Distribution of human articular chondrocytes cultured on the epithelial cells from the extra-embryonic ectoderm of human amniotic membranes (HAMs). H–E (hematoxylin–eosin) (**a**, **b**, **c**) and M–T (Masson's Trichrome) (**d**) staining. The monolayer of chondrocytes is separated or raised from the epithelial cells of the extra-embryonic ectoderm. CH, monolayer of cultured cells (chondrocytes) on the epithelial cells from the extra-embryonic ectoderm of HAMs; *BM* basement membrane; *EC* epithelial cells from the extra-embryonic ectoderm

formed from the HAM with chondrocytes showed a tendency towards a linear way, providing a superficial cell cover that decreased the irregularities of the damaged OA cartilage surface. Generally, this newly-formed tissue showed good integration with the OA cartilage. Some newly-formed tissue had cells in monolayers while others had bi-layers or even multiple layers when there were deep cracks in the OA cartilage (Fig. 6a). The cells had rounded morphology with the characteristics of chondrocytes (Fig. 6a).

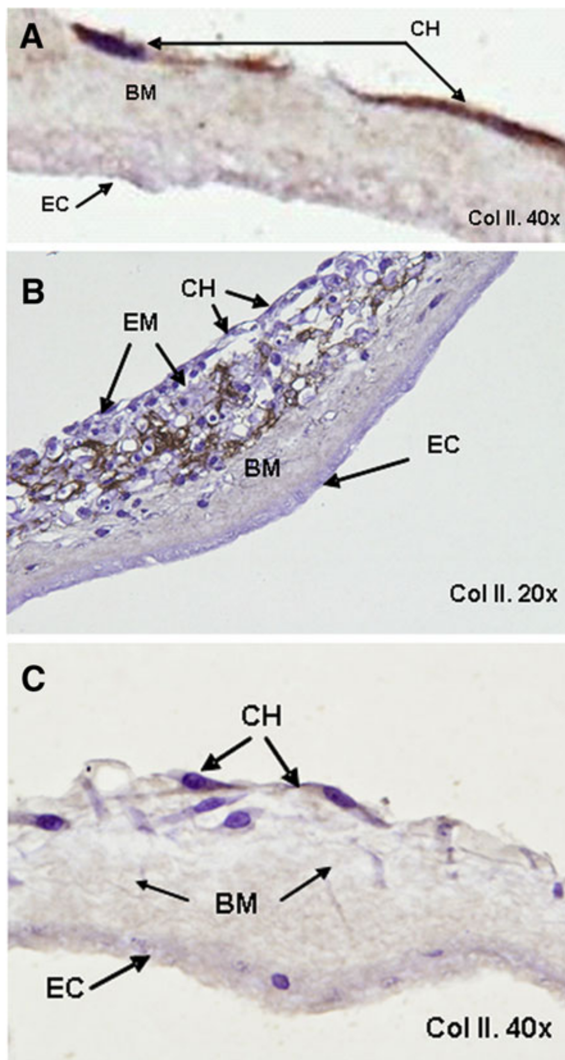

Fig. 4 Immunohistochemistry of the monolayer of human articular chondrocytes grown on the basement membrane of human amniotic membranes (HAMs) showed positive staining for type II collagen (Col II) (**a**, **b**, **c**). The thick basement membrane of the HAM also expresses Col II. CH, monolayer of human articular chondrocytes cultured on the basement membrane HAMs; *BM* basement membrane; *EC* epithelial cells from the extra-embryonic ectoderm. *ECM* extracellular matrix formed by cultured cells

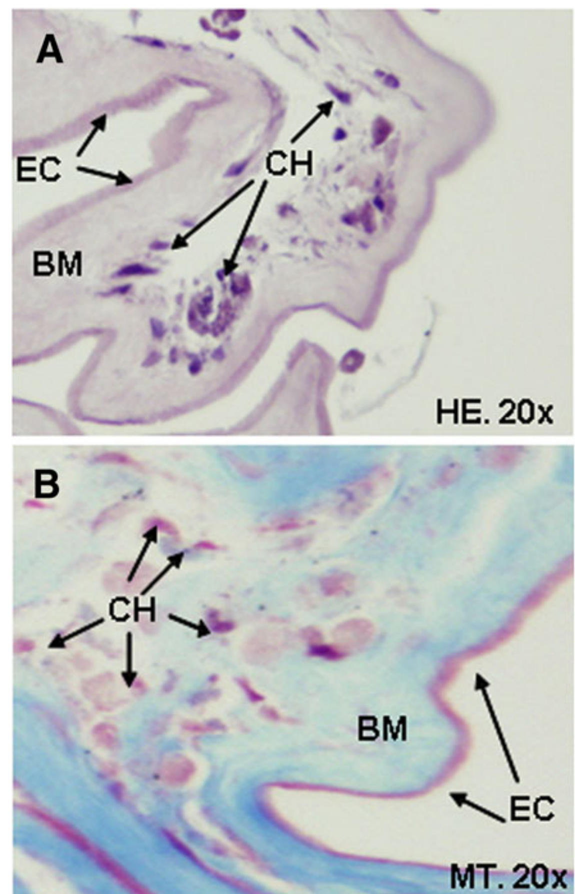

Fig. 5 Treatment of human amniotic membranes (HAMs) with trypsin 0.25% (**a**, **b**). Human articular chondrocytes grown on the thick basement membrane of HAMs have disintegrated cytoplasmic membranes, most cells are necrotic, and some are separated from the HAM. Epithelial cells from the extra-embryonic ectoderm of the HAM suffered a massive necrosis. CH, monolayer of human articular chondrocytes cultured on the basement membrane of HAMs; *BM* thick basement membrane; *EC* epithelial cells from the extra-embryonic ectoderm. H–E (hematoxylin–eosin) staining (**a**) and Masson's Trichrome staining (**b**)

In many of the transplants examined, chondrocytes from the HAM migrated to penetrate into the depths of the cavities or fissures in the OA cartilage (Fig. 6b, c).

The morphology of the repair tissue in the majority of experiments exhibited a fibrous appearance and high cellularity. We could not define the boundary between native cartilage and new tissue because that boundary appeared to be much diffused and not easily differentiated.

In most cases the newly-formed tissue was so cellular that its cell density was even higher than that of the native cartilage (Fig. 6d). Some of the transplants showed a thickening of the basement membrane of the HAM, this may be because chondrocytes grown on the basement membrane penetrate, infiltrate and spread in a uniform manner throughout the thickness of the stromal matrix (Jin et al. 2007) (Fig. 6d). In the repair tissue we saw the

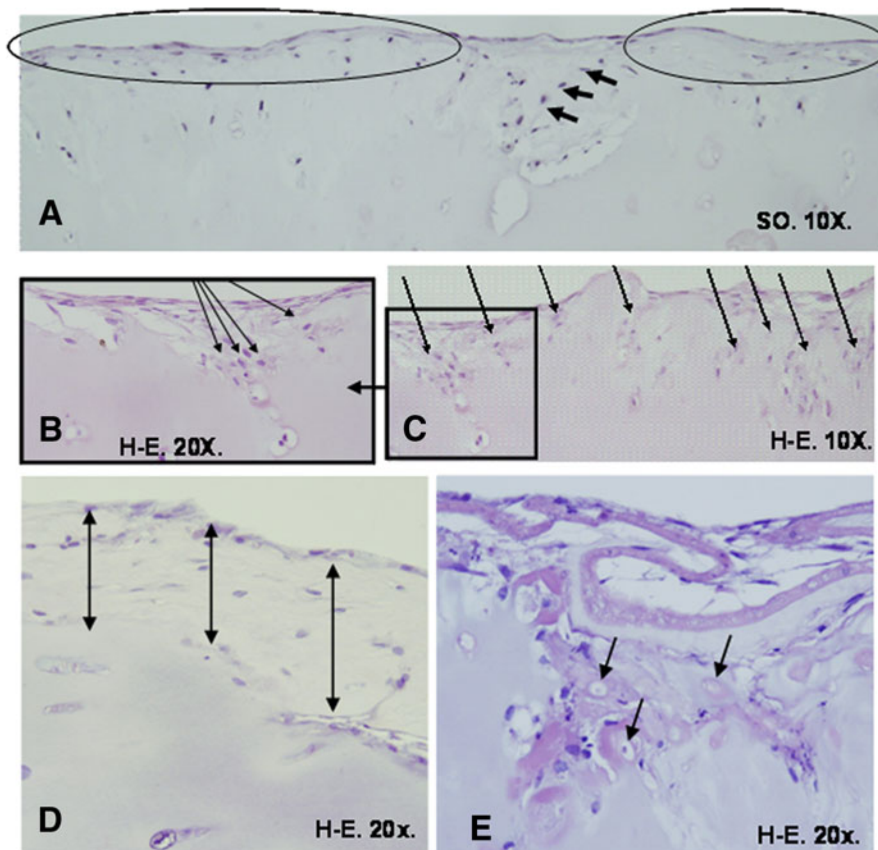

Fig. 6 In vitro repair model of human osteoarthritis (OA) articular cartilage. Human articular chondrocytes cultured on human amniotic membranes (HAMs) provided a smooth surface that filled the fissures and cavities providing a superficial cell cover that decreased the irregularities of the damaged cartilage surface of human articular OA cartilage. The newly-formed HAM tissue integrated well with native cartilage and formed bi-layers and monolayers of cells with a round morphology similar to chondrocytes (**a**). Chondrocytes, from the HAM, migrated and penetrated into the depth of the cavities or fissures of the OA cartilage (**b** and **c**). The newly-formed tissue showed high cell density and a thickening of the basement membrane of the HAM (**d**). Repair tissue also showed formation of lacunae that contained cells with a round morphology similar to chondrocytes (**e**). Safranin O staining (**a**), H–E (hematoxylin–eosin) staining (**b**, **c**, **d**, **e**)

formation of lacunae containing cells with round morphology similar to chondrocytes that were well integrated with neighbouring cartilage. H–E staining corroborated that some repair areas appeared similar to hyaline cartilage (Fig. 6e).

Staining to identify the specific and main components of the cartilage ECM was performed. Specifically, we sought to determine the presence of molecules characteristic of hyaline cartilage, such as proteoglycans by staining with Safranin O and types I and II collagen using immunohistochemistry. Safranin O staining revealed no proteoglycans in nearly all instances. There were only a few showing positive staining, notably in areas where the newly-formed HAM tissue showed good integration with the native cartilage (Fig. 7a, b). The deepest areas of native cartilage showed positive staining with Safranin O, which disappeared in the more superficial areas. The low proteoglycan content would indicate that the quality of the repair tissue is low and that the newly-formed tissue was fibrocartilage. The newly-formed HAM tissue showed such good integration with native cartilage that in some of the in vitro transplants it was impossible to distinguish the border between the newly-formed and native tissues (Fig. 7c, d). In contrast to the lack of staining for proteoglycans that indicates fibrocartilage formation, the newly-formed tissues showed a positive immunoreaction for type II collagen (Fig. 7e) while immunohistochemical staining for collagen type I was weak or absent (Fig. 7f).

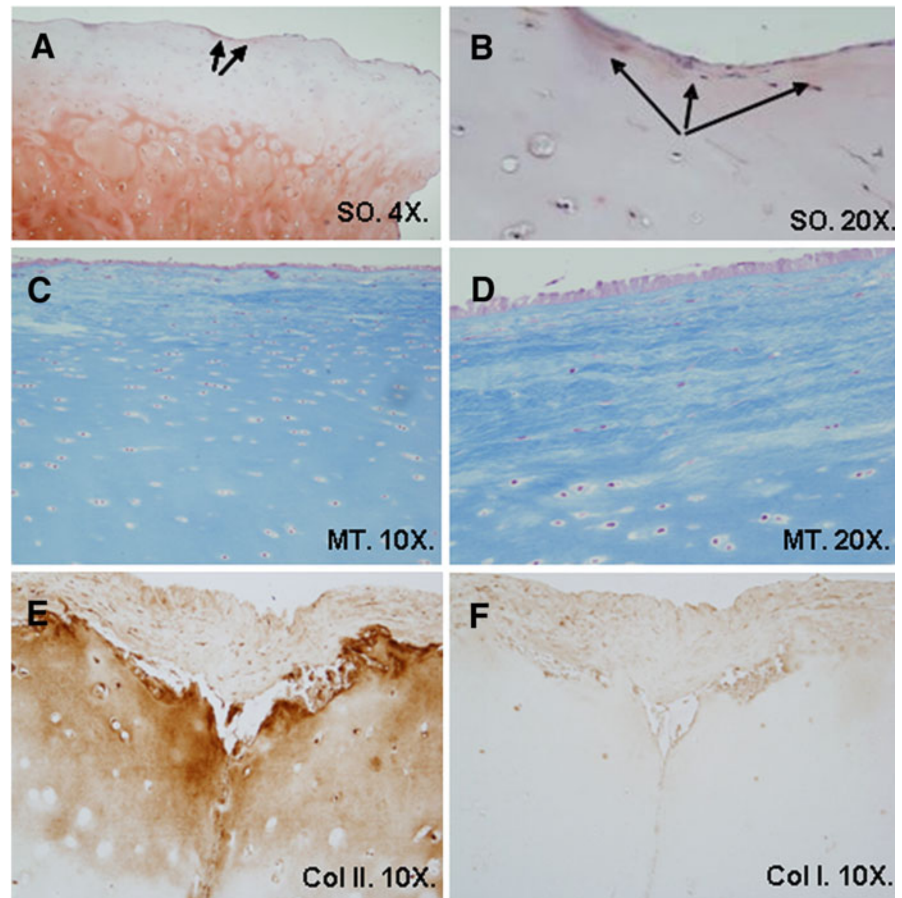

Fig. 7 Histology of the in vitro repair model of human osteoarthritis articular cartilage. Safranin O staining (**a**, **b**). Masson's Trichrome staining (**c**, **d**). Type II and I collagen staining respectively (**e**, **f**)

In the group of controls, amniotic membrane without chondrocytes was adhered to the surface of OA articular cartilage but it was not observed any newly-formed tissue neither in the fissures nor in the small cavities distributed along the edge of the lesion.

Discussion

The prevalence of OA in the human population underscores the importance of developing an effective and functional articular cartilage replacement. Recent research efforts have focused on tissue engineering as a promising approach for cartilage regeneration and repair (Kuo et al. 2006). Cartilage tissue engineering is critically dependent on the selection of appropriate cells, suitable scaffolds for cell delivery and biological stimulation with chondrogenically bioactive molecules (Kuo et al. 2006). A major prerequisite for choosing a scaffold is its biocompatibility, the property of not producing toxic, injurious, carcinogenic, or immunological responses in living tissue (Niknejad et al. 2008). New tissue regeneration should occur as the scaffold degrades, so the new tissue assumes the shape and size of the original scaffold. Design criteria for scaffolds include controlled biodegradability, suitable mechanical strength and surface chemistry, ability to be processed in different shapes and sizes, and the ability to regulate cellular activities such as differentiation and proliferation (Kuo et al. 2006). For cartilage tissue engineering, scaffolding has been fabricated from both natural and synthetic polymers, such as fibrous structures, porous sponges, woven or non-woven meshes and hydrogels (Kuo et al. 2006). Researchers have recently proposed that the HAM is suitable as a scaffold for tissue engineering (Niknejad et al. 2008; Wilshaw et al. 2006).

The ECM components of the basement membrane from the HAM include collagen, fibronectin, laminin

and other proteoglycans important for overlying cell growth. Other properties of the HAM include anti-inflammation, anti-fibrosis, anti-scarring, anti-microbial, low immunogenicity and adequate mechanical properties, all important requirements for tissue engineering (Niknejad et al. 2008). The HAM can produce a wide array of growth factors and provide a healthy new substrate suitable for re-epithelization and epithelial healing (Wilshaw et al. 2006). Amnion allografts are widely applied in ophthalmology, plastic surgery, dermatology, and gynecology (Tejwani et al. 2007; Santos et al. 2005; Rinastiti et al. 2006; Meller et al. 2000; Morton and Dewhurst 1986). The low cost of amnion graft preparation and the very good clinical results in multipurpose applications have made it a valuable material for tissue banking and a viable alternative to other natural (i.e., preserved human skin) and synthetic wound dressings.

The purpose of this study was to evaluate the potential use of cryopreserved HAMs as a support for human chondrocytes to repair human articular cartilage lesions. Recent studies have found that limbal, corneal and chondrocyte stem cells rapidly proliferate on HAMs (Koizumi et al. 2002; Galindo et al. 2003; Jin et al. 2007). We have determined that human chondrocytes were able to grow on both the epithelial and chorionic sides of the HAM. The chondrocytes showed a characteristic monolayer cell growth. However, when grown on the epithelial side of the HAM the monolayer of chondrocytes separated from the extra-embryonic ectoderm, suggesting a possible competition between the chondrocytes and the epithelial cells. This indicates that the chorionic surface of the HAM is more suitable than the epithelial side for human chondrocyte growth. We propose that the HAM could be an excellent candidate for use as a scaffold for cell delivery and migration in order to achieve bonding to the adjacent host tissue, but when the cells are grown on the chorionic surface. The utility of this new biomaterial may be because the HAM promotes epithelialization and neovascularization and possesses immune privilege (Sippel et al. 2001). It has been previously documented that HAMs may accelerate epithelialization of gingival wounds and ocular chemical and thermal injures when reconstructing damaged organs and corneal tissue (Rinastiti et al. 2006; Madhira et al. 2008; Sangwan et al. 2007; Tejwani et al. 2007). Also, Santos et al. (2005) have demonstrated the suitability of HAMs for treating limbal stem cell deficiency (LSCD). Although we found the chorionic surface of the HAM most suitable for chondrocyte growth in the in vitro repair model for human articular cartilage, other studies indicate that when HAM is used for support of human limbal epithelial cells (HLEC), the epithelial side of the HAM is more appropriate (Li et al. 2006; Meller et al. 2002).

Human chondrocytes are known to de-differentiate toward fibroblasts when cultured (Gimeno Longas and de la Mata Llord 2007). Using immunohistochemistry, we have demonstrated that human chondrocytes cultured on HAMs expressed type II but not type I collagen. This confirms that the chondrocytes did not de-differentiate to fibroblasts or to a different cell type, but maintained the characteristic phenotype of human chondrocytes.

Human chondrocytes cultured on HAM and transplanted onto human osteoarthritis articular cartilage produced a more regular surface, filling the fissures and cavities of the OA cartilage. The chondrocytes grew in a linear arrangement and decreased the degree of damages of the OA articular cartilage surface. Also, HAM with cultured chondrocytes showed good integration with the native cartilage and the newly synthesized tissue constituted bi-layers and monolayers of cells with round morphology and characteristics similar to chondrocytes. Chondrocytes migrated from the HAM to penetrate into the depths of the cavities and fissures in the OA cartilage. The morphology of the repair tissue exhibited a fibrous appearance and high cellularity. Also, we were not able to delineate the boundary between native cartilage and newly-formed tissue.

In most cases the newly-formed tissue was so cellular that it had a higher cell density than the native cartilage. Some of the transplants showed a thickening of the basement membrane of the HAM, probably because chondrocytes grown on the basement membrane penetrate, infiltrate and spread in a uniform manner throughout the thickness of the stromal matrix as previously described (Jin et al. 2007). In the newly-formed tissue, we observed the formation of lacunae containing cells with the round morphology of chondrocytes to be well integrated with neighbouring cartilage. In fact, using H–E staining, it was possible to corroborate that some repair areas appeared similar to hyaline cartilage.

Staining was done to detect specific major components of the ECM to obtain more detailed information about the structure and composition of the repair tissue (Fuentes Boquete and López Armada 2007). Safranin O staining showed no proteoglycans in almost all cultures, although a few did show positive staining, notably in areas where the HAM showed good integration with the native cartilage. The deepest area of the native cartilage showed positive staining with Safranin O, but this disappeared in the superficial area. The absence of proteoglycan in the outer areas of OA cartilage has been previously described (Fuentes Boquete and López Armada 2007). The low content of proteoglycan indicates that the quality of the repair tissue is low, the newly-formed tissue being hyaline-like cartilage. Importantly, the newly-formed tissue showed a positive immunoreactivity for type II collagen, while the immunohistochemical staining for type I collagen was weak or absent.

Conclusion

Our results indicate that cryopreserved HAMs are useful for the support of human chondrocyte proliferation in cell therapy to repair human OA cartilage.

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Amniotic Fluid: Not Just Fetal Urine Anymore

Mark A. Underwood, MD
William M. Gilbert, MD
Michael P. Sherman, MD

Amniotic fluid (AF) is a complex substance essential to fetal well-being. This article reviews recent discoveries and the current understanding of the origin and circulation of AF and its nutritive, protective, and diagnostic functions. Future directions for AF research are also discussed.
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INTRODUCTION

Amniotic fluid (AF) is a marvelously complex and dynamic milieu that changes as pregnancy progresses. AF contains nutrients and growth factors that facilitate fetal growth, provides mechanical cushioning and antimicrobial effectors that protect the fetus, and allows assessment of fetal maturity and disease. This article will review the development, content, and clinical significance of AF and its essential role in helping the fetus become a newborn.

DEVELOPMENT OF AF AND THE AF CIRCULATION

A fluid-filled extracelomic cavity which will eventually become the amnionic space is identified near the time of implantation, even before the embryo is recognizable. During embryogenesis, AF volume increases faster than embryonic size. The water in AF originally comes from maternal plasma and passes through the fetal membranes based on hydrostatic and osmotic forces. As the placenta and fetal vessels develop, water and solute from maternal plasma pass across the placenta to the fetus and then to the AF. In the early fetal period, AF volume and fetal size are related in a linear fashion. AF volume increases from about 25 ml at 10 weeks to about 400 ml at 20 weeks. During this period, AF composition is similar to fetal plasma. There is rapid bi-directional diffusion between the fetus and the AF across the not-yet-keratinized fetal skin and the surfaces of the amnion, placenta, and umbilical cord, each being freely permeable to water and solutes. During this phase of pregnancy, the AF serves both as a physiologic buffer and an extension of the fetal extracellular compartment. By 8 weeks of gestation, the urethra is patent and the fetal kidneys make urine. Shortly thereafter fetal swallowing begins; however, neither fetal urination nor swallowing contributes significantly to the content or volume of AF until the second half of pregnancy. Keratinization of fetal skin begins at 19 to 20 weeks of gestation and is usually complete at 25 weeks after conception. When keratinization is complete, the relationship between fetal size and AF volume is no longer linear. By 28 weeks of gestation, AF volume reaches a volume of ∼800 ml where it plateaus near term gestation and thereafter declines to ∼400 ml at 42 weeks.[1]

After the fetal skin is fully keratinized, AF volume is determined by factors that comprise the AF circulation. Five pathways of exchange have been identified between the amniotic space and the surrounding tissues (see Figure 1). Production of AF is predominately accomplished by excretion of fetal urine (∼300 ml/kg fetal weight/day or 600 to 1200 ml/day near term) and the secretion of oral, nasal, tracheal, and pulmonary fluids (∼60 to 100 ml/kg fetal weight/day).[2] Fetal breathing movements contribute to the efflux of lung fluid into the AF, but about half of the effluent is swallowed rather than entering the AF. While volume changes with each fetal breath are small, <5 ml per breath, and fetal breathing occurs only for 20 to 30 min of each hour in late gestation, the overall contribution of fetal breathing to AF volume is significant. Removal of AF is predominately accomplished by fetal swallowing (∼200 to 250 ml/kg fetal weight/day). Additionally, a significant intramembranous pathway transfers fluid and solutes from the amniotic cavity to the fetal circulation across the amniotic membranes.[3] The human amnion is a single layer of epithelial cells separating the amniotic cavity from the vascularized chorion. Early in gestation these amniocytes are flattened, but 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. The amount of fluid that passes through the intramembranous pathway is highly variable and has been estimated at 200 to 500 ml/day.[4] The transmembranous pathway, the movement of AF across the fetal membranes and into the maternal circulation within the lining of the uterus, affects AF volume only minimally. While this process has not been directly measured, it is estimated to be ∼10 ml/day at term.[2] Sherer[5] is an excellent review of AF dynamics.

Department of Pediatrics (M.A.U., M.P.S.), University of California, Davis School of Medicine, Davis, CA, USA; Department of Obstetrics and Gynecology (W.M.G.), University of California, Davis School of Medicine, Davis, CA, USA.

Address correspondence and reprint requests to Mark Underwood, MD, UCD School of Medicine, Neonatology, TB 193, One Shields Avenue, Davis, CA 95616, U.S.A.

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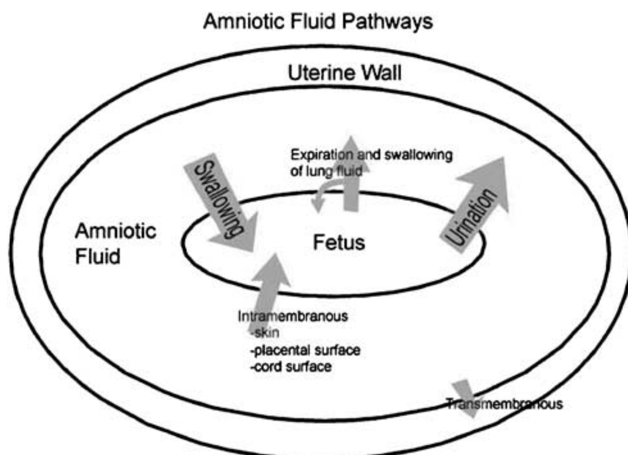

Figure 1. Amniotic fluid pathways.

The relative stability of AF volume in spite of large fluid shifts suggests that control mechanisms exist. It is noteworthy that only half of fetuses with esophageal atresia, and two-thirds of fetuses with duodenal or proximal jejunal atresia develop polyhydramnios; this suggests that other mechanisms besides swallowing are involved in AF volume regulation. Compensation via the intramembranous pathway is the best explanation for the significant number of fetuses with upper intestinal atresia who do not develop polyhydramnios. Compensation is evident in sheep where ligation of the esophagus leads to increased absorption of AF into the fetal circulation with no change in the total AF volume. Intramembranous absorption occurs against a hydrostatic gradient and had been assumed to be driven by passive diffusion due to an osmotic gradient. More recent studies show that passive diffusion accounts for only part of the intramembranous fluid absorption and that many solutes diffuse in the opposite direction (from fetus to AF). It is likely that much larger shifts of fluid and solutes occur by bulk transfer of AF with all of its dissolved solutes into the fetal circulation perhaps via a trans-cellular vesicular transport mechanism.[6] Vascular endothelial growth factor (VEGF) in the ovine fetal membranes appears to be a mediator of this process. VEGF promotes blood vessel development within the amnion and influences the permeability of the microvessels, which perfuse the fetal and placental surfaces.[7] The demonstration of aquaporin proteins in fetal membranes suggests the possibility of water channels as another potential regulator.[8]

Hormonal changes may also play a role in AF volume regulation. There are not significant numbers of receptors for estrogen or progesterone in fetal membranes after early pregnancy. Receptors for decidual prolactin, however, are widely expressed by both fetal and maternal tissues with increasing numbers as pregnancy progresses. There is evidence that decidual prolactin has an effect on amniotic permeability, although this is probably not the only hormonal or growth factor-related mechanism.[9]

Uterine perfusion also impacts AF volume. Maternal dehydration leads to increased fetal plasma osmolality and increased fetal production of arginine vasopressin. This causes an increase in the osmolality of both fetal urine and to a lesser extent AF. When arginine vasopressin is injected directly into ovine AF, fetal urine and AF osmolality increase and fetal urine output decreases significantly, and yet AF volume does not change suggesting reverse intramembranous flow from the isotonic fetal circulation to the hypertonic AF.[10]

The composition of AF changes with gestational age. In the second half of pregnancy, there is a decrease in sodium and chloride concentrations, an increase in urea and creatinine concentrations, and an overall decrease in AF osmolality. Many studies suggest that AF composition is more highly regulated than AF volume. Wintour and Shandley[4] has an excellent summary of these studies.

NUTRITIVE FUNCTIONS OF AF

AF contains carbohydrates, proteins and peptides, lipids, lactate, pyruvate, electrolytes, enzymes, and hormones. Prior to keratin production in fetal skin, amino acids diffuse from the placenta through the placental membranes into AF and from the fetal circulation through the fetal skin into AF. Later in pregnancy diffusion through the placental membranes persists and is augmented by fetal urinary excretion of amino acids.[11] Like breast milk, AF is rich in taurine which is found in greater quantity in AF than in maternal serum, while most other amino acids have lower concentrations in AF than in maternal and fetal blood. Glutamine is an essential precursor for nucleic acid biosynthesis in all cells and is particularly important in rapidly dividing cells such as intestinal mucosa cells. In fetal sheep, the uptake of glutamine from the AF by the fetal intestine is an active process.[12] Arginine also plays an essential role in fetal and placental development. Arginine is hydrolyzed to ornithine, which is then converted into the polyamines, putrescine, spermine, and spermidine, which are key regulators of placental angiogenesis, trophoblast growth, and embryogenesis. In sheep, the concentrations of arginine, ornithine, and polyamines increase rapidly in both allantoic and amniotic fluids early in gestation and remain elevated in AF throughout pregnancy. As gestational age increases, the swallowed polyamines in AF support proliferation and differentiation of intestinal epithelial cells.[13]

The role of swallowed carbohydrates and lipids in AF is less well defined. Growth-restricted rabbit fetuses were given infusions of dextrose or dextrose with amino acids directly into AF, and there was no improvement in growth, while an infusion of bovine AF did improve organ and somatic growth.[14] In a fetal rabbit model with esophageal ligation, the infusion of graded amounts of glucose or glucose with amino acids into AF enhanced organ weights and fetal

growth.[15] No studies have yet demonstrated reversal of fetal growth restriction (FGR) by intra-amniotic infusion of nutrient solutions.

Ligation of the esophagus in fetal rabbits to prevent swallowing followed by infusion of various solutions into the gut distal to the ligature has been performed to demonstrate the nutritive value of fetal swallowing. Those animals infused with lactated Ringer's solution had poor gut development whereas those infused with bovine AF showed more normal gut maturation.[16] Improved fetal organ growth with esophageal infusions of AF has also been shown in fetal sheep.[17] Trophic effects of AF have further been demonstrated on cultured human fetal small intestinal cells.[18] These studies suggest that growth factors found in AF, comparable to those in human milk, play a role in fetal growth and development. These trophic mediators are discussed below.

High levels of *epidermal growth factor* (EGF) are found in human milk and AF but not in standard infant formula. The concentration of EGF in amniotic fluid is four-fold higher than that found in fetal urine suggesting that the site of production is the amnionic membranes. EGF in human AF increases significantly during the second trimester, but is reduced in FGR. The function of EGF in the human fetus is largely unknown. In monkeys, in utero treatment with EGF improves lung maturity.[19] In fetal rabbits, enteral infusions of EGF reverse the effects of esophageal ligation.[16] EGF injected into the AF of pregnant rabbits increases small intestinal length and lactase and maltase activity compared to controls.[20] EGF receptors are present in the human stomach from the 18th week of gestation onward.

Transforming growth factor alpha (TGF-α) has a structure similar to EGF and binds to the same receptor. TGF-α is present in AF and human milk and, like EGF, is found in higher concentrations in human milk from women delivering prior to 27 weeks of gestation compared to those delivering after 27 weeks.[21] TGF-α is also produced in the fetal intestine. Recombinant TGF-α elicits a synergistic trophic response on cultured intestinal cells when combined with recombinant EGF, insulin-like growth factor-1, fibroblastic growth factor, and hepatocyte growth factor, but the trophic response is not as strong as either AF or breast milk.[18] The amnion cells of the umbilical cord express EGF, TGF-α, and the functional EGF/TGF-α receptor suggesting the possibility of a regulating role of the amnion in fetal growth and development. EGF and TGF-α have also been shown to stimulate the production of surfactant components.

Transforming growth factor beta-1 (TGF-β1) is found in rat AF and human breast milk, but is found in human AF only during the late stages of gestation. TGF-β1 is believed to induce terminal differentiation of intestinal epithelial cells and to accelerate the rate of healing of intestinal wounds by stimulating cell migration. TGF-β1 may also stimulate IgA production. Thus, TGF-β1 may prepare the fetal intestine for the extrauterine environment that is experienced after parturition at term.

Insulin-like growth factor I (IGF-I) is found in human milk and AF. When infused into the esophagus of fetal sheep, IGF-I improves somatic growth, spleen weight, and bowel wall thickness compared to control.[22] A single injection of labeled IGF-I into ovine AF demonstrated sustained delivery of IGF-I from the AF to the fetal gut and then into the systemic circulation over a 7-day period.[23] IGF-I and IGF-II receptors, as well as insulin receptors, are found throughout the human neonatal gut. IGF-I in AF may also increase the uptake of swallowed glutamine by the ovine gut.[12]

Erythropoietin (EPO) is found in human AF, colostrum, and mature milk. In the neonatal rat, enteral EPO is absorbed, stimulates erythropoiesis, and is a trophic factor for intestinal growth. The role of swallowed EPO in the human fetus and neonate is not clear. It is puzzling that concentrations of EPO are significant in AF and actually increase in human milk with the length of breast feeding, yet EPO is not absorbed from the gastrointestinal tract even though it is protected from digestion in the stomach.[24] This suggests the possibility of a local intestinal effect.

Granulocyte colony-stimulating factor (G-CSF) is found in human AF. When given enterally to suckling mice G-CSF enhances intestinal growth, suggesting that swallowed G-CSF in AF, colostrums, and breast milk may act as a topical growth factor in the fetal and neonatal intestine.

PROTECTIVE ROLE OF AF

AF plays an important protective role by providing a supportive cushion allowing fetal movement and growth. The oligohydramnios sequence and its related fetal deformations demonstrate the importance of this protective cushion.

AF also has a significant defensive role as a part of the innate immune system. The innate immune system is the first line of defense against pathogens and includes anatomic and physiologic barriers, enzymes and antimicrobial peptides, as well as phagocytosis and release of proinflammatory mediators by neutrophils and macrophages. Many of the substances that comprise the innate immune system have been identified in AF and vernix and have been shown to have significant antimicrobial properties; these include the α-defensins [HNP1-3], lactoferrin, lysozyme, bactericidal/permeability-increasing protein, calprotectin, secretory leukocyte protease inhibitor, psoriasin [S100A7], and a cathelicidin [LL-37].[25–27] These potent antimicrobials show broad-spectrum activity against bacteria, fungi, protozoa, and viruses. Perhaps the most important of these are the α-defensins [HNP1-3], which are found in significant concentrations in AF of women without evidence of infection and likely originate from the fetal skin and lung. AF concentrations of HNP1-3 increase with preterm labor, preterm premature rupture of membranes (PPROM), and chorioamnionitis probably due to release from neutrophils.

Lactoferrin (LF) is a glycoprotein with two binding sites for ferric ion. LF is found in human milk and appears in human AF at 20 weeks gestation increasing in concentration with gestation. Elevated levels of LF have been noted with preterm labor and with amnionitis. In pregnancies complicated by intra-amniotic infection (IAF), LF is likely secreted by neutrophils in the AF and by amniotic cells. LF has both bacteriostatic activity, due to sequestration of iron which is then unavailable for microbial growth, and bacteriocidal activity, due to binding to bacterial outer membranes triggering release of lipopolysaccharide. Enzymatic digestion of LF at acid pH releases a potent cationic, microbicidal peptide called lactoferricin. Lactoferricin shows antimicrobial effects against viruses, protozoa, and fungi.[28] Lactoferrin levels decrease with the onset of term labor.

The activity of the ''cellular'' innate immune system within AF as a protective mechanism for the fetus is less well defined. The numbers of mononuclear phagocytes (i.e., monocytes, macrophages, histiocytes) in AF are limited in normal pregnancies, while their numbers are increased in fetuses with neural tube defects. Whether these macrophages are present to prevent infection because of a disruption of the fetal skin or as scavenger cells to clean up neural debris is uncertain. Neutrophils are not normally identified in the AF of healthy fetuses, but are useful as a marker of AF infection. These cells are fetal in origin and appear to originate in the fetal vessels of the chorionic plate. It is interesting that meconium stained AF shows chemotactic activity for neutrophils in utero, although the meconium itself is not the likely chemotactic factor.[29] Two hematopoietic growth factors, G-CSF and macrophage colony-stimulating factor (M-CSF), are found in AF of healthy term and preterm fetuses. G-CSF is elevated in the serum of women with subclinical chorioamnionitis, in the cord blood of neonates with infection, fetal distress, premature rupture of membranes, and meconium staining of AF, and in the AF, neonatal urine and neonatal bronchoalveolar fluid of newborns after IAI. Whether G-CSF and M-CSF actually play a preventive host defense role in the AF or are just excreted by-products of the immune response during infection is not known.

There may also be nonimmune components of AF that protect the fetus from injury. For example, amniotic fluid may protect the fetal gut from the effects of platelet activating factor (PAF). PAF is a potent vasoconstrictor and has been strongly implicated in the pathophysiology of necrotizing enterocolitis in preterm infants.[30] PAF levels in human AF are low throughout gestation, but at term, PAF content undergoes an eight-fold increase with the onset of labor. PAF is elevated in AF of preterm fetuses whose mothers have failed tocolysis as well as AF of complicated pregnancies. The major PAF degrading enzymes are platelet activating factor acylhydrolase and platelet activating factor acetyl transferase; both show activity in AF, although their exact role is still unclear.[31] In addition, significant amounts of polyamines are found in AF; these have a cationic charge and may play both a nutritive and an antimicrobial role.

AF AS A DIAGNOSTIC MEDIUM

Amniocentesis has been a valuable tool in assessing fetal well-being since the 1970s. The most common evaluation of AF in the US is assessment of fetal chromosomes. Amniocentesis is commonly offered to women who will be at least 35 years of age at the time of full-term delivery or who have other risk factors for a chromosomal abnormality. As the diagnosis of aneuploidy moves into the first trimester with ultrasound assessment of nuchal translucency and more useful maternal serum markers, the use of amniocentesis will decrease with a corresponding increase in chorionic villus sampling. Amniocentesis is also offered when a previous child has a chromosomal abnormality, a parent carries a balanced chromosomal rearrangement or an autosomal recessive disorder, a mother carries an X-linked disorder, or a major structural abnormality or group of anomalies is identified on ultrasound. Assessment of AF is also helpful in the prenatal diagnosis of neural tube defects and an impressive array of inborn errors of metabolism and hematologic and genetic diseases (excellent reviews can be found in Wilson[32] and Kramer and Cohen[33]).

Evaluation of AF bilirubin level based on optical density has been an important tool to predict the severity of fetal hemolysis in red-cell alloimmunized pregnancies. Currently, the combination of amniocentesis to assess optical density, Doppler flow studies of the intra-hepatic umbilical vein and the middle cerebral artery and fetal blood sampling by cordocentesis are recommended to closely monitor the isoimmunized anemic fetus.[34] Allele-specific polymerase chain reaction of AF fetal cells can also be used to identify fetuses at risk for hemolytic disease of the newborn due to minor blood group incompatibilities.[35]

AF assessment has been studied in patients with preterm labor and/or PPROM to investigate possible IAI. AF indicators suggestive of infection include elevated levels of matrix metalloproteinase (e.g., MMP-9), interleukins (e.g., IL-6 and IL-1β), tumor necrosis factor (TNF-α), G-CSF, elevated white blood cell count, low glucose, and the presence of bacteria identified by Gram stain or culture. When preterm labor occurs with intact membranes, the rate of documented IAI is consistently lower than when preterm labor occurs with PPROM. While routine amniocentesis in preterm labor/PPROM has not been shown to be effective in decreasing perinatal mortality, there is still disagreement as to its optimum role in identification of IAI. Amniocentesis has also been helpful in prenatal diagnosis of cytomegalovirus, toxoplasma and parvovirus B-19 infection; this has become particularly relevant with the increasing use of the polymerase chain reaction allowing earlier diagnosis.

Assessment of fetal lung maturity by determination of the lecithin/sphingomyelin ratio and/or the presence of phosphatidyl glycerol in AF has become a well-accepted procedure. The assessment of lamellar body counts in AF,[36] the surfactant to albumin ratio in AF,[37] and electrical conductivity of AF[38] have

more recently been proposed as potentially superior methods for evaluation of fetal lung maturity.

A search for substances in AF that indicate fetal well-being has been ongoing since the 1980s. Changes in levels of inhibin-related proteins in both maternal serum and AF throughout pregnancy have been proposed as indicators of good fetal health. While the studies are contradictory, elevated levels of inhibin-A and activin-A may be useful markers related to fetal well-being during pre-eclampsia, trisomy 21, preterm delivery, and intrauterine growth restriction.[39] More research in this area is needed. A recent review of evaluation of AF S100B protein concentration as an early marker for brain injuries and/or brain maturation also merits further study.[40]

WHEN AF BECOMES PROBLEMATIC

Human AF may also contain substances that are potentially harmful. Perhaps the most concerning AF contaminant is meconium. There is good evidence that defecation in utero is a universal phenomenon occurring occasionally in the second trimester and frequently in the third trimester.[41] This is the likely explanation for the presence of bile pigments and enteric enzymes in AF. Meconium-stained AF occurs in about 13% of live deliveries. Most of these babies do well without associated acidosis or clinical illness. The combination of perinatal asphyxia, passage of meconium, and fetal gasping may lead to meconium aspiration syndrome (MAS), a potentially life threatening pulmonary disease caused by the combination of mechanical obstruction, inflammatory response, disruption of surfactant function, and often pulmonary hypertension (a recent review of MAS is found in Gelfand et al.[42]). MAS is uncommon in preterm infants but when present is associated with an increased risk of intraventricular hemorrhage. Meconium may also play a role in stimulating bacterial growth in the AF, perhaps by serving as an exogenous iron source. A recent study found a correlation between the presence and severity of meconium-stained AF and the rates of both chorioamnionitis and endomyometritis.[43]

AF demonstrates an irritant effect to exposed neural tissue, particularly after 34 weeks gestation. The precise identity of the irritant(s) is unclear, but there are several candidates. Tissue factor (TF), a procoagulant and initiator of disseminated intravascular coagulation, is found in high concentrations in AF at term, while TF pathway inhibitor, a natural inhibitor of TF, is found in relatively low concentrations. It is likely that TF plays a significant role in the devastating effects of AF embolism.[44] Late in pregnancy, elevated levels of activin-A and inhibin-A stimulate production of prostaglandin E2. As noted above, AF of pregnancies with premature rupture of membranes contains elevated levels of inflammatory cytokines (e.g. IL-1, IL-6, TNF-α, and interferon gamma). Whether this represents a fetal immune response or a preparatory step for the initiation of labor is not yet clear. The presence of PAF in AF with the onset of labor has been previously noted. Late in gestation AF contains vernix. While vernix contains antimicrobial substances and may be a contributor in protecting the fetus from IAI, it also has potent inflammatory properties and has been implicated as a cause of maternal antenatal peritonitis.[45] TGF-β, present late in gestation, may also play a role as a potential irritant.[46]

AF plays a major role in the gastrointestinal inflammatory changes associated with gastroschisis. An aseptic peritonitis leads to a fibrous peel, which has also been referred to as perivisceritis. The result is edema and thickening of the serosa, subserosa, and submucosa. This process has been attributed to an increase in the concentration of urea and nitrogenenous products and a decrease in the sodium and osmolality of AF that occurs at $\sim$30 weeks of gestation. Gastrointestinal waste products (i.e., bilirubin, bile acids, and meconium) have been shown to be elevated in the AF of human gastroschisis patients.[47] and in animal models are partly responsible for the perivisceritis seen in gastroschisis.[48] Amnioinfusion[49] and serial amnioexchanges[47] have both been performed in an attempt to minimize gastrointestinal inflammatory changes with preliminary results that are encouraging.

OTHER INTERESTING ASPECTS OF AF

Human AF contains factors that appear to minimize scarring.[50] It is interesting that a fetal incision made early in gestation will heal without a scar whereas one made in late gestation heals with scar formation. Two theories predominate: the first is that hyaluronic acid, which is found in high levels in AF, inhibits collagen synthesis. This hyaluronic acid-rich environment is due to a relative lack of hyaluronidase in AF and to the presence of hyaluronic acid-stimulating factor in AF. In one study looking at the effect of AF on proteases important to wound healing, human AF was shown to enhance collagenase activity, but to inhibit activities of hyaluronidase, elastase, and cathepsin.[51] The second theory is that TGF-β, which is absent from AF early in gestation but present late in gestation, plays a major role in scar formation.[46] Disagreement remains as to whether healing occurs without scar formation during early pregnancy because of a favorable fetal environment (i.e., fetal serum and AF) or because of the properties of fetal skin.

AF has been investigated as a potential way to deliver therapeutic agents to the fetus. Instillation of antibiotics, thyroxine, nutrients (i.e., dextrose, amino acids, and lipids), glucocorticoids, growth factors, surfactants, and beta-adrenergic-receptor agonists directly into the AF for delivery to the fetal circulation by either fetal swallowing or via the intramembranous route has been tried with mixed results. A 1999 National Institutes

of Health (NIH) conference on AF biology superbly summarizes this field.[52]

Human AF also contains factors that alter metabolism of opiates. Placental opioid enhancing factor has been found in placentae and AF of rats, and in placentae of humans and dolphins.[53] In cows and rats, maternal ingestion of AF enhances opioid-mediated analgesia. This effect has not been studied in humans.

Human AF has been evaluated as a source for stem cells with initial encouraging results.[54] The potential to develop a noncontroversial source of stem cells may stimulate research in this area.

UNANSWERED QUESTIONS AND FUTURE DIRECTIONS FOR AF-RELATED RESEARCH

Many research questions about AF remain unanswered. The 1999 NIH conference sponsored by the National Institute of Child Health and Development reviewed the current understanding of AF biology and important future directions for research. The conference summary called for more research in the areas of polyhydramnios, oligohydramnios, AF pressure determinations, embryonic and early fetal kidney development and function, control of lung liquid secretion, development of fetal swallowing and gastrointestinal motility, the dynamics of intramembranous absorption at the cellular and molecular level, AF pharmacokinetics and the potential therapeutic use of the amniotic space, and computer and mathematical models of AF dynamics.[52]

The functions and significance of individual growth factors in human AF remain incompletely described. It is interesting to note that some infants with esophageal atresia have malabsorption of intestinal nutrients. Other infants have a well functioning gut at birth without having swallowed significant amounts of AF. This disparity suggests that there is a redundancy of mediators that promote fetal gut growth with some effectors being swallowed in AF, while others arrive via the hematogenous route. Investigators have speculated that components of AF may protect the preterm infant against NEC or enhance intestinal recovery when NEC is in its healing stages. Components of AF that may promote these effects include glutamine,[55] arginine,[56] EGF,[57] EPO,[58] PAF-AH,[59] and LF.[60] Could harvested or synthetic AF be used as an enteral infusion in the preterm neonate at risk for or recovering from NEC? Would scarring of the gut be decreased? A recent ‘‘simulated AF’’ containing G-CSF and EPO was fed enterally to human neonates and was ‘‘well tolerated’’ at a dose of 20 ml/kg/day.[61] A follow-up study by the same investigative group showed infants tolerated simulated AF as an initial feeding when they were recovering from NEC.[62]

The skin is a major barrier to bacterial infection except in very preterm infants. Whether harvested or synthetic AF could be used to bathe and protect the not-yet-keratinized skin of the extremely preterm neonate is an appealing question. There is also much to be learned about the immunoprotective properties of AF and whether these can be enhanced to prevent IAI. There is really little information regarding how the innate host defenses of AF interact with the adaptive immune system of the mother and fetus.

Can significant amounts of AF be harvested at elective caesarean section in non-laboring women without harm to the fetus? Would this harvested AF be safe and free of infectious agents or could AF be processed (e.g., pasteurization) to render it free of infectious agents without inactivation of the desired host defense molecules? Storage and processing of AF has been investigated.[63] It is unclear whether trophic factors in AF would survive processes such as pasteurization, freezing, and storage. Given the apparent ease with which the fetus can absorb large volumes of AF in utero, would babies who are unable to tolerate regular enteral feeding (e.g. short gut, lymphatic disruption sequence, gastroschisis) be able to tolerate enteral AF infusion and thus nourish and stimulate the mucosa and minimize villous atrophy? The value of early trophic feedings in preterm infants has been well established. It is also clear that human milk is superior to premature infant formulas for these feedings. Unfortunately, breast milk is not always available. Preterm infants for whom breast milk is not available might benefit from a formula containing growth factors like those in AF and/or human milk.

Finally, as the role of VEGF in control of human AF volume becomes clear, it may be feasible to assess the role of VEGF inhibitors (e.g. bevacizumab) in the treatment of oligohydramnios and the role of VEGF receptor agonists in the treatment of polyhydramnios.

SUMMARY

AF is a wonderfully complex and unique body fluid that nourishes and protects the fetus. Just as breast milk is the optimum beverage for the newborn, AF is the ideal, germ-free bath, cushion and liquor for the fetus. Based on the significant contributions of AF to fetal and neonatal health, additional research is needed to better understand its functions and correct its disorders.

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19 Amniotic Fluid Cell Therapy to Relieve Disc-Related Low Back Pain and Its Efficacy Comparison with Long-Acting Steroid Injection

Niranjan Bhattacharya

Introduction

Human intervertebral disc undergoes multifactorial, biochemical, and morphologic degenerative changes during the process of aging. Surgically removed human discs show an active inflammatory process proceeding from the outside-in. The pathogenesis of discogenic acute nonspecific low back pain is mostly considered to be a re-rupture in an asymptomatic ruptured region in the posterior annulus, repaired by granulation tissue, in a moderately degenerated intervertebral disc with a radial tear [1].

The clinical manifestation of the process starts with acute nonspecific low back pain that is characterized by the sudden onset and severe unendurable low back pain without radicular pain or neurological deficit in the lower extremities. The background pathophysiology indicates that degeneration of the painful disc may originate from an injury and subsequent repair of annulus fibrosus. Growth factors, such as bFGF and TGF-beta1, macrophages, and mast cells might play a key role in the repair of the injured annulus fibrosus and subsequent disc degeneration [2].

Physical therapies should aim to promote healing in the disc periphery, by stimulating cells, boosting metabolite transport, and preventing adhesions and reinjury. Such an approach has the potential to accelerate pain relief in the disc periphery, even if it fails to reverse age-related degenerative changes in the nucleus [3]. The biochemical and molecular background behind the disease process revealed that type II collagen and proteoglycan (predominantly aggrecan) content is crucial to proper disc function, particularly in the nucleus pulposus. In degeneration, synthesis of matrix molecules changes, leading to an increase in the synthesis of collagens types I and III and a decreased production of aggrecan. Linked to this is an increased expression of matrix-degrading molecules including MMPs (matrix metalloproteinases), aggrecanases, and ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) 1, 4, 5, 9, and 15, all of which are produced by native disc cells. Growth factors and cytokines (particularly TNF alpha [tumor necrosis factor alpha] and IL-1 [interleukin 1]) have been implicated in the regulation of this catabolic process. Investigators have shown that in degenerate discs, there is an increase in IL-1, but no corresponding increase in the inhibitor IL-1 receptor antagonist. Inhibition of IL-1 would therefore be an important therapeutic target for preventing/reversing disc degeneration [4]. Another group of investigators have suggested that ADAMTS-5 is probably involved in the process of IVD degeneration and that IL-1β-induced expression of ADAMTS-5 is mediated by NO [5].

N. Bhattacharya, D.Sc., M.D., M.S., FACS (USA)
Department of Regenerative Medicine and Translational Science, Calcutta School of Tropical Medicine, Calcutta, West Bengal, India
e-mail: sanjuktaniranjan@gmail.com

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In a nutshell, traumatic or age-induced degeneration is the key for this condition. Treatment practiced globally is analgesic (nonsteroidal anti-inflammatory) drug and its different permutations and combinations with rest initially, followed by physiotherapy by different approaches. The other option is surgical decompression. But in any of the approaches, the core problem, of degeneration, is not solved. Treatment is aimed to treat the effect of degeneration, not the cause. Cell therapy is a modern new approach which can take care of the main issue of degeneration either by injection of autologous mesenchymal stem cells at the degenerated disc site of the bone after its collection from bone marrow avoiding the immunological problems. The other approach of live cell therapy is a simple solution to the degeneration of the disc problem by treatment with freshly collected amniotic fluid, which is a rich source of epithelial progenitor cells and mesenchymal cells apart from its intrinsic antibacterial component like properdin-like substances embedded in it. This amniotic fluid is collected from consenting mothers undergoing hysterotomy and ligation for family planning purpose approved by the Government of India, Ministry of Health.

Materials and Methods

Fresh amniotic fluid was collected from women admitted for hysterotomy and ligation at Bijoygarh State Hospital (2001–2006) and was used for the present study for the treatment of patients with disc-related pain. As per the standing direction of the State Family Planning Department, hysterotomy and ligation may be allowed up to 20 weeks of pregnancy, provided the mother has two or more healthy children. For the present study, 10 mL amniotic fluid was collected aseptically in the OT from each mother undergoing hysterotomy and ligation. The collection of the amniotic fluid is done always from an intact sac after opening the uterus, when the amniotic membrane containing the amniotic fluid generally herniates outside the uterus. The sac was gently punctured, and the amniotic fluid was sucked out aseptically with a wide-bore size 16 needle and syringe. The collection protocol initiates, after getting the donor's consent and the recipient's informed consent and also the approval of the hospital ethical committee.

Initially, 51 patients volunteered for this project of amniotic fluid cell therapy after confirmation of the degenerative lumber intervertebral disc in magnetic resonance imaging (MRI) of the lumbosacral joint with clinical discogenic pain. Four cases were discarded from the study due to advanced prolapsed intervertebral disc (PID) with serious nerve compression sequel who needed immediate decompression. Another five cases were discarded from the study due to the association of neurodegenerative diseases such as parkinsonism, cerebral atrophy with dementia of varying etiology, and other chronic disease burdens.

These patients were randomized for age and sex and eventually divided in two equal groups: Group A received long-acting steroid and Group B received 10 mL of amniotic fluid as a source of cell therapy. The donor of the amniotic fluid were (HIV 1 and 2 and hepatitis B and C negative) mothers carrying pregnancy (14–20 weeks gestation) and admitted for hysterotomy and ligation from the Family Planning Department. Only informed and consenting mothers were enrolled for the procedure after passing through the institutional ethical committee.

Before the procedure, a thorough history of all the patients was taken, i.e., age; sex; height; weight; menstrual history; history of chronic disease like tuberculosis, hypothyroid, frank diabetes, or even altered glucose tolerance; history of diabetes in the family; lipid profile including uric acid level; apart from a history of specific involvement of cancer; systemic lupus erythematosus; and ankylosing spondylitis. Specific rheumatological history with history of oral or intra-articular steroid intake, degree, and pattern of joint involvement with the duration of affection was noted. The discogenic pain was noted on a 100-mm horizontal visual analog pain scale (VAS). The other parameters that were assessed included the distance walked in 1 min (WD) and also a locally modified and local (Bengali)

language-translated modified Health Assessment Questionnaire that was to be filled up.

At follow-up visits (1st–6th, 9th, 12th, 18th, and 24th month), a specialist doctor made an objective assessment of the clinical condition with subjective correlation, as much as it was practicable, for all the patients enrolled for the type of treatment. The idea is to clinically assess the overall status of cell therapy treatment and its comparison with standard long-acting steroid treatment for relief of the discogenic pain.

Pain score (VAS), WD, and HAQ were recorded. Student's paired test (p value) was also conducted. Analysis of variance for repeated measures was used to compare differences that were assessed by simple regression analysis. The differences in patient opinion of overall change and relationship between clinical evidences were calculated by contingency table analysis incorporating mean with standard deviation (SD). Differences that were significant at the 5 % confidence interval are quoted in the follow-up chart record. At the completion of the study after 3 years of follow-up, patients who received cell therapy were offered steroid therapy if they voluntarily requested for that procedure, and vice versa.

In order to quantify the overall impression of improvement or deterioration of the low back pain with the treatment offered, we have a disability scoring system known as the Oswestry low back pain disability questionnaire. This scoring system has ten components like assessment of (1) pain intensity, (2) personal care capability, (3) lifting capability, (4) walking capability, (5) sitting capability, (6) standing capability, (7) sleeping, (8) sex life, (9) social life, and (10) traveling capability. Each parameter has many components, viz., 0 (normal) to 5 (highest pain) when pain totally prevents that specific intended activity (0–5), viz., (a) 0–20 %: minimal disability: The patient can cope with most living activities. Usually no treatment is indicated apart from advice on lifting, sitting, and exercise. (b) 21–40 %: moderate disability: The patient experiences more pain and difficulty with sitting, lifting, and standing. Travel and social life are more difficult and they may be disabled from work. Personal care, sexual activity, and sleeping are not grossly affected, and the patient can usually be managed by conservative means. (c) 41–60 %: severe disability: Pain remains the main problem in this group, but activities of daily living are affected. These patients require a detailed investigation. (d) 61–80 %: crippled: Back pain impinges on all aspects of the patient's life. Positive intervention is required. (e) 81–100 %: These patients are bed bound [6].

Table 19.1 The patients selected for the study (epidemiological profile) ($N=42$)

1. Age of group: 36–82 years
2. Sex: males 21 and females 21
3. Weight: 44.8–112.6 kg
4. Height: 4 ft 6 in. to 6 ft 3 in.
5. Duration: 1–11 years
6. Low back pain not relieved with lumbosacral corset: 16
7. Low back pain with radiation to leg, not relieved with lumbosacral corset: 26
8. Treatment with analgesic including NSAID and physiotherapy: all of them (42)
All cases were randomized and divided equally into Group A ($n=21$) and Group B ($n=21$)

Result and Analysis

In the present series, 42 patients of age varying from 36 to 82 years were enrolled, vide details on Table 19.1. There was definite history of fall or trauma that was present in 7 cases; the residual 35 cases did not have a similar history. These 42 cases, who did not respond to conventional pharmacological or nonpharmacological treatment, were ultimately enrolled for this trial.

The pharmacological treatment had included use of NSAIDs, i.e., naproxen, ibuprofen as well as the cyclooxygenase-2 inhibitor group of drugs like celecoxib with supporting drugs such as glucosamine, chondroitin, and opiates, only to name a few. The nonpharmacological treatment had included special exercises under supervision and lumbosacral support (corset). Those patients suffering from disc-related pain and difficulty in

Table 19.2 The value of the VAS (visual analog pain scale), WD (walking distance in meters), and HAQ (Health Assessment Questionnaire) assessments in steroid (Group A) and cell therapy (Group B)

(Pretreatment mean ± SD) VAS (mm)	(Third month mean ± SD) VAS (mm)	(Sixth month mean ± SD) VAS (mm)	*p* value
Mean Group A values with SD: 29 ± 7.3	19 ± 6.47	22 ± 3.8	($p < 0.02$)
Mean Group B values with SD: 31 ± 6.2	17 ± 3.3	11 ± 4.6	($p < 0.002$)
Walking distance in meters (WD)			
Mean Group A values with SD 36.4 ± 4.8 m	52 ± 3.7 m	55 ± 4.2 m	($p < 0.01$)
Mean Group B values with SD 34.8 ± 3.9 m	58.6 ± 6.9 m	69.4 ± 7.2 m	($p < 0.01$)
Local language Modified Health Analysis Questionnaire (1–11)			
Mean Group A values with SD 2.2 ± 0.2	2.4 ± 0.2	2.3 ± 0.4	($p < 0.002$)
Mean Group B values with SD 2.4 ± 0.3	1.9 ± 0.12	1.6 ± 0.31	($p < 0.01$)

(The *t*-test, one-way analysis of variance [ANOVA], and a form of regression analysis)

walking not effectively reduced with rest, analgesic, and muscle relaxant were divided in two equal groups for inclusion in either of the following protocols of aseptically, intradiscal C-arm-guided injection in operation theater of 10 mL freshly collected amniotic fluid for cell therapy (Group B) or intradiscal instillation of long-acting steroid, i.e., methylprednisolone acetate injectable suspension of 80 mg dissolved in 5 mL water + 5 mL of 1 % Xylocaine is infiltrated slowly at the site of maximum tenderness of the patient in a sterile manner, (USP Depo-Medrol, 40 mg/1 mL) under X-ray guidance (Group A), depending on the patient's voluntary informed consent.

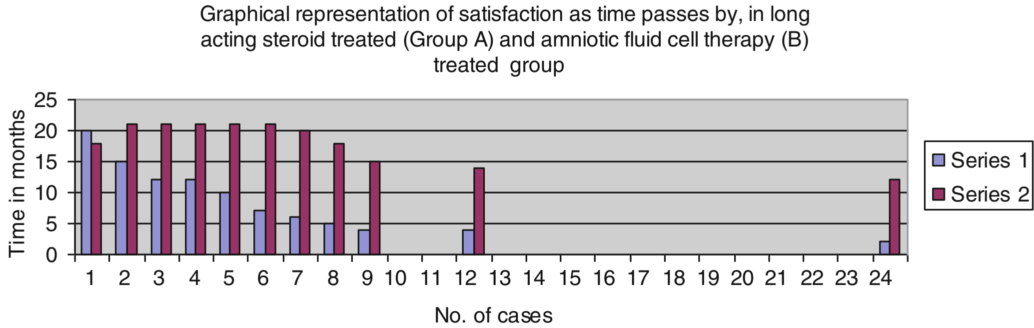
Epidemiological details are noted in Table 19.1. The subsequent follow-up was noted with the response to treatment as seen on the visual analog pain scale (VAS), walking distance in meters (WD), and modified local language Health Assessment Questionnaire (HAQ) in Table 19.2. In Table 19.3, the response to therapy as per Oswestry low back pain disability and follow-up is shown and graphically represented in Graph 19.2. If we study the Tables 19.1, 19.2, and 19.3 and the impact and comparison of the treatment in Graphs 19.1 and 19.2, one conclusion is quite obvious and is statistically significant ($p < 0.01$), i.e., amniotic fluid cell therapy in its own niche is highly effective and much superior to the globally practiced C-arm-guided long-acting steroid in relieving discogenic pain.

If we study further, the relief with steroid is found to be temporary and there is a great possibility of recurrence; however, cell therapy with its rich epithelial and mesenchymal cell component can help regeneration of the injured tissue with its stem cell-related regenerative potential. Sometimes for immediate pain relief, surgery is effective, but it is highly technical, hence costly. There is also high morbidity and mortality directly and indirectly related to the procedure. Hence, the surgical approach which can have the potentiality for relief is often short lived in most cases, and surgery does not offer a cure or permanent repair for this chronic painful condition.

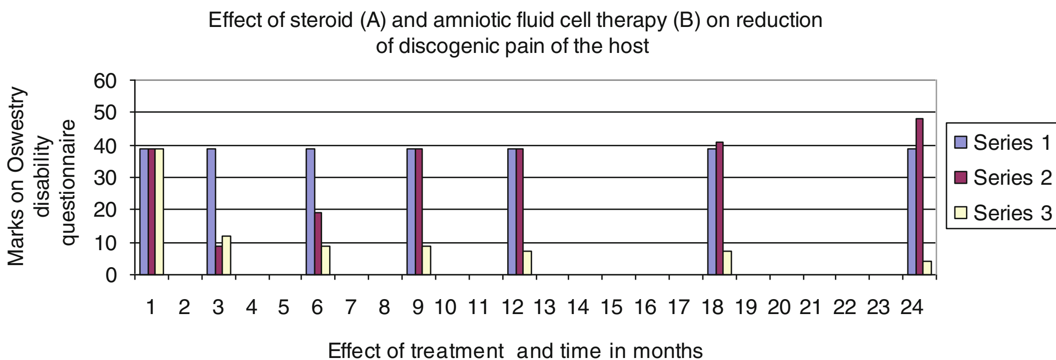
As a source of cell therapy, amniotic fluid is being studied in great detail by many global investigators. One researcher has analyzed the number and size of viable amniotic fluid cells (AFCs). Small AFCs (SAFCs) and large AFCs (LAFCs) were isolated using a sterile 10-micron pore-size strainer. Flow cytometry analyses showed that cell surface antigen expression on LAFCs and SAFCs was positive for CD29, CD44, CD73, CD90, CD166, and HLA-I, but negative for CD31, CD34, CD45, CD117, and

Table 19.3 The follow-up of Group A and B with the comparison of the results

Group A treated with intra-articular steroid $N=21$	Group B treated with cell therapy $N=21$	Satisfaction after 1 month	Special comment
Assessment after 1 month showed improvement, i.e., mean subjective and objective assessment of definite relief in 20 cases lost follow-up (LFU) = Nil	Assessment after 1 month showed improvement, i.e., mean subjective and objective assessment of definite relief in 18 cases lost follow-up (LFU) = Nil	Group A = 20 Group B = 18	($p<0.01$)
Assessment after 2 months showed improvement, i.e., subjective and objective assessment of definite relief in 15 cases (LFU) = Nil	Assessment after 2 months showed improvement, i.e., mean subjective and objective assessment of definite relief in 18 cases (LFU) = Nil	Satisfaction after 2 months Group A = 15 Group B = 21	($p<0.01$)
Assessment after 3 months showed improvement, i.e., subjective and objective assessment of definite relief in 12 cases (LFU) = Nil	Assessment after 3 months showed improvement, i.e., mean subjective and objective assessment of definite relief in 21 cases (LFU) = Nil	Satisfaction after 3 months Group A = 12 Group B = 21	($p<0.01$)
Assessment after 4 months showed improvement, i.e., subjective and objective assessment of definite relief in 12 cases (LFU) = Nil	Assessment after 4 months showed improvement, i.e., subjective and objective assessment of definite relief in 21 cases (LFU) = Nil	Satisfaction after 4 months Group A = 12 Group B = 21	($p<0.01$)
Assessment after 5 months showed improvement, i.e., subjective and objective assessment of definite relief in 10 cases (LFU) = Nil	Assessment after 5 months showed improvement, i.e., subjective and objective assessment of definite relief in 21 cases (LFU) = Nil	Satisfaction after 5 months Group A = 10 Group B = 21	($p<0.01$)
Assessment after 6 months showed improvement, i.e., subjective and objective assessment of definite relief in 7 cases (LFU) = Nil	Assessment after 6 months showed improvement, i.e., subjective and objective assessment of definite relief in 21 cases (LFU) = Nil	Satisfaction after 6 months Group A = 7 Group B = 21	($p<0.01$)
Assessment after 7 months showed improvement, i.e., subjective and objective assessment of definite relief in 6 cases (LFU) = Nil	Assessment after 7 months showed improvement, i.e., subjective and objective assessment of definite relief in 20 cases (LFU) = Nil	Satisfaction after 7 months Group A = 6 Group B =20	($p<0.01$)
Assessment after 8 months showed improvement, i.e., subjective and objective assessment of definite relief in 5 cases (LFU) = Nil	Assessment after 8 months showed improvement, i.e., subjective and objective assessment of definite relief in 18 cases (LFU) = Nil	Satisfaction after 8 months Group A = 5 Group B = 18	($p<0.01$)
Assessment after 9 months showed improvement, i.e., subjective and objective assessment of definite relief in 4 cases (LFU) = Nil	Assessment after 9 months showed improvement, i.e., subjective and objective assessment of definite relief in 15 cases (LFU) = Nil	Satisfaction after 1 year Group A = 4 Group B = 15	($p<0.01$)
Assessment after 12 months showed improvement, i.e., subjective and objective assessment of definite relief in 4 cases (LFU) = Nil)	Assessment after 12 months showed improvement, i.e., subjective and objective assessment of definite relief in 14 cases (LFU) = Nil)	Satisfaction after 2 years Group A = 4 Group B = 14	($p<0.01$)
Assessment after 24 months showed improvement, i.e., subjective and objective assessment of definite relief in 2 cases (LFU) = Nil	Assessment after 24 months showed improvement, i.e., subjective and objective assessment of definite relief in 12 cases (LFU) = Nil	Satisfaction after 2 years Group A = 2 Group B = 12	($p<0.01$)



Graph 19.1 The comparison and follow-up results of Group A and Group B treatment up to 24 months. *Series 1* is treated with long-acting steroid. *Series 2* is treated with freshly collected second trimester amniotic fluid cell therapy



Graph 19.2 The comparison and follow-up results of Group A and Group B treatment as per scoring by Oswestry low back pain disability questionnaire up to 24 months. *Series 1*: pretreatment. Scoring as per Oswestry low back pain disability questionnaire. *Series 2*: posttreatment. Scoring as per Oswestry low back pain disability questionnaire. Group A (long-acting steroid injection). *Series 3*: posttreatment. Scoring as per Oswestry low back pain disability questionnaire. Group B (amniotic fluid cell therapy)

HLA-II. Importantly, Nanog, Oct-4, ABCG2, and SOX2 expressions in cells were easily detectable among the SAFC population. Expression of Nanog and ABCG2 was not observed among LAFCs [7].

The discovery of amniotic fluid stem cells has initiated a new and very promising field in stem cell research. In the last 4 years, amniotic fluid stem cells have been shown to express markers specific to pluripotent stem cells, such as Oct-4. Due to their high proliferation potential, amniotic fluid stem cell lineages can be established. Meanwhile, they have been shown to harbor the potential to differentiate into cells of all three embryonic germ layers [8].

Amniotic fluid stem cells have more recently been isolated. They represent a novel class of pluripotent stem cells with intermediate characteristics between embryonic and adult stem cells, as they are able to differentiate into lineages representative of all three germ layers but do not form tumors when injected in vivo. These characteristics, together with the absence of ethical issues concerning their employment, suggest that stem cells present in the amniotic fluid might be promising candidates for tissue engineering and stem cell therapy in several human disorders [9].

In recent years, various types of stem cells have been characterized, and their potential for cardiac regeneration was investigated by a prominent group of investigators, who had previously described the isolation of broadly multipotent cells from amniotic fluid, defined as amniotic fluid stem (AFS) cells [10].

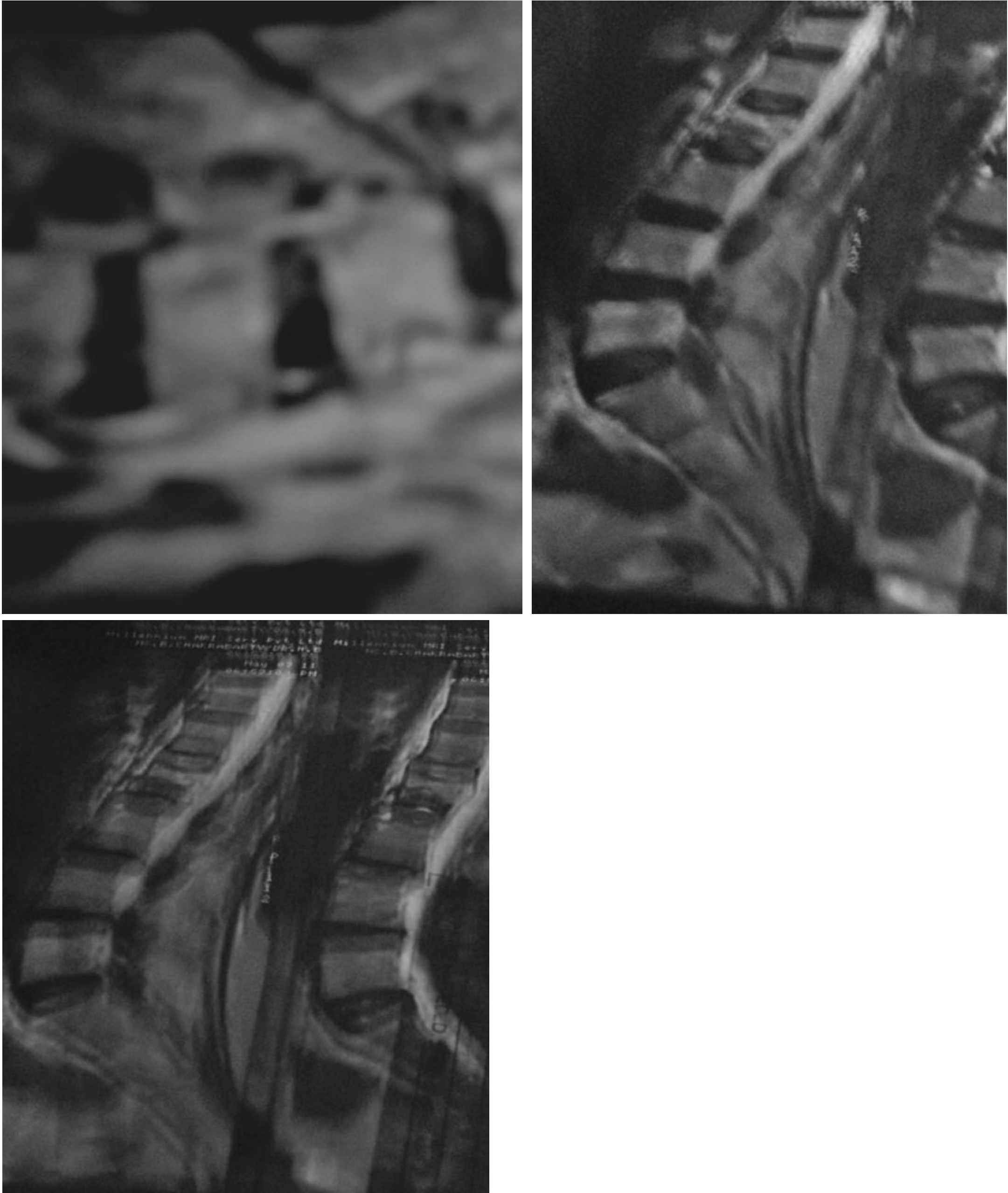
Cell-based therapies for bone regeneration are an exciting emerging technology, but the availability of osteogenic cells is limited, and an ideal cell source has not been identified. Amniotic fluid-derived stem cells (AFS) and bone marrow-derived mesenchymal stem cells (MSCs) were compared to determine their osteogenic differentiation capacity in both 2-D and 3-D environments in one study. It was found that MSCs would be a good choice for immediate matrix production, but the AFS cells would continue robust mineralization for an extended period of time. This study demonstrates that stem cell source can dramatically influence the magnitude and rate of osteogenic differentiation in vitro [11].

The problem lies with the treatment attempt of the patients reporting with degenerated disc with or without disc prolapse, desiccation, bulge, or compression of the adjacent nerves and its implications [12]. Most of the patients with chronic discogenic back pain, without specific history of trauma, are in the geriatric age group. In this age group, low back pain is associated with varying degree of age-induced degenerative osteoporosis, spondylosis, spondyloarthrosis, intervertebral disc prolapse, or even compression collapse apart from other problems like diabetic background, hypertension, ischemic heart disease, chronic obstructive pulmonary disorder, dyslipidemia, and hypothyroidism. MRI presentation of a typical geriatric manifestation of low back pain is shown (Figs. 19.1, 19.2, and 19.3).

If we see the epidemiological profile of the present study groups in Table 19.1, we can see the age of the group (36–82 years), sex of the group suggested (males 21 and females 21), weight of the group (varied from 44.8 to 112.6 kg), and the duration of illness (varying from duration 1 to 11 years). The clinically manifested effects of treatment can be easily noticed from Table 19.2. Here, both steroid (Group A) and cell therapy (Group B) patients showed improvement from the pretreatment value; however, Group B scoring is much better (p, 0.01), as seen and assessed from the value of the VAS (visual analog pain scale), WD (walking distance in meters), and HAQ (Health Assessment Questionnaire) assessments mentioned in the same Table. Further, if we examine the clinical assessment of pain relief and patient's satisfaction as seen from Table 19.3 and Graph 19.1, in case of Group A (long acting steroid group), it was 20/21 cases in the 1st month which became 12/21 in the 3rd month, 6/21 in the 6th month, 4/21 in the 12th month, and 2/21 after the 24th month follow-up. Similarly, in Group B (cell therapy patients), the identical values after the 1st month were 18/21, which became 21/21 in the 3rd month, 21/21 in the 6th month, 14/21 in the 12th month, and 12/24 after the 24th month follow-up. Another globally practiced guideline for pain assessment or scoring for comparison is the Oswestry low back pain disability questionnaire. Here in Table 19.4 and Graph 19.2, we have again compared the effect of treatment of Group A (steroid) and Group B (cell therapy with fresh amniotic fluid) and followed up the results of Group A and Group B treatment as per scoring by Oswestry low back pain disability questionnaire up to 24 months. Here, postinjection with long-acting steroid (Group A) suggested a mean scoring of 9 ± 1.2 % SD after 3 months, which became mean 1 9 ± 1.2 % SD after 6 months, mean 39 ± 9.2 % SD after 9 months, mean 39 ± 8.2 % SD after 12 months, mean 41 ± 7.2 % SD after 18 months, and then ultimately mean 48 ± 12.2 % SD after 24 months.

Similarly, in case of the cell therapy group (Group B), the mean scoring was 11.7 ± 1.6 % SD after 3rd month follow-up which became mean 9.4 ± 0.6 % SD after the 6th month, mean 9.1 ± 0.96 % SD after the 9th month, mean 7.1 ± 0.6 % SD after the 12th month, mean 6.7 ± 0.4 % SD after the 18th month, and ultimately mean 4.1 ± 0.96 % SD after the 24th month follow-up.

If we analyze the results, we can see long-acting steroid, due to its anti-inflammatory and other activities, causes some improvement in the patients; however, it is ill sustained as noted from the follow-up. But freshly collected simple amniotic fluid cell therapy has a much more sustained effect apart from remarkable improvement; the question remains as to why in the long-term follow-up there is a reappearance of pain in some of the victims? Can psychosomatic treatment or recurrent cell therapy or an increase in cell dosage have a more sustained



Figs. 19.1, 19.2, and 19.3 A typical presenting patient with low back pain is noted in the following (Figs. 19.1, 19.2, and 19.3). Here, the patient is 79 years old, female in the background of diabetes, chronic obstructive pulmonary disorder, ischemic heart disease, and aortic and mitral calcification of the valves with resulting incompetence. The MRI (Figs. 19.1, 19.2, and 19.3) suggested: (a) Compression fracture of L2 vertebral body with partial rupture of L2/L3 disc. (b) Disc desiccation at L1–L2 to L5–S1. (c) Disc protrusion causing secondary spinal canal and bilateral neuronal foramina narrowing and thecal compression at L4–L5 level. (d) Mild diffuse disc bulge causing mild thecal compression at L3–L4 level

Table 19.4 The comparison and follow-up results of Group A and Group B treatment as per scoring by Oswestry low back pain disability questionnaire up to 24 months

Group A treated with intra-articular steroid	Group B treated with cell therapy	Pretreatment scoring as per Oswestry low back pain disability questionnaire Group A	Pretreatment scoring as per Oswestry low back pain disability questionnaire Group B	Posttreatment scoring as per Oswestry low back pain disability questionnaire Group A	Posttreatment scoring as per Oswestry low back pain disability questionnaire Group B
N = 21 *M* = 12 Female = 9 Age 38–78 Mean age 48 ±6.4 SD	*N* = 21 *M* = 10 Female = 11 Mean age 56.4 ± 4.8 SD	12–84 %, mean 39 ± 11.2 % SD	16–88 %, mean 41 ± 9.6 % SD after 3rd month	12–34 %, mean 9 ± 1.2 % SD after 3rd month	6–18 %, mean 11.7 ± 1.6 % SD after 3rd month
Do	Do	Do	Do	22–67 %, mean 1 9 ± 1.2 % SD after 6th month	6–12 %, mean 9.4 ± 0.6 % SD after 6th month
Do	Do	Do	Do	31–69 %, mean 39 ± 9.2 % SD after 9th month	9–11 %, mean 9.1 ± 0.96 % SD after 9th month
Do	Do	Do	Do	32–74 %, mean 39 ± 8.2 % SD after 12th month	6–9 %, mean 7.1 ± 0.6 % SD after 12th month
Do	Do	Do	Do	33–75 %, mean 41 ± 7.2 % SD after 18th month	4–8 %, mean 6.7 ± 0.4 % SD after18th month
Do	Do	Do	Do	26–88 %, mean 48 ± 12.2 % SD after 24th month	4–6 %, mean 4.1 ± 0.96 % SD after 24th month

effect? These are some of the questions for the future investigators in this frontline area of cellular therapy.

From an overall point of view, regeneration can only treat the root cause of degeneration of the whole lumbosacral region. Cell therapy is the only curative approach for such a generalized multisystemic deterioration of the region, and the palliative approach to pain relief with anti-inflammatory drug including steroid is short lived and has longtime use and may lead to drug-induced problems in addition of the recurrence of the symptoms.

Surgical options are mainly aimed at decompression procedure so as to relieve the compression through different neurosurgical and orthopedic combined technical procedures like facetectomy (to remove part of the facet), foraminotomy and laminotomy (to enlarge the vertebral foramen), intervertebral disc annuloplasty (a procedure of heating the disc to 90 °C for 15 min in an effort to seal the disc), intervertebral disc arthroplasty (also called artificial disc replacement), laminoplasty, microdisectomy, percutaneous laser disc decompression, spinal decompression, and spinal laminectomy, only to name a few such procedures which should be individualized for proper application.

Other strategies for disc degeneration have included attempts to upregulate the production of key matrix proteins or reduce the proinflammatory cytokines, interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α) which cause the inflammation. In order to achieve that, protein injection and viral or nonviral gene transfer has been attempted without the desired success. Subsequently, there were attempts to inject anabolic factors or recombinant growth factors, stereotactically at the site of inflammation or injury of the disc, again, without the desired success.

The expression of tumor necrosis factor alpha in adult discs is statistically associated with disc degeneration. Its occurrence in adults of more advanced age suggests that tumor necrosis factor alpha is not involved in the initiation of disc degeneration but may be associated with further promotion of degenerative disarrangement and pain induction [13].

Basic science research has demonstrated that the intervertebral disc is an avascular tissue element occupied by inadequately characterized cells in an extensive extracellular matrix. While the annulus fibrosus is predominantly collagenous, the matrix of the central nucleus pulposus is rich in proteoglycans. A variety of inflammatory mediators have been implicated in the degeneration of the intervertebral disc including nitric oxide (NO), interleukins, matrix metalloproteinases (MMP), prostaglandin E2 (PGE2), tumor necrosis factor alpha (TNF-alpha), and a group of cytokines. MMPs, PGE2, and a variety of cytokines have already been shown to play a role in the degradation of articular cartilage. Nitric oxide is a novel mediator that has recently drawn much attention for its role in disc abnormalities. Elevated nitric oxide production derived from NO synthase activity has been manifested in cerebrospinal fluid in patients with degenerative lumbar disease. However, the regulatory mechanism of NO and its relationship to the clinical manifestations are unclear [14].

Discussion

The investigators mentioned earlier in the chapter have stressed on the effect of disc degeneration and have attempted to understand the process of degeneration in order to find strategies to relieve pain. However, few researchers have tried to attack the root cause of disc degeneration through attempts at regeneration.

Live cell therapy can treat the process of degeneration by reversing it or by an attempt to regenerate those affected cells. Treatment with autologous mesenchymal stem cell from bone marrow, stromal cells from the same source, adipose tissue stem cells, growth factors etc for regeneration of the damaged disc, have been attempted by many investigators with varying degrees of success [15–23].

The present study is the first global report on the treatment of discogenic pain with a biological waste, i.e., amniotic fluid.

The present group of researchers have earlier reported on amniotic fluid cell therapy in advanced arthritis and its regenerative effects [24].

Physical therapies should aim to promote healing in the disc periphery by stimulating cells, boosting metabolite transport and preventing adhesions and reinjury. Such an approach has the potential to accelerate pain relief in the disc periphery, even if it fails to reverse age-related degenerative changes in the nucleus.

Tumor necrosis factor alpha is substantially expressed in disc material of symptomatic patients (surgical specimens) in comparison to samples taken at autopsy. The expression of tumor necrosis factor alpha in early fetal/infantile nucleus pulposus may indicate "physiologic" tissue disarrangement with closure of the blood vessel canals. Human intervertebral disc undergoes multifactorial, biochemical, and morphologic degenerative changes during the process of aging. The frequency of degeneration, especially lumbar degeneration, increases sharply with age and is regarded as a major cause of discogenic low back pain. Since degenerative discs are often asymptomatic, the pathobiology of discogenic back pain remains unclear. Degenerated discs spontaneously produce increased amounts of inflammatory mediators suggesting their role in the degenerative process of the intervertebral disc. However, the relationship between aging, degenerative processes, and actual illness is far from clear.

Randomized clinical trials (RCTs) are regarded as the "golden standard" for providing research evidence for interventions in evidence-based health care [25]. The validity and reliability of trial results are, however, largely dependent on the study design and the methodology in its conduct. Jadad [26] has defined the quality of a trial, with emphasis on the methodological quality, as "the confidence that the trial design,

conduct, and analysis have minimized or avoided biases in its treatment comparisons." In this chapter, an attempt was made to follow the basic guidelines to minimize investigator or other biases as far as practicable. Our subjective assessment of that score in this study is possibly four on the Jadad scale. The present study is the first global report of a clinical comparison of the effect of amniotic fluid cell therapy and the impact of standard intra-articular palliative treatment in case of varying degrees of discogenic low back pain. Under normal circumstances, the fetus and the amniotic fluid-containing sac are immediately disposed for eventual clearance through the incinerator of the hospital. To recapitulate, amniotic fluid is to be found in the amniotic cavity that protects the fetus as a buffer and also helps growth and movement and prevents adherence to the placenta or the surrounding structures. This clear watery fluid is contributed principally from the maternal blood via the amniotic fluid epithelium but freely intermixes with secretions from the fetal lung, kidney, gastrointestinal tract, and the skin; hence, the properties of this specialized fluid compartment are quite complex with contributions from both the maternal and the fetal side. Toward the outside, the amniotic cavity is delimited by the amniotic epithelium, the chorion laeve, and the decidua capsularis. The main constituents are water and electrolytes (99 %) together with glucose, lipids from the fetal lungs, proteins with bactericide properties, and fetal epithelium cells. Pluripotent progenitor cells isolated from the amniotic fluid and the placenta possibly present an exciting contribution to the field of stem cell biology and regenerative medicine. Compared with embryonic stem cells, progenitor cells isolated from the amniotic fluid have many similarities: they can differentiate into all three germ layers, they express common markers, and they preserve their telomere length. However, progenitor cells isolated from the amniotic fluid and placenta have considerable advantages. They easily differentiate into specific cell lineages, and further, they avoid the current controversies associated with the use of human embryonic stem cells. Pregnancy results in the acquisition of specialized and unique cells that may have clinical applications and therapeutic potential. Whether the pregnancy-associated progenitor cells (PAPCs) are hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), or are a new population of stem cells is an unresolved issue. It is also unknown whether PAPCs respond to all types of maternal injury or only those injuries that recruit stem cells. It is possible that these cells, since they are fetal in origin, have a higher proliferative capacity or more plasticity than their equivalent adult (maternal) cells.

In the current debate over the use of embryonic stem cells for treatment of disease, the discovery of a population of fetal stem cells that apparently differentiate from the ones in adult women and can be acquired without harming the fetus may be significant [27, 28]. The growing fetus in the womb is an eternal source of stem cells. Meanwhile, scientists have been able to isolate and differentiate only 30 % of mesenchymal stem cells (MSCs) on an average, extracted from a newborn's umbilical cord jelly-like material shortly after birth. The success rate for amniotic fluid-derived stem cells, on the other hand, is close to 100 %. Analysis of surface markers shows that progenitor cells from amniotic fluid express human embryonic stage-specific marker SSEA4 and the stem cell marker Oct-4 and do not express SSEA1, SSEA3, CD4, CD8, CD34, CD133, C-MET, ABCG2, NCAM, BMP4, TRA1-60, and TRA1-81 [29, 30].

Differentiation of Amniotic Fluid- and Placenta-Derived Progenitor Cells

The progenitor cells derived from amniotic fluid and the placenta are pluripotent and have been shown to differentiate into osteogenic, adipogenic, myogenic, neurogenic, endothelial, hepatic, and renal phenotypes in vitro. Each differentiation has been performed through proof of phenotypic and biochemical changes consistent with the differentiated tissue type of interest. In 2007, Perin et al. showed that AFSC (amniotic fluid stem cells) could be induced to

differentiate into renal cells when placed into an in vitro embryonic kidney environment [31]. In this preliminary clinical study, freshly collected amniotic fluid has been utilized as a source of cell therapy with the hypothetical assumptions that the mesenchymal cells of the AF (amniotic fluid) will participate in the degenerated disc repair process, the viscosity of the amniotic fluid will assist lubrication, and the bactericidal property of the amniotic fluid will guard against inadvertent infection. The idea was to match/compare this new therapeutic protocol (cell therapy for regeneration) with the globally accepted standard protocol of intra-articular injection of long-acting steroid triamcinolone.

New Horizon for Offering a Cure (Repair) for Discogenic Pain with Simple Cell Therapy

In the developing world, surgical abortion as a method of family planning is practiced widely. Hysterotomy and ligation is a standard surgical method of termination in government hospitals in India. Aseptic collection of the amniotic fluid is not a difficult job for experienced gynecologists and obstetricians who perform this simple surgery with skill and dedication. The aseptically collected amniotic fluid can be easily preserved in special containers in the vapor phase of liquid nitrogen chambers or jars. This may work as an amniotic fluid bank that can supply amniotic fluid on demand. Amniotic fluid is a unique fluid made by nature; it is a cocktail of mesenchymal stem cells with antibacterial property, which is used in the present study as the cell therapy source for the repair of damaged cartilage, synovial membrane, supporting muscles, and supporting ligaments, as per the niche provided to these specialized stem cells for regeneration purposes, in advanced and degenerative osteoarthritis with satisfying results. The amniotic fluid, because of its increased viscosity due to protein and other cellular suspension, differs from the steroid-treated fluid (normal saline) and may act as a lubricant that diminishes the irritation at the initial phase; the mesenchymal cells, which do not express HLA antigens, may possibly help in the repair process of the adjacent structures in the joint space as a whole. Though the epidemiological background (Table 19.1) of Groups A and B is grossly randomized, the result of the therapy (shown in Tables 19.2 and 19.3, which is graphically represented in Graphs 19.1 and 19.2) strongly supports the potential of this new form of cell therapy in case of advanced disc-related pain. Lastly, it may be noted with interest that in this simple method of cell therapy, Group B maintained superior patient satisfaction in 12 cases only out of 26 enrolled patients, after completion of the 24-month follow-up period. The corresponding number for the standardized universally practiced protocol of intra-articular long-acting steroid (Group A) therapy for advanced discogenic pain is a pathetic figure of four cases only. The results are further supported by the VAS, WD, and HAQ assessments as mentioned in Table 19.2. The results of these tests reiterated the observation that there was a significant improvement in VAS in the third month and this improvement was sustained at the 6-month interval assessments in both groups but more so in the cell therapy Group B ($p<0.001$). The present treatment proved to be much superior to, and lasted longer than, the conventional widely practiced therapy with corticosteroid instillation at the joint.

Summary and Conclusion

An analysis of the results show that long-acting steroid, due to its anti-inflammatory and other activities, causes some improvement in the disc related pain of the patients; however, it is ill sustained as noted from the follow-up. Freshly collected amniotic cell therapy has a much more sustained effect. However, despite remarkable improvement, long-term follow-up indicates a return of some degree of pain. The issue is whether the symptoms are psychosomatic, or whether other methods like recurrent cell therapy or an increased cell dosage can have a more sustained effect. These questions may perhaps be answered by investigators working in this

frontline area of cell therapy in the future someday.

Regeneration treats the root cause of degeneration in the entire lumbosacral region. While palliative treatment with pain-relieving drugs including steroid has only a short-time effect and can also lead to drug-induced problems, cell therapy has a curative approach for the multisystemic degeneration of the region, which is the actual cause of the pain in most cases.

Discussion and Conclusion

Intradiscal C-arm-guided amniotic fluid instillation is a new method of treatment in advanced disc degeneration-related pain irrespective of traumatic or age-induced degeneration background, especially in cases where the patient is not getting any relief with sustained conventional analgesic and physiotherapeutic support. The long-term follow-up result of this type of cell therapy justifies its procedural superiority over conventionally and universally practiced intra-articular long-acting corticosteroid (methylprednisolone) ($p<0.001$).

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8735 W. Higgins Road, Suite 300
Chicago, IL 60631-2738
847-375-4731 Phone
info@painmed.org
www.painmed.org

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Contact Information
Email: info@painmed.org
Attn: Director of Communications
American Academy of Pain Medicine
Phone: 847-375-4731

Amniotic Fluid May be Safe and Effective Alternative to Hyaluronic Acid for Osteoarthritis Pain: Interim Results

March 19, 2015, NATIONAL HARBOR, Md. – An early snapshot of study outcomes suggests that the use of a processed amniotic fluid allograft may be safe and effective for the treatment of knee osteoarthritis (OA) as an alternative to hyaluronic acid (HA). Longer-lasting benefits with less risk of complications were reported today in a scientific poster presented at the 31st Annual Meeting of the American Academy of Pain Medicine.

"AmnioClear LCT is demonstrated in this study to offer pain and functional improvement that is greater at 13 weeks than at 30 days; thus it appears to offer longer-lasting relief at a higher level," said lead author Didier Demesmin, M.D., a pain management specialist with the University Pain Medicine Center in Somerset, N.J.

"It also demonstrated much lower incident of pain, swelling or inflammation compared to other injections," said Noreen Rana, M.P.H., research director at the center.

The most common form of knee arthritis is OA in which the cartilage wears away in a gradual process with pain that worsens over time, according the American Academy of Orthopaedic Surgeons (AAOS). Steroids, which may offer quick transient pain relief but are not recommended for repetitive use, and HA are standard alternatives to surgery. HA is a naturally occurring substance found in the synovial fluid, which lubricates the cartilage and reduces friction in the joint. The FDA approved HA knee injections starting in the 1990s, and they are frequently performed.

Yet the effects of HA decline after 7 weeks for a single injection or 12 weeks with multiple injections, the study authors said. Further, the Centers for Medicare and Medicaid Services (CMS) and AAOS have questioned the effectiveness of hyaluronic acid in the treatment of knee OA in patients over 65 and in the general population (Newberry et al, *AHRQ Technology Assessment, Draft*: Project ID: DJDTO913). In a 200-page Technical Assessment of HA in OA knees, CMS collaborated with the Agency for Healthcare Research and Quality to perform a meta analysis of the literature. They concluded that evidence was inconclusive to determine whether HA knee injections led to clinically meaningful improvement.

-More-

"Payer coverage has started to decline as a result of the AAOS recommendation, and many believe the CMS Tech Assessment will eventually cause further and more severe decline in HA coverage," Demesmin said.

As an alternative, investigators looked at amniotic fluid, noting its similarity to the synovial fluid in that both protect and lubricate the contents of a closed environment. Furthermore, the transplant of fetal membranes and fluid from one individual to another is not new and has been used to treat orthopedic conditions (Trelford et al, *Am J Obstet Gynecol* 1979;134(7):833-45). The cushioning action of amniotic fluid for the fetus is the same – "homologous," as the FDA terms it -- function in a recipient's knee, Demesmin said.

"This all-natural supplement alternative to synthetic treatments and the anti-inflammatory nature of amniotic fluid is precisely what painful OA knees need." Demesmin added that HA is FDA-cleared only for use in the knee, while the amniotic injection can be used in any synovial joint.

In this single-arm, prospective, multi-center, post-marketing study, a cohort of registry enrollees with a diagnosis of Grade 1, 2 or 3 OA and no recent HA, steroid or platelet-rich plasma injections were assessed for pain with the visual analogue scale (VAS) and the Western Ontario and McMaster Universities Arthritis Index (WOMAC) at baseline and at 30, 90 and 180 days. The registry was underwritten by Liventa Bioscience, which is based in West Conshohocken, Penn., and run under institutional review board guidance.

The interim report presented data from the first 15 of 23 investigative sites. Results observed in the first 170 amniotic fluid-treated patients showed their VAS and WOMAC scores improved an average of 68.1 percent (44mm) and 70.9 percent (812mm), respectively, at 30 days. Improvements increased at 90 days to 82 percent for WOMAC and 74 percent for VAS.

Like other commonly used surgical allografts, the amniotic fluid injection does not require pre-market approval, thus it is currently marketed and being used in clinics across the country. Liventa Bioscience elected to conduct a post-market (non-FDA) study to confirm efficacy and safety and to inform clinicians of expected outcomes, prior to a full market launch, which is taking place at the AAPM annual meeting. Additional studies, including randomized controlled trials, are planned.

Poster LB004 – Amniotic Fluid as a Homologue to Synovial Fluid: Interim Analysis of Prospective, Multi-Center Outcome Observational Cohort Registry of Amniotic Fluid Treatment for Osteoarthritis of the Knee

About AAPM

The American Academy of Pain Medicine is the premier medical association for pain physicians and their treatment teams with over 2,500 members. Now in its 32nd year of service, the Academy's mission is to optimize the health of patients in pain and eliminate pain as a major public health problem by advancing the practice and specialty of pain medicine through education, training, advocacy and research. Information is available on the Academy's website at www.painmed.org.

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Clinical Study

Amniotic Tissues for the Treatment of Chronic Plantar Fasciosis and Achilles Tendinosis

Bruce Werber[1,2]

[1] *Summit Foot and Ankle Specialists, 9188 E. San Salvador Drive, Scottsdale, AZ 85258, USA*
[2] *Amnio Technology, 22510 N 18th Drive, Phoenix, AZ 85027, USA*

Correspondence should be addressed to Bruce Werber; docwerber@gmail.com

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Introduction. Allogeneic amniotic tissue and fluid may be used to treat chronic plantar fasciosis and Achilles tendinosis. This innovative approach involves delivering a unique allograft of live human cells in a nonimmunogenic structural tissue matrix to treat chronic tendon injury. These tissues convey very positive regenerative attributes; procurement is performed with maternal consent during elective caesarian birth. *Materials and Methods*. In the present investigation all patients were unresponsive to multiple standard therapies for a minimum of 6 months and were treated with one implantation of PalinGen SportFLOW around the plantar fascia and/or around the Achilles paratenon. The patients were given a standard protocol for postimplant active rehabilitation. *Results*. The analogue pretreatment pain score (VAS) of 8. By the fourth week after treatment, all patients had significantly reduced self-reported pain. Twelve weeks following the procedure the average pain level had reduced to only 2. No adverse reactions were reported in any of the patients. *Conclusion*. All patients in this study experienced heel or Achilles pain, unresponsive to standard therapy protocols. After treatment all patients noted significant pain reduction, indicating that granulized amniotic membrane and amniotic fluid can be successfully used to treat both chronic plantar fasciosis and Achilles tendinosis.

1. Introduction

Heel pain is a common problem that may be due to a variety of soft-tissue abnormalities. Plantar fasciosis is one of the most common causes of heel pain and affects approximately two million people in the US, resulting in one million visits to primary care physicians and foot specialists [1]. Plantar fasciosis is the result of chronic overload from either lifestyle or exercise that promotes tissue degeneration [1]. Similarly, Achilles tendinosis affects both inactive and active individuals and is thought to result from changes in tissue structure [2]. Recent studies have shown that both plantar fasciosis and Achilles tendinitis involve degenerative fibrosis, rather than inflammation. While patients with plantar fasciosis and Achilles tendinitis experience severe, long-term pain, current treatments have limited efficacy, treating only the acute inflammation and pain and failing to address the underlying cause.

In such cases where no effective treatment options exist, engineered *ex vivo* tissues offer promising alternative regenerative therapies. Such tissues can deliver growth factors, fibroblasts, collagen, and extracellular matrix (ECM) on which cells can grow facilitating tissue healing and wound repair. However, many of these tissues are difficult to obtain and can elicit a negative immunogenic response. Embryonic stem cells possess the potential for differentiation into a wide range of cell lineages and hold immense promise for regenerative medicine; however, they are associated with a number of technical difficulties and ethical concerns. Currently, bone marrow (BM) is the most common source of adult stem cells for hematopoietic stem cell transplants and cellular therapies. The mesenchymal stem cells (MSCs) obtained from BM are pluripotent and able to differentiate into many different cell types, including osteoblasts, chondrocytes, adipocytes, neurons, cardiac myocytes, and vascular endothelial cells. BM harvest is an invasive surgical procedure that usually requires general anesthesia or sedation. Additionally, the proliferative potential and differentiation capacity of the BMMSCs from older donors appears reduced. Thus, other sources of stem cells from adult or fetal tissue are sought [3].

The amniotic membrane (AM) or amnion is a tissue of particular interest as a source of readily obtained, multipotent stem cells and factors that promote tissue healing [4]. The 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 [5, 6]. It also contains fibroblasts and growth factors and has been shown to have unique properties, including the ability to suppress pain, fibrosis, and bacteria and to promote wound healing [7, 8]. The AM contains two cell types of different embryologic origin, specifically amnion epithelial cells, derived from the embryonic ectoderm, and amnion mesenchymal cells, derived from embryonic mesoderm [9]. Recently, the International Society for Cellular Therapy recommended that mesenchymal cells derived from amnion be referred to as amniotic membrane-human mesenchymal stromal cells (AM-hMSCs) [10]. Importantly, amnion is easily obtained after caesarian delivery because the placenta, amniotic fluid, and membrane are typically discarded after childbirth. This procurement avoids the controversies associated with obtaining human embryonic stem cells and BMMSCs. Thus, the use of AM and amniotic fluids (AF) is highly promising innovative allografts and stem cell therapies for degenerative disorders where existing treatments have failed, such as plantar fasciosis and Achilles tendinosis.

AF derived cells are able to replicate rapidly and take 20–24 hours to double in cell number, faster than both umbilical cord stem cells (28–30 hours) and BMMSCs (30+ hours) [11]. The progenitor cells also have a high self-renewal capacity with more than 300 population doublings [12]. In addition, only 30% of MSCs extracted from a child's umbilical cord shortly after birth can be extracted and differentiated. In contrast, the success rate for AF derived MSCs has been close to 100%. Importantly, unlike other multipotent stem cells, particularly those with high self-renewal capacity, the risk of cancer development is low, and AF progenitor cells do not form teratomas *in vivo* [13].

In addition to multipotent stem cells, AF also contains a number of nutrients and growth factors that encourage fetal growth and protection. These factors are highly advantageous in regenerative clinical applications and aid tissue repair. Specifically, AF contains carbohydrates, proteins and peptides, lipids, lactate, pyruvate, electrolytes, enzymes, and hormones, transforming growth factor alpha (TGF-a), transforming growth factor beta 1 (TGF-b1), and fibroblast growth factor (FGF). A recent study demonstrated the effectiveness of FGF in restoring the morphologic and biomechanical properties of injured tendons in rabbits [14].

AF and AM have also been shown to have significant antimicrobial properties, mediated by a-defensins (human neutrophil defensins 1–3), lactoferrin, lysozyme, bactericidal/permeability-increasing protein, calprotectin, secretory leukocyte protease inhibitor, psoriasin, and cathelicidin [15]. Human beta-defensin-2 is another natural antimicrobial peptide present in the AF that may account for much of its antimicrobial activity [16]. Furthermore, lactoferrin, a glycoprotein secreted into the AF by neutrophils and amniotic cells, has bacteriostatic and bactericidal activity [17]. Human AF also contains factors known to minimize scarring. Hyaluronic acid (HA) is abundant in AF and fetal HA is thought to inhibit collagen deposition to prevent fibrotic tissue formation [18, 19]. In recent studies addressing the effect of AF on proteases important for wound healing, human AF was shown to enhance collagenase activity but to inhibit activation of hyaluronidase, elastase, and cathepsin [20, 21].

Due to its regenerative, anti-microbial and anti-scarring properties the amnion has been used as an effective wound dressing and as a graft for skin wound coverage. Several studies have highlighted the low immunogenicity of human amniotic epithelial cells following transplantation into human volunteers. For example, no signs of acute rejection were observed after amnion was transplanted into subcutaneous pouches in normal human volunteers [22]. Following transplantation of amniotic tissues HLA antibodies are absent in serum samples [23]. In addition, amnion surface epithelial cells do not express HLA-A, HLA-B, HLA-C, or HLA-DR or b2-microglobulin [23, 24]. This, at least in part, explains why amniotic tissues can be used successfully as a skin graft without concern for tissue typing and matching of the donor to the host [8]. This lack of immunogenicity has been described in numerous clinical studies and is termed immune privilege [25]. Collectively, these studies suggest that acute immune rejection does not occur after transplantation of human amniotic epithelial cells, and granulized AM and AF (gAM-AF) are a suitable treatment option for all patients, even those who are severely immunocompromised.

2. Allograft Procurement

In the present study, patients experiencing heel pain caused by chronic plantar fasciosis and Achilles tendinosis and who were unresponsive to standard therapies for a minimum of 6 months were treated with PalinGen SportFLOW (Amnio Technology, llc. Phoenix, AZ) to promote tissue repair and regeneration. The PalinGen SportFLOW allograft was generated from human amniotic membrane and amniotic fluid (hAM-AF), harvested from females undergoing elective caesarian section. PalinGen SportFlow is a human allograft and is processed and packaged at an FDA registered tissue bank accredited by the American Association of Tissue Banks (AATB). PalinGen SportFLOW is regulated by the FDA under Title 21 Part 1271 Section 361 of the Public Health Service Act. Tissues were tested extensively to ensure the absence of communicable diseases and other abnormalities. After testing, the tissues were aseptically processed and cryopreserved to preserve cell viability. Cryopreservation of the hAM-AF yielded a multifactorial tissue matrix containing viable pluripotent mesenchymal stem cells, fibroblasts, keratinocytes, epithelial cells, cytokines, proteins, growth factors, and multipotent cells, all required for fetal growth and development and able to stimulate tissue repair and regeneration. In this study, allografts were used to create a microenvironment suitable for regeneration of tendons and fascia that had become chronically thickened due to abnormal function and healing. Allograft was also used as

a potent anti-inflammatory and to create the appropriate conditions in which to drive poorly formed tendons and fascia to a normal state.

The PalinGen SportsFLOW allograft most important components are a wide spectrum of growth factor proteins, that are, VEGF, TGF-beta1, EGF, PDGF-AA, PDGF-BB, FGFb, extracellular matrix (cryofractured amnion membrane) and amniotic fluid derived cells.

Amnion donors were subject to a thorough prescreening process performed by the Medical Director. Eligibility was confirmed through behavioral risk assessment, medical history, hematology, and communicable disease testing. Procurement of the amnion tissues was done with an aseptic recovery technique during cesarean section, using standard sterile techniques. Procurement of hAM-AF does not require fetal death, and its recovery was performed with maternal consent during an elective caesarian section live birth.

3. Patients, Methods, and Techniques

Chronic plantar fasciosis patients were chosen from a pool of patients with chronic heel pain that had failed a variety of noninvasive therapies, including custom and/or prefab orthotics, stretching, steroid injections, physical therapy, and night splints (used for 1-2 hours in evening with leg extended). These patients additionally had a thickened fascia on diagnostic ultrasound of at least 4.0 mm.

4. Plantar Fascia Technique

(1) ZimmerWave radial pulse therapy was applied to the painful area, typically 1500 pulses, at 10 hertz and 110 mJ.

(2) The plantar fascia was visualized under ultrasound imaging, and the area was aseptically prepared with Betadine or alcohol.

(3) 0.5 mL of PalinGen SportFLOW and 0.5 mL of 1% lidocaine were drawn into a 3 mL syringe with a 22-Gauge needle.

(4) Observing the plantar fascia under ultrasound guidance, the approach was from medial to lateral. The needle was directed to the superior surface of the plantar fascia, not directly into the plantar fascia, and 0.3 mL was deposited along the medial and central bands of the fascia.

(5) The needle was then redirected to the plantar aspect of the plantar fascia and another 0.3 mL was deposited along the fascial band.

(6) The needle was then redirected towards the central plantar calcaneal bursa between the medial and lateral tubercles and the remainder of the allograft was implanted.

(7) The patient was instructed to stretch every 30 minutes with a traditional runners calf stretch during waking hours.

(8) Patients were also instructed to wear lace up stable shoes, to minimize time being barefoot and to minimize wearing flip-flops or slip-on shoes.

(9) No anti-inflammatory medication was taken for 3–6 weeks.

(10) No ice was applied to the affected area.

Achilles tendinopathy patients were chosen from a pool of patients with Achilles pain, who were unresponsive to a variety of therapies including stretching, physical therapy, and modified shoe gear and in some cases low energy radial pulse therapy. Imaging confirmed that there was no rupture using either MRI or diagnostic ultrasound.

5. Achilles Tendon Technique

(1) ZimmerWave radial pulse therapy was applied to the painful area, typically 1500 pulses, at 10 hertz and 110 mJ.

(2) The Achilles tendon was visualized under ultrasound imaging, and the area was aseptically prepared with Betadine or alcohol.

(3) 1.0 mL of PalinGen SportFLOW and 1.0 mL of 1% lidocaine were drawn into a 3 mL syringe with a 22-Gauge needle.

(4) Observing the Achilles tendon under ultrasound guidance, the needle was directed along the paratenon starting on the medial aspect, not into the tendon substance, and 0.5 mL was deposited along the medial aspect of the tendon. The needle was then redirected to the posterior aspect of the tendon and the remainder was deposited along the lateral aspect.

(5) Patient was instructed to stretch every 30 minutes with a traditional runners calf stretch during waking hours.

(6) Patients were also instructed to wear lace up stable shoes, to minimize time being barefoot and to minimize wearing flip-flops or slip-on shoes.

(7) No anti-inflammatory medication was taken for 3–6 weeks.

(8) No ice was applied to the affected area.

6. Results

In total, 44 patients experiencing chronic plantar fasciosis and Achilles tendinosis, with a mean age of 55.1 and 47.7 years, respectively, who were all unresponsive to multiple standard therapies for a minimum of 6 months, were treated with one implantation of PalinGen SportFLOW around the plantar fascia and/or into and around the Achilles paratenon. Following treatment they were instructed to wear laced shoes and perform posterior muscle group stretching exercises or rolling pin massage instructions. No changes were made to their exercise routines.

The visual analog scale (VAS) is an instrument used to quantify the level of pain reported by patients. The VAS

Table 1: Baseline statistics and time course postoperative visual analogue pain scores (VAS) for patients with either plantar fasciosis (PF) or Achilles tendinosis (AF). Data is presented as mean with SD. $^{***}p < 0.001$ versus preop VAS (mean age compared with Mann-Whitney U-test).

	Patient age	Preop	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
PF	55.11 ± 5.9	8.1 ± 1.4	6.2 ± 1.8	5.2 ± 1.6	4.2 ± 1.2	3.6 ± 0.7***	2.5 ± 0.9***	1.5 ± 1.4***
AT	47.69 ± 3.3	8.2 ± 1.2	6 ± 1.9	4.7 ± 1.6***	4 ± 1.0***	3.6 ± 0.9***	2.9 ± 1.0***	2.3 ± 1.3***

ranges from 0 to 10 with 0 representative of no pain, 1–3 indicating mild pain, 4–6 indicating moderate pain, and 7–10 representative of severe pain. Preoperative pain was self-reported as severe in all patients, with a mean of 8.2 (Table 1). Changes in self-reported pain were monitored every 2 weeks for 12 weeks after procedure. Changes in pain over time were statistically determined using the Freidman nonparametric repeated measures ANOVA with Dunn's post hoc test for multiple comparisons.

For patients experiencing plantar fasciosis there was a significant improvement in pain scores in all patients by postoperative week four ($p < 0.05$, Figure 1), with a mean pain score of 5.2 (Table 1) indicative of moderate pain. By postoperative week 10 the pain scores were markedly reduced ($p < 0.0001$, Figure 1) and the average self-reported scores indicated that the majority of patients experienced only mild pain.

Similar results were observed in patients experiencing Achilles tendinosis, and all patients gave self-reported pain scores not higher than moderate pain by postoperative week 6, with an average pain score of 4.7 (Table 1) ranging from 1 to 6 (Figure 2). By 12 weeks after treatment the average pain score had reduced to only 2.3 (Table 1) indicating that the majority of patients were experiencing mild pain. Therefore, after treatment with granulized amniotic membrane and amniotic fluid pain was significantly reduced compared to preoperative pain, with the majority of patients reporting only mild pain.

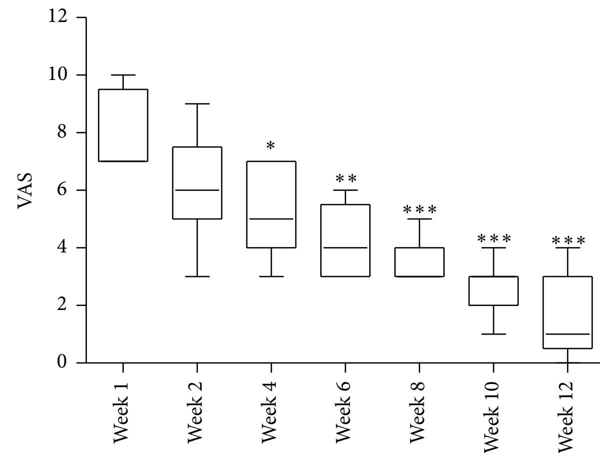

Figure 1: Time course self-reported postoperative visual analogue pain score (VAS) from patients with plantar fasciosis, showing median and minimum and maximum scores, where $^{***}p < 0.001$ versus preop (week 1) VAS.

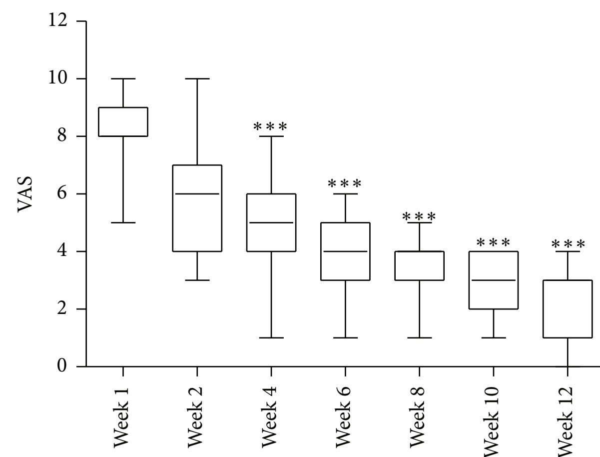

Figure 2: Time course self-reported postoperative visual analogue pain score (VAS) from patients with Achilles tendinosis, showing median and minimum and maximum scores, where $^{***}p < 0.001$ versus preop (week 1) VAS.

7. Discussion and Conclusion

Heel pain is a common problem that may be present in 15% of patients presenting to their primary care physician [26]. In this study we show for the first time to our knowledge that a single injection of hAM-AF allograft is sufficient to significantly reduce heel pain caused by plantar fasciosis and Achilles tendinosis. At the end of the study all patients showed a significant improvement in pain, and on average self-reported pain had reduced from severe to mild. Our findings suggest that amniotic allografts create the appropriate environment needed to promote tissue repair and healing in complex soft-tissue disorders such as plantar fasciosis and Achilles tendinosis.

Plantar fasciosis is the most common cause of inferior heel pain and is often due to repetitive mechanical stress, producing microtears and inflammation of the fascia and perifascial soft tissues. The condition is commonly seen in individuals who are susceptible to injury such as runners and obese patients [27]. To date there is no definitive treatment proven to be the best option for plantar fasciosis. Treatment is patient dependent and commonly requires a combination of different therapies to successfully alleviate symptoms [28]. In many cases patients do not respond to current treatments and symptoms persist. This is likely due to the fact that plantar fasciosis is not simply the product of mechanical stress and is actually the result of a number of contributing factors associated with aberrant tissue development and healing. Factors include enthesopathy in association with seronegative

spondyloarthropathies, such as ankylosing spondylitis, Reiter syndrome, or psoriatic arthritis [29]. Findings from MRI studies have shown a number of other tissue abnormalities associated with plantar fasciosis, including plantar fascial thickening and intrafascial edema [29].

Achilles tendinosis is also a common cause of heel pain in a sport-active population and is responsible for reduced physical performance and increased severe pain over several years [30]. Despite being associated with mechanical stress, recent studies have shown that this pathology also affects an older population with less involvement in sporting activities, suggesting that tissue degeneration, in some cases age-associated, contributes to its pathogenesis. Recent reports also highlight the heterogeneity of Achilles tendinopathy pathogenesis and have identified multiple synergistic risk factors including genes, age, circulating and local cytokine production, sex, biomechanics, and body composition [31].

Current conventional treatments for heel pain include physical therapy, rest, stretch exercise, nonsteroidal anti-inflammatory drugs (NSAIDs), and steroid injections. Steroid injection is one of the most popular options [32]; however, it may produce serious side effects such as a recognized risk of subsequent plantar fascia rupture [33]. Consequently, treatments that only address the symptoms of plantar fasciosis and Achilles tendinosis are often unsuccessful, and treatments able to stimulate wound healing are highly sought.

Provision of factors that provide a regenerative stimulus is an emerging treatment strategy which aims at alleviating chronic tendinopathies characterized by a poor healing ability. Recent studies have shown that provision of platelet rich plasma (PRP)—rich in platelet derived growth factors—can provide a local regenerative stimulus for tissue healing. Achilles tendinopathy patients receiving PRP injections showed significant improvements after treatment; however, these improvements took several months to occur [34]. MSCs are an emerging alternative option to promote tissue regeneration. Recently, several studies in animal models have shown that administration of hMSCs can improve healing in tendon injuries. Specifically, hMSCs can support tendon healing through better vascularization, larger deposits, and better organization of the extracellular matrix [35]. Although overall this treatment procedure may be clinically safe, cartilage and bone formation at the implantation site is an expected adverse event [35]. In addition procurement of hMSCs is associated with invasive surgical procedures and ethical concerns.

Amniotic tissue allografts are also associated with soft-tissue repair and regeneration. Specifically, recent studies have shown that amniotic allografts contain angiogenic growth factors that promote amplification of angiogenic cues by inducing endothelial cell proliferation and migration to promote the formation of blood vessels *in vivo* [36]. Such grafts offer promising stem cell therapies with the potential to promote revascularization and tissue healing within poorly vascularized, nonhealing wounds. In addition, amniotic allografts are not associated with problematic procumbent procedures and contain additional factors with anti-inflammatory and anti-microbal properties. However, preservation of these properties during processing remains a challenge.

To date, the efficacy of amniotic tissue allografts in rescuing chronic heel pain has not been demonstrated. In the present study, cryopreserved (PalinGen SportFLOW) hAM-AF was injected into the tissues of patients who experienced severe heel pain and who were unresponsive to existing therapies. Significant improvements in pain were observed 4 weeks after treatment in all patients, with almost complete pain recovery in many patients by the end of the study. Our observations suggest that cryopreserved hAM-AF mediates the biological properties required for effective and rapid tissue healing and repair. Our findings support the use of PalinGen SportFLOW allograft as a promising therapy for plantar fasciosis and Achilles tendinosis and other soft-tissue disorders associated with deficiencies in the normal wound healing processes.

Conflict of Interests

Bruce Werber DPM, FACFAS, does have a financial relationship with Amnio Technology.

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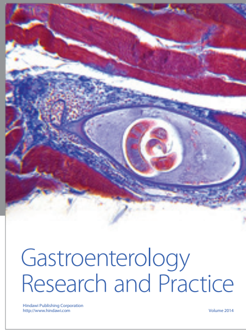

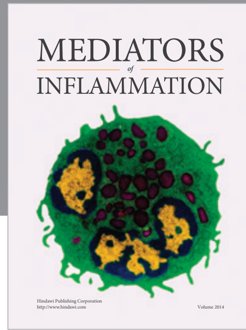

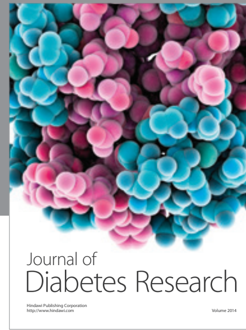

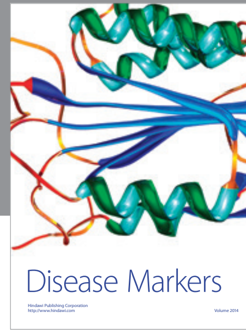

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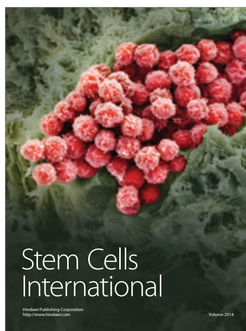

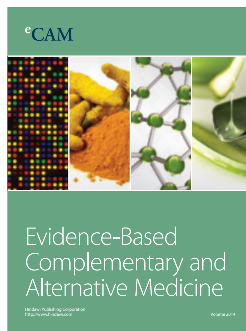

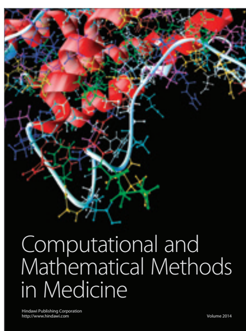

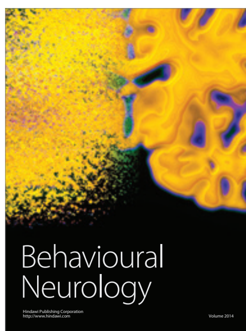

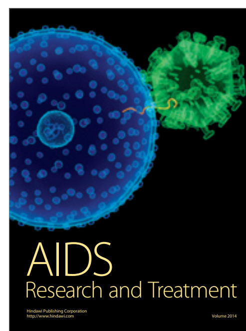

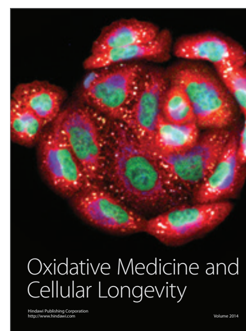

Sports Medicine, Orthopaedics & Pain Management

Amniotic Fluid Cell Therapy to Relieve Disc-Related Low Back Pain and Its Efficacy Comparison with Long-Acting Steroid Injection

Introduction: There are many problems in case of treatment of the patients reporting with degenerated disc with or without disc prolapse, desiccation, bulge, or compression of the adjacent nerves and its implications. Most of the patients with chronic discogenic back pain, without specific history of trauma, are on geriatric age group. In this age group, low back pain is associated with varying degree of age-induced degenerative osteoporosis, spondylosis, spondyloarthrosis, intervertebral disc prolapse, or even compression collapse apart from other problems like diabetic background, hypertension, ischemic heart disease, chronic obstructive pulmonary disorder, dyslipidemia, and hypothyroidism. MRI presentation of a typical geriatric presentation of low back pain is shown (Figs. 19.1, 19.2, and19.3). Materials and methods: 42 patients participated and randomized in two equal groups. Group A (N = 21, male 10 and female 11, mean age 56.4 ± 8.9 year) was treated with 80 mg methylprednisolone in 10 mL water for injection under C-arm guidance in the operation theater (OT) after 1 % infiltration with Xylocaine at the site of maximum tenderness in the back. Similarly, Group B (N = 21, male 12 and female 9, mean age 59.4 ± 6.4 year) was also treated in the OT with similar protocol with 10 mL of freshly collected amniotic fluid from mothers undergoing hysterotomy and ligation. All the procedures passed through the donor and recipient's informed consent protocol and vetted by the institute-based ethical committee. Result and analysis: Studying and comparing the clinically manifested effect of treatment, it can be easily seen that both steroid (Group A) and cell therapy (Group B) patients showed improvement of pain and distress from the pretreatment value; however, Group B scoring is much better (p, 0.01), as seen and assessed from the value of the VAS (visual analog pain scale), WD (walking distance in meters), and HAQ (Health Assessment Questionnaire). If we see further the clinical assessment of pain relief and patient's satisfaction as seen from Table 19.3 and Graph 19.1 in case of Group A (long-acting steroid group), it was 20/21 cases in 1st month which became 12/21 in 3rd month, 6/21 in 6th month, 4/21 in 12th month, and 2/21 after 24-month follow-up. Similarly in Group B (cell therapy patients), the identical values after the 1st month were 18/21, which became 21/21 in 3rd month, 21/21 in 6th month, 14/21 in 12th month, and 12/24 after 24-month follow-up. Another globally practiced guideline for pain assessment or scoring for comparison is Oswestry low back pain disability questionnaire. Here in Table 19.4 and Graph 19.2, we have again compared the effect of treatment of Group A (steroid) and Group B (cell therapy with fresh amniotic fluid) and followed up the results of Group A and Group B treatment as per scoring by Oswestry low back pain disability questionnaire up to 24 months. Here, postinjection with long-acting steroid (Group A) suggested a mean scoring of 9 ± 1.2 % SD after 3 months, which became mean 1.9 ± 1.2 % SD after 6 months, mean 39 ± 9.2 % SD after 9 months, mean 39 ± 8.2 % SD after 12 months, mean 41 ± 7.2 % SD after 18 months, and then ultimately mean 48 ± 12.2 % SD after 24 months. Similarly in case of cell therapy group (Group B), the mean scoring was 11.7 ± 1.6 % SD after 3rd month follow-up, which became mean 9.4 ± 0.6 % SD after 6th month, mean 9.1 ± 0.96 % SD after 9th month, mean 7.1 ± 0.6 % SD after 12th month, mean 6.7 ± 0.4 % SD after 18th month, and ultimately mean 4.1 ± 0.96 % SD after 24th month follow-up. Discussion and conclusion: If we analyze the results, we can see long-acting steroid, due to its anti-inflammatory and other activities, causes some improvement of the patients; however, it is ill sustained as noted from the follow-up. But freshly collected simple amniotic fluid cell

therapy has a much more sustained effect apart from the remarkable improvement, but the question remains why in long-term follow-up there is reappearance of pain in some of the victims〗 Is it psychosomatic aspects or a recurrent cell therapy or increasing the cell dosage that can have a more sustained effect〗 These are some of the questions for the future investigators in this frontline area of cellular therapy. But from an overall point of view, regeneration can only treat the root cause of degeneration of the whole lumbosacral region. Cell therapy is the only curative approach for such a generalized multisystemic deterioration of the region, and the palliative approach of pain relief with anti-inflammatory drug including steroid is short lived and has longtime use and may lead to drug-induced problems in addition of the recurrence of the symptoms.

Bhattacharya, Niranjan. (2012, December 5). Human Fetal Tissue Transplantation. Amniotic Fluid Cell Therapy to Relieve Disc-Related Low Back Pain and Its Efficacy omparison with Long-Acting Steroid Injection. 2013, pp 251-264

Amniotic Fluid Stem Cells: a Promising Therapeutic Resource for Cell-Based Regenerative Therapy

Stem cells have been proposed as a powerful tool in the treatment of several human diseases, both for their ability to represent a source of new cells to replace those lost due to tissue injuries or degenerative diseases, and for the ability of produce trophic molecules able to minimize damage and promote recovery in the injured tissue. Different cell types, such as embryonic, fetal or adult stem cells, human fetal tissues and genetically engineered cell lines, have been tested for their ability to replace damaged cells and to restore the tissue function after transplantation. Amniotic fluid -derived Stem cells (AFS) are considered a novel resource for cell transplantation therapy, due to their high renewal capacity, the "in vitro" expression of embryonic cell lineage markers, and the ability to differentiate in tissues derived from all the three embryonic layers. Moreover, AFS do not produce teratomas when transplanted into animals and are characterized by a low antigenicity, which could represent an advantage for cell transplantation or cell replacement therapy. The present review focuses on the biological features of AFS, and on their potential use in the treatment of pathological conditions such as ischemic brain injury and bone damages.

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Mesenchymal stem cells in arthritic diseases

Mesenchymal stem cells (MSCs), the nonhematopoietic progenitor cells found in various adult tissues, are characterized by their ease of isolation and their rapid growth in vitro while maintaining their differentiation potential, allowing for extensive culture expansion to obtain large quantities suitable for therapeutic use. These properties make MSCs an ideal candidate cell type as building blocks for tissue engineering efforts to regenerate replacement tissues and repair damaged structures as encountered in various arthritic conditions. Osteoarthritis (OA) is the most common arthritic condition and, like rheumatoid arthritis (RA), presents an inflammatory environment with immunological involvement and this has been an enduring obstacle that can potentially limit the use of cartilage tissue engineering. Recent advances in our understanding of the functions of MSCs have shown that MSCs also possess potent immunosuppression and anti-inflammation effects. In addition, through secretion of various soluble factors, MSCs can influence the

local tissue environment and exert protective effects with an end result of effectively stimulating regeneration in situ. This function of MSCs can be exploited for their therapeutic application in degenerative joint diseases such as RA and OA. This review surveys the advances made in the past decade which have led to our current understanding of stem cell biology as relevant to diseases of the joint. The potential involvement of MSCs in the pathophysiology of degenerative joint diseases will also be discussed. Specifically, we will explore the potential of MSC-based cell therapy of OA and RA by means of functional replacement of damaged cartilage via tissue engineering as well as their anti-inflammatory and immunosuppressive activities.

Chen, F. H., & Tuan, R. S. (2008). Mesenchymal stem cells in arthritic diseases. Arthritis Research & Therapy, 10(5), 223-223. doi:10.1186/ar2514

Amniotic fluid-derived stem cells in regenerative medicine research

The stem cells isolated from amniotic fluid present an exciting possible contribution to the field of regenerative medicine and amniotic fluid-derived stem (AFS) cells have significant potential for research and therapeutic applications. AFS cells are multipotent, showing the ability to differentiate into cell types from all three embryonic germ layers. They express both embryonic and adult stein cell markers, expand extensively without feeder cells, double in 36 h, and are not tumorigenic. The AFS cells can be maintained for over 250 population doublings and preserve their telomere length and a normal karyotype. They differentiate easily into specific cell lineages and do not require human embryo tissue for their isolation, thus avoiding the current controversies associated with the use of human embryonic stem (ES) cells. The discovery of the AFS cells has been recent, and a great deal of work remains to be performed on the characterization and use of these cells. This review describes the various differentiated lineages that AFS cells can form and the future of these promising new stem cells in regenerative medicine research.

Joo, S., Ko, I. K., Atala, A., Yoo, J. J., & Lee, S. J. (2012). Amniotic fluid-derived stem cells in regenerative medicine research. Archives of Pharmacal Research, 35(2), 271-280. doi:10.1007/s12272-012-0207-7

Mesenchymal Stromal Cells in Rheumatoid Arthritis: Biological Properties and Clinical Applications

Mesenchymal stromal cells (MSC) isolated from a variety of adult tissues including the bone marrow (BM), have the capacity to differentiate into different cell types such as bone and cartilage and have therefore attracted scientific interest as potential therapeutic tools for tissue repair. MSC display also immunosuppressive and anti-inflammatory properties and their putative therapeutic role in a variety of inflammatory autoimmune diseases is currently under investigation. Joint destruction, caused by persistent inflammation, renders rheumatoid arthritis (RA) a possible clinical target for cartilage and bone repair using BM MSCs for their tissue repair and immunoregulatory effects. A number of studies, based mainly on experimental animal models, have recently provided interesting data on the potential of BM-MSCs to suppress local inflammation and tissue damage in RA whereas tissue engineering and cell-scaffold technology represents an emerging field of research. This review deals with the biological repair/regeneration of joint tissues in RA via MSC-based therapies. In view of the current interest in the autologous usage of BM MSC in RA, all available data on the biological properties of patient MSCs including the immunoregulatory characteristics, differentiation capacity towards osteocytes/chondrocytes,

clonogenic/proliferative potential and molecular/protein profile and the possible influence of the RA milieu will be also summarized.

Kastrinaki, M., & Papadaki, H. (2009). Mesenchymal stromal cells in rheumatoid arthritis: Biological properties and clinical applications. CURRENT STEM CELL RESEARCH & THERAPY, 4(1), 61-69. doi:10.2174/157488809787169084

Local Adherent Technique for Transplanting Mesenchymal Stem Cells as a Potential Treatment of Cartilage Defect

Introduction Current cell therapy for cartilage regeneration requires invasive procedures, periosteal coverage and scaffold use. We have developed a novel transplantation method with synovial mesenchymal stem cells (MSCs) to adhere to the cartilage defect. Methods For ex vivo analysis in rabbits, the cartilage defect was faced upward, filled with synovial MSC suspension, and held stationary for 2.5 to 15 minutes. The number of attached cells was examined. For in vivo analysis in rabbits, an autologous synovial MSC suspension was placed on the cartilage defect, and the position was maintained for 10 minutes to adhere the cells to the defect. For the control, either the same cell suspension was injected intra-articularly or the defects were left empty. The three groups were compared macroscopically and histologically. For ex vivo analysis in humans, in addition to the similar experiment in rabbits, the expression and effects of neutralizing antibodies for adhesion molecules were examined. Results Ex vivo analysis in rabbits demonstrated that the number of attached cells increased in a time-dependent manner, and more than 60% of cells attached within 10 minutes. The in vivo study showed that a large number of transplanted synovial MSCs attached to the defect at 1 day, and the cartilage defect improved at 24 weeks. The histological score was consistently better than the scores of the two control groups (same cell suspension injected intra-articularly or defects left empty) at 4, 12, and 24 weeks. Ex vivo analysis in humans provided similar results to those in rabbits. Intercellular adhesion molecule 1-positive cells increased between 1 minute and 10 minutes, and neutralizing antibodies for intercellular adhesion molecule 1, vascular cell adhesion molecule 1 and activated leukocyte-cell adhesion molecule inhibited the attachment. Conclusion Placing MSC suspension on the cartilage defect for 10 minutes resulted in adherence of >60% of synovial MSCs to the defect, and promoted cartilage regeneration. This adherent method makes it possible to adhere MSCs with low invasion, without periosteal coverage, and without a scaffold.

Koga, H., Shimaya, M., Muneta, T., Nimura, A., Morito, T., Hayashi, M., . . . Sekiya, I. (2008). Local adherent technique for transplanting mesenchymal stem cells as a potential treatment of cartilage defect. Arthritis Research & Therapy, 10(4), R84-R84. doi:10.1186/ar2460

Human Amnion Tissue Injected with Human Umbilical Cord Mesenchymal Stem Cells Repairs Damaged Sciatic Nerves in Rats

Human umbilical cord mesenchymal stem cells, incorporated into an amnion carrier tubes, were assessed for nerve regeneration potential in a rat nerve defect model. Damaged nerves were exposed to human amnion carriers containing either human umbilical cord mesenchymal stem cell (cell transplantation group) or saline (control group). At 8, 12, 16 and 20 weeks after cell implantation, the sciatic functional index was higher in the cell transplantation group compared with the control group. Furthermore, electrophysiological examination showed that threshold stimulus and maximum stimulus intensity gradually decreased while compound action potential amplitude gradually increased. Hematoxylin-

eosin staining showed that regenerating nerve fibers were arranged in nerve tracts in the cell transplantation group and connective tissue between nerve tracts and amnion tissue reduced over time. Gastrocnemius muscle cell diameter, wet weight and restoration ratio were increased. These data indicate that transplanted human umbilical cord mesenchymal stem cells, using the amnion tube connection method, promote restoration of damaged sciatic nerves in rats.

Li, D., Wang, C., Shan, W., Zeng, R., Fang, Y., & Wang, P. (2012). Human amnion tissue injected with human umbilical cord mesenchymal stem cells repairs damaged sciatic nerves in rats. NEURAL REGENERATION RESEARCH, 7(23), 1771-1778. doi: 10.3969/j.issn.1673-5374.2012.23.002

Tendon Regeneration and Repair with Stem Cells

The use of stems cells in tendon repair is of particular interest given the frequency of tendon injuries worldwide together with the technical difficulty often encountered when repairing or augmenting tendons. Stems cells have the capability to differentiate into a variety of different cell types including osteocytes and tenocytes, and if normal architecture of damaged tendon (either macroscopic or microscopic) could be restored, this would significantly improve the management of patients with these injuries. There is already encouraging research on the use of stems cells clinically although considerable further work is required to improve knowledge and clinical applications of stem cells in tissue engineering.

MacLean, S., Khan, W. S., Malik, A. A., Snow, M., & Anand, S. (2012). Tendon regeneration and repair with stem cells. Stem Cells International, 2012, 316281. doi: 10.1155/2012/316281

Effects of Human Amniotic Fluid on Cartilage Regeneration From Free Perichondrial Grafts in Rabbits

After the chondrogenic potential of free grafts of perichondrium was shown in several experimental studies, perichondrium has been used to reconstruct cartilage tissue in various clinical situations. This study investigates the effects of human amniotic fluid on neochondrogenesis from free perichondrial grafts in a rabbit model. Since this fluid contains high concentrations of hyaluronic acid, hyaluronic acid-stimulating activator, growth factors, and extracellular matrix precursors during the second trimester, it may have a stimulating effect on neochondrogenesis. Perichondrial grafts, measuring 20x20 mm super(2) were obtained from the ears of 144 New Zealand young rabbits and were sutured over the paravertebral muscles. The rabbits were randomly divided into three groups with 48 rabbits per group. In group 1, 0.3 ml human amniotic fluid, and in group 2, 0.3 ml saline were injected underneath the perichondrial grafts. Group 3 formed the control group in which no treatment was given. Histologically, neochondrogenesis was evaluated in terms of cellular form and graft thickness at 2, 4, 6, and 8 weeks after surgery. In group 1, the mature cartilage was generated quickly and the cartilage plate in this group was significantly thick and extensive when compared with groups 2 and 3 at 8 weeks ($p<0.05$, ANOVA). In conclusion, our study shows that human amniotic fluid enhances neochondrogenesis from free perichondrial grafts. The rich content of hyaluronic acid and growth factors possibly participate in this result.

Ozgenel, G. Y., Filiz, G., & Ozcan, M. (2004). Effects of human amniotic fluid on cartilage regeneration from free perichondrial grafts in rabbits. British Journal of Plastic Surgery, 57(5), 423-428. doi:10.1016/j.bjps.2003.12.021

Meniscal Tears Respond to Cell Injections

Vangsness reported that a few patients in the low-dose MSC group also showed evidence of meniscal regeneration in MRI scans taken after one year.

Young, B. (2012) Meniscal Tears Respond to Cell Injections. ORTHOPEDICS THIS WEEK. Retrieved from Single Source Surgical: http://www.singlesourcesurgical.com/wp-content/uploads/2014/01/amniotic-tissue.pdf

Platelet-Rich Plasma Therapy - Future or Trend?

Chronic complex musculoskeletal injuries that are slow to heal pose challenges to physicians and researchers alike. Orthobiologics is a relatively newer science that involves application of naturally found materials from biological sources (for example, cell-based therapies), and offers exciting new possibilities to promote and accelerate bone and soft tissue healing. Platelet-rich plasma (PRP) is an orthobiologic that has recently gained popularity as an adjuvant treatment for musculoskeletal injuries. It is a volume of fractionated plasma from the patient's own blood that contains platelet concentrate. The platelets contain alpha granules that are rich in several growth factors, such as platelet-derived growth factor, transforming growth factor-β, insulin-like growth factor, vascular endothelial growth factor and epidermal growth factor, which play key roles in tissue repair mechanisms. PRP has found application in diverse surgical fields to enhance bone and soft-tissue healing by placing supra-physiological concentrations of autologous platelets at the site of tissue damage. The relative ease of preparation, applicability in the clinical setting, favorable safety profile and possible beneficial outcome make PRP a promising therapeutic approach for future regenerative treatments. However, there is a large knowledge gap in our understanding of PRPs mechanism of action, which has raised skepticism regarding its potential efficacy and use. Thus, the aim of this review is to describe the various factors proposed to contribute to the biological activity of PRP, and the published pre-clinical and clinical evidence to support it. Additionally, we describe the current techniques and technology for PRP preparation, and review the present shortcomings of this therapy that will need to be overcome if it is to gain broad acceptance.

Dhillon, R. S., Schwarz, E. M., & Maloney, M. D. (2012). Platelet-rich plasma therapy - future or trend? Arthritis Research & Therapy, 14(4), 219-219. doi:10.1186/ar3914

Human Stem Cell Delivery for Treatment of Large Segmental Bone Defects

Local or systemic stem cell delivery has the potential to promote repair of a variety of damaged or degenerated tissues. Although various stem cell sources have been investigated for bone repair, few comparative reports exist, and cellular distribution and viability postimplantation remain key issues. In this study, we quantified the ability of tissue-engineered constructs containing either human fetal or adult stem cells to enhance functional repair of nude rat critically sized femoral defects. After 12 weeks, defects treated with cell-seeded polymer scaffolds had significantly higher bone ingrowth and torsional strength compared to those receiving acellular scaffolds, although there were no significant differences between the cell sources. Next, stem cells were labeled with fluorescent quantum dots (QDs) in an attempt to noninvasively track their distribution after delivery on

scaffolds. Clear fluorescence was observed at implantation sites throughout the study; however, beginning 7-10 days after surgery, signals were also observed at contralateral sites treated with acellular QD-free scaffolds. Although immunostaining for human nuclei revealed retention of some cells at the implantation site, no human cells were detected in the control limb defects. Additional histological analysis of implantation and control defect tissues revealed macrophages containing endocytosed QDs. Furthermore, QD-labeling appeared to diminish transplanted cell function resulting in reduced healing responses. In summary, augmentation of polymeric scaffolds with stem cells derived from fetal and adult tissues significantly enhanced healing of large segmental bone defects; however, QD labeling of stem cells eliminated the observed therapeutic effect and failed to conclusively track stem cell location long-term in vivo.

Dupont, K. M., Sharma, K., Stevens, H. Y., Boerckel, J. D., García, A. J., & Guldberg, R. E. (2010). Human stem cell delivery for treatment of large segmental bone defects. Proceedings of the National Academy of Sciences, 107(8), 3305-3310. doi: 10.1073/pnas.0905444107

Metabolic Functions of Myostatin and GDF11

Myostatin is a member of the transforming growth factor β superfamily of secreted growth factors that negatively regulates skeletal muscle size. Mice null for the myostatin gene have a dramatically increased mass of individual muscles, reduced adiposity, increased insulin sensitivity, and resistance to obesity. Myostatin inhibition in adult mice also increases muscle mass which raises the possibility that anti-myostatin therapy could be a useful approach for treating diseases such as obesity or diabetes in addition to muscle wasting diseases. In this review I will describe the present state of our understanding of the role of myostatin and the closely related growth factor growth/differentiation factor 11 on metabolism.

Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland USA. (2010) Metabolic Functions Of Myostatin AND GDF11. Immunol Endocr Metab Agents Med Chem. 2010 Dec; 10(4): 217-231.

Potential Applications for Using Stem Cells in Spine Surgery

While the use of biologics as adjuncts for spine surgery is growing annually stem cells have yet to be approved for this clinical application. Stem cells have the unique ability to differentiate into a variety of musculoskeletal tissues including bone or cartilage. Moreover they have been shown to secrete growth factors that promote matrix repair and regeneration and can down regulate inflammation and immune cell functions. It is these combined activities that make stem cells attractive candidates for advancing current techniques in spine surgery and possibly mitigating those pathologies responsible for tissue degeneration and failure thereby minimising the need for surgical intervention at a later date. This review focuses on the characteristics of progenitor cells from different sources and explores their potential as adjuncts for both current and future applications in spine surgery. Where possible we draw on the experimental outcomes from our own preclinical studies using adult mesenchymal progenitor stem cells, as well as related studies by others to support our contention that stem cell based therapies will play a significant role in spine surgery in the future.

Goldschlager, T., Jenkin, G., Ghosh, P., Zannettino, A., & Rosenfeld, J. (2010). Potential applications for using stem cells in spine surgery. CURRENT STEM CELL RESEARCH & THERAPY, 5(4), 345-355. doi: 10.2174/157488810793351686

Initial Clinical Experience with The Use of Human Amniotic Membrane Tissue During Repair of Posterior Tibal and Achilles Tendon

The demonstrated anti-adhesive, anti-inflammatory and anti-microbial properties of amniotic membrane tissue make this a potentially unique alternative to biologically inert collagen matrix products currently available for use in foot and ankle surgery and possible for tendon repair surgery of the upper extremities.

Jay, R. (n.d.). Amniotic Tissue. Retrieved from Single Source Surgical: http://www.singlesourcesurgical.com/wp-content/uploads/2014/01/amniotic-tissue.pdf

Applying Stem Cells to Orthopaedic Conditions: using bone marrow stromal cells to treat nonunions and osteonecrosis

... of infection, makes stem cell therapy appealing to both patients and physicians. Although not yet widespread in orthopaedics, the use of adult stem cells to address...

Kelly, F. B., & Porucznik, M. A. (2014). Applying stem cells to orthopaedic conditions: Using bone marrow stromal cells to treat nonunions and osteonecrosis. AAOS Now, , 1.

Effects of Human Amniotic Fluid on Fracture Healing in Rat Tibia

Human amniotic fluid (HAF), including hyaluronic acid (HA) and several growth factors, has been used experimentally in tendon, nerve, and cartilage regeneration and in bone defects because of its positive stimulating effects on regeneration potential. This study was performed to investigate whether HAF was effective on fracture healing.

Kerimoğlu, S., Livaoğlu, M., Sönmez, B., Yuluğ, E., Aynacı, O., Topbas, M., & Yarar, S. (2009). Effects of human amniotic fluid on fracture healing in rat tibia. Journal of Surgical Research, 152(2), 281-287. doi: 10.1016/j.jss.2008.02.028

Summary of Clinical Outcome Related to The Use of Human Amnion Soft Tissue Allograft in Right L4-L5 Decompression Procedure

The use of the nonadherent barrier significantly reduced both scar tissue formation and adherence to the underlying dura in this patient. The lack of scar tissue and associated plane preservation between the dural sac and the surrounding soft tissue significantly decreased the operative time required to perform the revision procedure.

Ploska, P. (2010) Summary of Clinical Outcome Related to The Use of Human Amnion Soft Tissue Allograft in Right L4-L5 Decompression Procedure. Retrieved from Single Source Surgical: http://www.singlesourcesurgical.com/wp-content/uploads/2014/01/amniotic-tissue.pdf

Implantation of Amniotic Membrane to Reduce Postlaminectomy Epidural Adhesions

Postlaminectomy epidural adhesion is implicated as a main cause of "failed back surgery syndrome" and associated with increased risk of complications during revision surgery. Various materials acting as mechanical barriers to reduce fibroblasts infiltration into epidural space have met with limited success. In present research, amniotic membrane (AM) was studied to investigate its effects on reducing epidural scar adhesion after laminectomy in a canine model. Laminectomy sites were created at L-1, L-3, L-5, and L-7 levels in 24 adult mongrel dogs. Freeze dried AM (FAM), cross-linked AM (CAM), and autologous free fat (AFF) were implanted, respectively, at a randomly assigned site in each dog with the remaining untreated site serving as internal control. The animals were sacrificed at 1, 6, and 12 weeks postoperatively. Then, gross pathologic observation including scar amount and adhesion tenacity, qualitative histology evaluation, and quantitative histology analysis were compared. Gross observation demonstrated that scar amount and adhesion tenacity of CAM group were significantly lower in comparison with those of FAM and non-treatment groups. A white, slightly vascularized CAM layer covered the dura mater without tenacious scar adhesion. The histology analysis also indicated reduced fibroblasts infiltration and consequent epidural fibrosis, which were similar to the results of AFF group. In conclusion, the CAM is effective in reducing epidural fibrosis and scar adhesion after laminectomy in canine model. It is a promising biomaterial for future clinical applications.

Tao, H., & Fan, H. (2009). Implantation of amniotic membrane to reduce postlaminectomy epidural adhesions. European Spine Journal : Official Publication of the European Spine Society, the European Spinal Deformity Society, and the European Section of the Cervical Spine Research Society, 18(8), 1202-1212. doi: 10.1007/s00586-009-1013-x

Mesenchymal Stem Cells for Bone Repair and Metabolic Bone Diseases

Human mesenchymal stem cells offer a potential alternative to embryonic stem cells in clinical applications. The ability of these cells to self-renew and differentiate into multiple tissues, including bone, cartilage, fat, and other tissues of mesenchymal origin, makes them an attractive candidate for clinical applications. Patients who experience fracture nonunion and metabolic bone diseases, such as osteogenesis imperfecta and hypophosphatasia, have benefited from human mesenchymal stem cell therapy. Because of their ability to modulate immune responses, allogeneic transplant of these cells may be feasible without a substantial risk of immune rejection. The field of regenerative medicine is still facing considerable challenges; however, with the progress achieved thus far, the promise of stem cell therapy as a viable option for fracture nonunion and metabolic bone diseases is closer to reality. In this review, we update the biology and clinical applicability of human mesenchymal stem cells for bone repair and metabolic bone diseases.

Undale, A. H., Westendorf, J. J., Yaszemski, M. J., & Khosla, S. (2009). Mesenchymal stem cells for bone repair and metabolic bone diseases. Mayo Clinic Proceedings, 84(10), 893-902. doi: 10.4065/84.10.893

Birth Tissue/Ankle Tendon Repair Study Released

The white, slighlty vascularized membrane was found between the dura matter and surrounding tissues to reduce scar intrusion. Furthermore, the CAM layer seldom adhered to the dura mater and was easily removed.

Young, R. (2012) Birth Tissue/Ankle Tendon Repair Study Released. ORTHOPEDICS THIS WEEK - EXTREMITIES. Retrieved from Single Source Surgical: http://www.singlesourcesurgical.com/wp-content/uploads/2014/01/amniotic-tissue.pdf

Using Birth Tissue in Spine Surgery

Fascia is one of the most important covering materials in the body and serves to protect virtually every structure in the body—bones, nerves, muscles, tendons, organs, the spinal cord and the brain. So when trauma or surgery disrupts that natural, protective fascia covering, amniotic membranes are structurally and by composition, extremely similar if not precise transplants

Young, R. (2012, August 20). Using Birth Tissues in Spine Surgery. Retrieved from Orthopedics This Week: http://ryortho.com/2012/08/using-birth-tissues-in-spine-surgery/

Anti-Aging

Inflammation Links Aging to the Brain

The authors go on to show that this feed-forward loop leads to epigenetic changes (chemical and structural modifications that alter gene expression without changing the DNA sequence) in the gene that encodes gonadotropin-releasing hormone (GnRH), leading to its reduced expression. [...]their studies in mice suggest that reduced hypothalamic release of GnRH could contribute to several systemic attributes of ageing, including declining muscle strength, skin atrophy, bone loss, reduced neurogenesis and memory impairment. A decrease in gonadal sex steroids is a well-established marker of ageing, but many other hormonal changes occur as well; and some of these ageregulated hormones (such as dehydroepiandrosterone) also regulate inflammation and other immune responses. [...]interplay between the hormonal and immune systems occurs at multiple levels.

Gabuzda, D., & Yankner, B. A. (2013). Inflammation links ageing to the brain. Nature, 497(7448), 197.

Aging

Overview of Human Aging

Ganz, P. (2014) Aging. Retrieved from http://www.goldlabcolorado.com/2014/Ganz_slides.pdf

Inflammation Links Aging to the Brain

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decrease in gonadal sex steroids is a well-established marker of ageing, but many other hormonal changes occur as well; and some of these ageregulated hormones (such as dehydroepiandrosterone) also regulate inflammation and other immune responses. [...]interplay between the hormonal and immune systems occurs at multiple levels.

Gabuzda, D., & Yankner, B. A. (2013). Inflammation links ageing to the brain. Nature, 497(7448), 197.

Other Therapies

Amniotic Fluid as a Rich Source of Mesenchymal Stromal Cells for Transplantation Therapy

Stem cells isolated from amniotic fluid are known to be able to differentiate into different cells types, thus being considered as a powerful tool for cellular therapy of different human diseases. In the last 4 years, amniotic fluid-derived stem (AFS) cells have been shown to express embryonic and adult stem cell markers. These cells can be considered an intermediate stage between embryonic stem cells and adult stem cells. AFS cells can give rise to adipogenic, osteogenic, myogenic, endothelial, neurogenic, and hepatic lineages, inclusive of all embryonic germ layers. AFS cells have a high renewal capacity and can be expanded for over 250 doublings without any detectable loss of chromosomal telomere length. Taken together, all these data provide evidence that amniotic fluid represents a new and very promising source of stem cells for research, as well as clinical applications. Certainly stem cells from amniotic fluid will be useful both for a customized cell supply for newly born children and for banking cells to be used for therapeutic cell transplantation in immunogically matched recipients. Further investigations are also warranted to fully explore the amniotic cells' potential for adult human disorders.

Antonucci, I., Stuppia, L., Kaneko, Y., Yu, S., Tajiri, N., Bae, E. C., . . . Borlongan, C. V. (2011). Amniotic fluid as a rich source of mesenchymal stromal cells for transplantation therapy. Cell Transplantation, 20(6), 789-795. doi: 10.3727/096368910X539074

Culture of Human Amniotic Fluid Stem Cells in 3D Collagen Matrix

Most of the researchers attribute amniotic fluid stem cells (AF SCs) to mesenchymal stem cells (MSCs). However, AF SCs express both mesenchymal and epithelial markers, which distinguishes them from postnatal MSCs. Cultivation in the three-dimensional (3D) matrix provides a different look at the nature of the cells. We showed that in 3D collagen gel AF SCs form epithelial structures (tubules and cysts). The active contraction of the gel during the first days of cultivation, which is characteristic of mesenchymal cells, does not occur. Electron microscopic study showed that adherent junctions typical to epithelial cells are formed between AF SCs. On the other hand, during culturing in the gel AF SCs continue to express MSCs markers. Thus, AF SCs may be not true mesenchymal cells because they can display properties of epithelial cells. Perhaps these cells undergo epithelial-mesenchymal transition, a process which actively takes place during embryogenesis.

Davydova, D. A., Vorotelyak, E. A., Bragina, E. E., Terskikh, V. V., & Vasiliev, A. V. (2011). Culture of human amniotic fluid stem cells in 3D collagen matrix. Cell and Tissue Biology, 5(4), 339-345. doi: 10.1134/S1990519X11040031

Isolation of Amniotic Stem Cells Lines with Potential for Therapy

Stem cells capable of differentiating to multiple lineages may be valuable for therapy. We report the isolation of human and rodent amniotic fluid-derived stem (AFS) cells that express embryonic and adult stem cell markers. Undifferentiated AFS cells expand extensively without feeders, double in 36 h and are not tumorigenic. Lines maintained for over 250 population doublings retained long telomeres and a normal karyotype. AFS cells are broadly multipotent. Clonal human lines verified by retroviral marking were induced to differentiate into cell types representing each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic lineages. Examples of differentiated cells derived from human AFS cells and displaying specialized functions include neuronal lineage cells secreting the neurotransmitter L-glutamate or expressing G-protein-gated inwardly rectifying potassium channels, hepatic lineage cells producing urea, and osteogenic lineage cells forming tissue-engineered bone.

De Coppi, P., Bartsch, J., Georg, Siddiqui, M. M., Xu, T., Santos, C. C., Perin, L., . . . Atala, A. (2007). Isolation of amniotic stem cell lines with potential for therapy. Nature Biotechnology, 25(1), 100-106. doi:10.1038/nbt1274

Effects of Platelet Growth Factors on Human Mesenchymal Stem Cells and Human Endothelial Cells In Vitro

The aim of the present in vitro study has been to investigate the effects of a enriched platelet derived growth factors on proliferation and migration of human endothelial and mesenchymal stem cells and on osteogenic differentiation of stem cells. Platelet rich plasma has been produced, yielding a four time higher number of thrombocytes than normal plasma. Degranulation of platelets has been performed by means of calcium and thrombin. Plasma has served as a control, whereas plasma in combination with calcium and thrombin was used to distinguish the difference between calcium and/or thrombin mediated effects and growth factor induced effects on the cells. The observed enhanced proliferation and migration of endothelial cells towards the platelet derived growth factors was driven by the plasma component of these preparations. However PDGF solely stimulated the migration and proliferation of mesenchymal stem cells. The increased osteogenic differentiation of growth factor treated mesenchymal stem cells was mostly driven by the high level 4 calcium used for the platelets degranulation. In summery, the different components of platelet derived growth factors work together to influence human endothelial and mesenchymal stem cells. This is of special clinically interest regarding the stimulation of bone healing in orthopaedic and traumatic surgery.

Kilian, O., Flesch, I., Wenisch, S., Taborski, B., Jork, A., Schnettler, R., & Jonuleit, T. (2004). Effects of platelet growth factors on human mesenchymal stem cells and human endothelial cells in vitro. European Journal of Medical Research, 9(7), 337-344.

Effect of Basic Fibroblast Growth Factor and Alpha-Melanocytic Stimulating Hormone on Nerve Regeneration Through a Collagen Channel

An experimental study on the rat sciatic nerve was performed to evaluate nerve regeneration through a collagen guide and to study the effects of -melanocytic stimulating hormone (-MSH) and basic fibroblast growth factor (b-FGF) in accel erating axonal elongation. After transection, nerves were repaired over a 7 mm gap using a placental collagen type IV guide. The channel was filled with either a b-FGF solution or an -MSH

solution or was produced with b-FGF incorporated into the guide. Four weeks later, only

groups in which b-FGF had been injected or incorporated displayed a significant somatosensory evoked potential response. Histological and quantitative analysis of nerve fibres confirmed the existence of nerve continuity in groups receiving an -MSH solution or a channel containing b-FGF. These results demonstrate that -MSH in solution and b-FGF incorporated into a collagen type IV channel enhance peripheral nerve regeneration. However, at 4 weeks, only b-FGF (3 ng) restores functional activity.

LAQUERRIERE, A., PEULVE, P., JIN, O., TIOLLIER, J., TARDY, M., VAUDRY, H., . . . TADIE, M. (1994). EFFECT OF BASIC FIBROBLAST GROWTH-FACTOR AND ALPHA-MELANOCYTIC STIMULATING HORMONE ON NERVE REGENERATION THROUGH A COLLAGEN CHANNEL. Microsurgery, 15(3), 203-210. doi: 10.1002/micr.1920150312

Properties of the Amniotic Membrane for Potential Use in Tissue Engineering

An important component of tissue engineering (TE) is the supporting matrix upon which cells and tissues grow, also known as the scaffold. Scaffolds must easily integrate with host tissue and provide an excellent environment for cell growth and differentiation. Most scaffold materials are naturally derived from mammalian tissues. The amniotic membrane (AM) is considered an important potential source for scaffolding material. The AM represents the innermost layer of the placenta and is composed of a single epithelial layer, a thick basement membrane and an avascular stroma. The special structure and biological viability of the AM allows it to be an ideal candidate for creating scaffolds used in TE. Epithelial cells derived from the AM have the advantages of stem cells, yet are a more suitable source of cells for TE than stem cells. The extracellular matrix components of the basement membrane of the AM create an almost native scaffold for cell seeding in TE. In addition, the AM has other biological properties important for TE, including anti-inflammatory, anti-microbial, anti-fibrosis, anti-scarring, as well as reasonable mechanical property and low immunogenicity. In this review, the various properties of the AM are discussed in light of their potential use for TE.

Niknejad, H., Peirovi, H., Jorjani, M., Ahmadiani, A., Ghanavi, J., & Seifalian, A. M. (2008). Properties of the amniotic membrane for potential use in tissue engineering. European Cells & Materials, 15, 88-99.

Pigs' Bladder Helps Patients' Stem Cells Grow Missing Muscles

Putic, G., (2014, August 04) Pigs' Bladder Helps Patients' Stem Cells Grow Missing Muscles. Retrieved from Voice of America News: http://www.voanews.com/content/pig-bladder-helps-patients-stem-cells-grow-missing-muscles/1971525.html

Amniotic Fluid Stem Cells: Future Perspectives

The existence of stem cells in human amniotic fluid was reported for the first time almost ten years ago. Since this discovery, the knowledge about these cells has increased dramatically. Today, amniotic fluid stem (AFS) cells are widely accepted as a new powerful tool for basic research as well as for the establishment of new stem-cell-based therapy concepts. It is possible to generate monoclonal genomically stable AFS cell lines harboring high proliferative potential without raising ethical issues. Many different groups have demonstrated that AFS cells can be differentiated into all three germ layer lineages, what is of relevance for both, the scientific and therapeutical usage of these cells. Of special importance for the latter is the fact that AFS cells are less tumorigenic than other

pluripotent stem cell types. In this paper, we have summarized the current knowledge about this relatively young scientific field. Furthermore, we discuss the relevant future perspectives of this promising area of stem cell research focusing on the next important questions, which need to be answered.

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Biochemical and Biological Characterization of a Crude Growth Factor Extract (EAP) From Human Term-Placental Tissue

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Regenerative Medicine Market on Growth Spurt

According to a new market research report published by Transparency Market Research, the regenerative medicine (bone and joint) market was valued at $2.6 billion in 2012 and is estimated to reach a market worth of $6.5 billion by 2019. That is a growth rate of 12.8% from 2013 to 2019...Young, B. (2013, July 30).

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Human Maternal Placentophagy

Maternal placentophagy, although widespread among mammals, is conspicuously absent among humans cross-culturally. Recently, however, advocates for the practice have claimed it provides human postpartum benefits. Despite increasing awareness about placentophagy, no systematic research has investigated the motivations or perceived effects of practitioners. We surveyed 189 females who had ingested their placenta and found the majority of these women reported perceived positive benefits and indicated they would engage in placentophagy again after subsequent births. Further research is necessary to determine if the described benefits extend beyond those of placebo effects, or are skewed by the nature of the studied sample.

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