2007

Osteoarthritis: clinical evaluation and cartilage tissue engineering

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by

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A dissertation submitted to the graduate faculty in partial

fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Biomedical Sciences (Cell Biology)

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Ames, Iowa

2007

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ACKNOWLEDGEMENTS

I would like to express my gratitude toward all those that collaborated on the projects in this dissertation. Special thanks to Heather Greenlee, my major professor, who welcomed me into her laboratory half way through my program. Additionally, I would like to extend my appreciation to Mike Conzemius who believed in my potential, provided research mentorship, monetary support, and created multiple opportunities for career development during my graduate education. Finally, I am grateful for the cooperation and expertise of my committee.

ABSTRACT

Osteoarthritis (OA) is the 2nd leading cause of long term disability in North America affecting 40 million Americans. The work presented in this manuscript addresses clinical outcome measures used to determine treatment effect and 2 *in vitro* studies using tissue engineering techniques to grow cartilage suitable for *in vivo* use. In the first study, radiographs of the stifle joint in dogs were scored for the severity of OA, and limb function was measured by force platform gait analysis. The 2 outcome measures were analyzed using regression methods. There was no correlation of the severity of radiographic OA and limb function; therefore, radiographs should not be used as a measure of functional outcome for the treatment of OA. Additionally, treatment for OA using tissue engineered tissue is a current research paradigm. The second study in the manuscript hypothesized that tantalum trabecular metal was chondroconductive. Canine chondrocytes were cultured *in vitro* under dynamic conditions. Histopathologic evaluation showed matrix stained with toluidine blue and safranin O. Biochemical evaluation of the exterior portion of cartilage showed 84% type II collagen. The results reflected the chondroconductive potential of the unique metal. In order to optimize the medium used for in vitro cartilage production, a pellet culture model was used to determine the effect of Fibroblast Growth Factor 2 (FGF2) followed by Transforming Growth Factor β1 (TGFβ1) on matrix production. Although TGFβ1 treated cultures were larger than the others, FGF2 treatment in monolayer decreased matrix production. Additionally, type II collagen was insufficient in all groups. Further study is needed to define the ideal culture requirements to produce cartilage from canine chondrocytes consistently with ideal matrix properties.

CHAPTER 1. INTRODUCTION

INTRODUCTION

Osteoarthritis (OA) affects 40,000,000 Americans, is the second most common cause for long term disability, and is expected to affect 18.2% of the population by 2020.[1] Similarly, 20% of the canine pet population show clinical signs of OA, and OA is the leading cause of euthanasia in military working dogs.[2,3,4] Regardless of species, osteoarthritis is caused by a disturbance in the homeostasis of normal cartilage. This interruption to homeostasis can be caused by trauma, joint instability, infectious or noninfectious inflammation or focal or generalized malformation of the articular surface. The pathogenesis of OA is complicated affecting multiple tissues and responses to injury. A cascade of inflammatory mediators and other factors affect the subchondral bone, cartilage surface, and synovial membrane. The disease is perpetuated over time causing degeneration of the joint and subsequent pain.

Clinically, radiographs are commonly used in both human and veterinary medicine to diagnose and monitor OA. Unfortunately, radiographic signs of disease are nonspecific (e.g. showing osteophytes), lack in sensitivity because they do not evaluate cartilage and may lag behind the progression or initiation of OA. Nonetheless, radiography is used as an outcome measure to distinguish between treatment modalities.

Treatment of OA can be frustrating and end in ultimate failure. The current research paradigm for development of new treatment methodologies includes cartilage tissue engineering for joint resurfacing. The premise for using tissue engineered cartilage for treating OA includes replacing damaged cartilage or biomechanically inferior repair tissue with either the building blocks for in vivo repair or in vitro generated normal cartilage.

THESIS ORGANIZATION

This dissertation addresses both the clinical and the basic sciences of OA. Chapters are 2-4 are organized as journal articles with an abstract, introduction, materials and methods, results, and discussion. The reproduction of this material in this thesis is done with permission from the publisher.

Chapter 2 of this thesis investigates the correlation between radiography and the function of osteoarthritic limbs. This study is published in *Veterinary Surgery* with permission from the publisher. The first author is the graduate student and primary author followed by the associate professor and those that contributed to data collection or statistical analysis.

Chapter 3 focuses on the properties of a metal with a unique surface as a scaffold for engineering articular cartilage. This is the first step in developing a cartilage based implant capable of replacing the joint surface. This study has also been published in *Journal of Biomedical Materials Research Part B: Applied Biomaterials*. The first author is the graduate student and primary author followed by the associate professor and those that provided collaboration making the project possible.

In addition to the importance of scaffolds, growth factors are imperative to develop mature, healthy, biomechanically stable cartilage. Chapter 4 investigates the best growth factors for extracellular matrix production using canine chondrocytes. The first author is the graduate student and primary author followed by those that contributed to the design of the experiment. The major professor is the last author.

LITERATURE REVIEW

Osteoarthritis

Articular or hyaline cartilage is the tissue that lines the joint surface. It is a connective tissue with low cellularity made up of predominantly type II collagen, glycosaminoglycans, and proteoglycans. These unique molecules give articular cartilage the mechanical properties necessary to support joint range of motion and the transfer of

force from one bone to another. The tissue is connected to the subchondral bone through a calcified cartilage layer. Blood vessels do not penetrate the subchondral bone; therefore, the chondrocytes receive nutrition via diffusion from synovial fluid. Unfortunately, the combination of the absence of a direct blood supply and the low cellularity of the tissue contributes to the limited capacity for repair of articular cartilage. Injuries that penetrate the subchondral bone undergo repair through the formation of tissues usually characterized as fibrous, fibrocartilagenous, or hyaline-like, depending upon the species, age of the animal, and the location and size of the injury.[5-7] However, these reparative tissues, including those that resemble hyaline cartilage histologically, differ from normal hyaline cartilage both biochemically and biomechanically, and by six months, fibrillation, fissuring, and extensive changes occur in the reparative tissues of approximately half of the full-thickness defects.[6,7] This often leads to secondary osteoarthritis resulting in pain and disability.

Osteoarthritis (OA) affects 40,000,000 Americans, is the second most common cause for long term disability, and is expected to affect 18.2% of the population by 2020.[1] Eighty percent of patients diagnosed with OA report limited ability and 25% cannot perform major daily activities.[8] Numerous surgical and medical treatments are used to treat OA; including autologous chondrocyte implantation, debridement arthroplasty, and conservative management (including weight loss, moderate exercise, and non-steroidal anti-inflammatory medication). Total joint arthroplasty, is the final treatment option for OA that has been resistant to other therapies.

Currently Available Treatments

Treatments have been developed to prevent or delay total joint arthroplasty. Initial treatment options for OA include transplantation of whole joints, partial joints, osteochondral segments, and autologous chondrocyte implantation.[9,10] The success of each of these techniques is limited. Although whole or partial joint transplantation may be used for any size defect, it requires a healthy cadaver donor that is size matched. Additionally, there is risk of disease transmission and rejection of the transplanted tissue. Decreased cell viability at the time of transplantation is also an issue creating morbidity.[10]

Osteochondral autografts or mosaicplasty is a procedure requiring harvest of a cartilage and subchondral bone graft from a portion of a joint with minimal weight bearing and transplantation to the affected portion. The disadvantages of this procedure include donor site degeneration and pain, insufficient graft to harvest, and ideally limitation to 1-2 cm lesions.[10] Additionally, the transplanted cartilage does not integrate with the surrounding cartilage.[11]

Autologous chondrocyte implantation requires an initial surgery to harvest chondrocytes by removing 200-300 mg of cartilage from a portion of the joint with minimal weight bearing.[10] The chondrocytes are released from the matrix and proliferated in culture. In a second surgery, the cells are placed into the defect under a periosteal flap sealed with fibrin. At first, repair tissue forms more quickly than control defects; however, integration with surrounding cartilage as well as biomechanical stability of the tissue remains problematic.[9,12] Additionally, this method has limited clinical application in the repair of large or multiple defects of articular cartilage because of the limited availability of donor material.[9,10,13]

Joint resurfacing with tissue engineered materials is a relatively new paradigm. Although preliminary results have shown promise in animal models, full regeneration of the normal cartilage surface has not been accomplished.

When the pain and debilitation of OA returns, patients are left with the choice of arthrodesis or arthroplasty. Arthrodesis entails fusion of the joint eliminating motion and limiting function. Total joint arthroplasty effectively replaces the articulating surfaces of a joint with synthetic materials designed to preserve range of motion providing a more favorable clinical outcome.

Total Joint Arthroplasty

Total joint arthroplasty is performed nearly 500,000 times per year in the hip alone, and its frequency increased 50% over 13 years as the mean age and activity of the general population increases in North America.[14,15] Arthroplasty generally restores function; however, the rate of total hip arthroplasty revision surgeries is up to 30% with

only 72% of implants surviving 10 years.[16] Aseptic loosening (ASL) is the leading cause of surgical revision (70% of total hip revision, 44% of total knee revisions).[17]

Aseptic loosening occurs because of the patient's natural remodeling response to a foreign material that has been surgically introduced. The foreign materials (metals, plastics, and ceramics) that currently represent the articular surface of all joint arthroplasty systems shed microscopic wear debris that cause an inflammatory response activating the cells that resorb bone (osteoclasts and macrophages) and suppress osteoblastic (bone forming) activity. Wear debris activates macrophages through several mechanisms. In turn, macrophages secrete interleukin 1 (IL1), tumor necrosis factor alpha (TNFα), prostaglandin E2 (PgE2), and interleukin 6 (IL6).[17,18] These cytokines inhibit osteoblastic bone forming activity and stimulate osteoclastic differentiation and activity. The overall effect is resorption of the bone-implant interface and ultimately an unstable implant.

Research has improved materials leading to a reduction in wear particles, but the rate of ASL has not significantly decreased. Although metal on metal or ceramic on ceramic implants produce less debris, metal debris can be carcinogenic, affect intellectual capacity, or decrease hematopoesis.[17] Ceramic particles have caused cytokine release from macrophages in vitro.[17] Zirconia ceramics may also be directly cytotoxic.[17] Other improvements in materials include porous ingrowth surfaces or hydroxyapatite coatings.[19] Hydroxyapatite lowers the histologic migration or wear debris, but has not been evaluated clinically in humans. Other efforts have been focused on improving high molecular weight polyethylene (commonly used in arthroplasty).[17,18] There is evidence to suggest that decreasing the oxidation of polyethylene will decrease the amount and reaction to debris.[17,18]

The fields of tissue engineering research and regenerative medicine pursue a solution to the problem by preventing the need for total joint arthroplasty by repairing focal defects or replacing the implant material interface with a cartilage on cartilage interface. This latter option would eradicate wear debris thereby eliminating or significantly reducing ASL.

Canine Model

The canine model is an appropriate clinical and laboratory model for several reasons. First, OA is a naturally occurring disease in the canine affecting 1 in 5 adult dogs, and causing disability in 62% of dogs affected.[14,15] Secondly, the canine is already an established model for hip arthroplasty, aseptic loosening and OA.[20-22] Joint articulation angles and range of motion are similar to humans. Although cattle and sheep are also used as models for arthroplasty because of larger joints, housing, handling, and expense are distinct disadvantages. The rabbit is commonly used to model cartilage tissue engineering due to housing and cost issues; however, the joint angles are not similar to humans, and articular cartilage is only <0.5 mm compared to 2.5 mm on average in human cartilage.[21,23] Dogs have articular cartilage thickness that is greater at 1 mm in depth.[24] The main disadvantage of a quadrupedal model is that it may not accurately represent bipedal loading. This is only alleviated by the use of non-mammals such as birds or non-human primates.

Another advantage of using a canine animal model is that clinical outcomes can be assessed objectively. Although it is often ignored, clinical outcome is as important and histologic or biochemical outcome when designing a treatment for OA.[24] Gait analysis is an established and accepted method of measuring clinical outcome related to the orthopedic procedures in dogs.[25,26] Objective clinical outcome measures of lameness are not commonly practiced in rabbits, cattle, or sheep.

Clinical Outcome Measures

There are several outcomes of OA treatment that are detectable from a variety of methods. However, from a patient's perspective, the desired outcome would be decreased pain and increased joint function. Evaluation of pain is subjective and variable. A visual assessment score (VAS) is a 10 mm line that represents a continuum from no pain to the most pain possible. The patient or pet owner marks on the line accordingly. Numerical rating scales (NRS) are similar except these scoring systems use a numerical score for specific aspects of pain or lameness.[27,28] This becomes more complicated when evaluating dogs that cannot verbally communicate with people. Instead, the owner or

veterinarian must use behavioral cues to determine the pain of the animal. The inherent variation produced with subjectivity compounded by difficulty interpreting canine behavior is not ideal for determining clinical outcomes.

Although pain cannot be truly known in animals, it is reasonable that degree of lameness detected would be correlated to pain. Force platform gait analysis is a well established method of determining limb function in human and veterinary medicine. More specifically, force platform gait analysis has been used to determine improvements in limb function after both surgical and medical interventions.[29-31] This outcome measure is also objective and non-invasive.

Although force platform gait analysis is easily performed, strict control of certain variables must be maintained in order to limit variation. In general, a valid trial is described as one in which the dog places a forelimb on the platform followed by the ipsilateral hindlimb without concurrent placement of contralateral paws. Additionally, velocity and acceleration are maintained within 0.3 m/s and 0.5m/s^2, respectively whether the gait chosen is at a walk or trot.[29,32,33] Five trials are collected for each dog. The number of trials was determined with mathematical modeling techniques using the data from normal dogs by Jevins in 1993, and has become convention for studies involving lame dogs.[29,31,33,34] Although the data used for determining the trial number is based on normal dogs, dogs with chronic OA have low intradog coefficients of variation.[35]

Additionally, the intraclass correlation using peak vertical force in lame dogs was 96.5%. (Unpublished data) This means that the intradog variation is much lower than the variation from dog to dog. Intraclass correlation was used to determine the estimate of the standard deviation. The estimate of standard deviation was relatively unchanged when using 5 and 50 trials (6.22% and 6.20%, respectively).

Additional sources of variation include handler, dog acclimation, day to day variation, and subject morphology.[36-38] Variation induced by different handlers can also be attenuated by experience.[34] However, subject morphology is a significant source of dog to dog variation in ground reaction forces.[33,39,40] To account for these differences, the ground reaction forces are standardized by subject weight.[33,39] Comparing the change in ground reaction forces from pre- to post-treatment values reduces this source of

variation further.[41] Additionally, block randomization may be used to evenly distribute dogs of different morphologies to treatment groups.

Radiographic evidence of OA and its progression is commonly used as an outcome measure in human and veterinary medicine.[27,42-45] Plain film radiography is routinely performed, takes minimal time, is relatively inexpensive and non-invasive. Disadvantages include variation in positioning and radiograph quality, subjectivity of evaluation, and x-ray exposure. Additionally, because radiographs are routinely performed for surgical follow-up, retrospective studies commonly use radiographic data for outcome.[42-45]

Radiographic data can be obtained through descriptive means or by using published scales to assign a number to the aspect being evaluated.[43] In osteoarthritic joints, swelling or joint effusion, subchondral sclerosis, osteophytosis and enthesiophytosis are often assessed as part of the scoring system.

Joint collapse is regarded in the human literature as an accurate measure of cartilage structural integrity.[46] However, controversy surrounds radiographic evidence of joint collapse because variation in positioning commonly causes artifactual radiographic collapse.[47] Additionally, the preferred views are not standardized, and there is high interobserver variation when assessing collapse.[46] This is accentuated when the limb is not weight bearing when radiographed, as occurs most often with veterinary patients.

Intuitively, since radiographs are an indicator of pathology, radiographic evidence of OA should correlate to pain and disuse. However, radiographic signs do not change as rapidly as other modes of detection (i.e. magnetic resonance imaging).[36,47,49] Nevertheless, the correlation of radiographic severity of OA to the pain and function remains controversial.[27,35,49-51] Several authors in the veterinary literature have commented on the apparent disconnect between radiographic severity and subjective evaluation of the patient's condition.[29,36,43,45,48,50-54] Furthermore, many noted an increase in radiographic severity of OA while subjectively noting functional improvement.[43,45,50,52] Despite common use as an indicator of disease, the presence of osteophytes is not correlated with subjective evaluation of pain.[27] In contrast, Evers et al. found a good correlation between radiographic score and limb function in the dogs with OA in the

hip.[51] The relationship between radiographic OA and limb function has not been objectively evaluated using force platform gait analysis.

Future Treatment: Joint Resurfacing

Regenerative medicine is an emerging field of research that includes the application of molecular and tissue engineering techniques to promote tissue repair with functional, and adaptable tissue instead of replacement with scar tissue. One approach to regenerating the cartilage surface of a joint is by combining cells with a 3-dimensional matrix. There are 3 major aspects to this approach: cell type, scaffolding, and culture conditions.

Cell Type and Source

The cell type most suitable for cartilage regeneration is debatable. The most commonly utilized cells for cartilage tissue engineering are chondrocytes and mesenchymal stem cells (MSCs) which show promise for rapid extracellular matrix production.[9,10,13,55-87] Mesenchymal stem cells can be harvested from the umbilical cord, bone marrow, and adipose tissue.[88] Each type of MSC has different characteristics. Umbilical cord MSCs are more difficult to successfully harvest than MSCs or adipose but they have greater capacity to proliferate prior to senescence.[88] Bone marrow derived MSCs senesce after fewer passages than the other 2 types.[88]

Mesenchymal Stem Cells can be harvested with minimal morbidity to the patient, and they can be easily proliferated to appropriate numbers in vitro for reimplantation.[55] However, the number of MSCs per 100,000 nucleated bone marrow cells decreases from 1 in a newborn to 0.1-0.05 in an 80 year old human.[89] Another disadvantage of MSCs is the time needed to proliferate and differentiate the cells.

Chondrocytes also have a finite number of passages prior to senescence.[57] Chondrocytes can be harvested from the ribs, articular surface, auricular cartilage and nasal septum.[90] As with the MSCs, there are differences in chondrocytes from different locations. For example, auricular and nasal chondrocytes produce elastic collagen in addition to the more desirable collagen type II.[90] However, chondrocytes harvested from

the nasal septum and ribs have produced more collagen type II and aggrecan than articular chondrocytes in direct comparisons.[90] Additionally, when using articular chondrocytes donor site morbidity may incite inflammation and exacerbate OA, and cells collected from osteoarthritic joints and/or the aged may not provide the same potential.[10,57,85] Despite the disadvantages, chondrocytes are a differentiated cell-type and have the potential for cartilage formation reducing the time needed to grow an implant. Chondrocytic potential is highlighted by the results of autologous chondrocyte implantation which shows short-term improvement in 62-89% of patients.[10]

The immunologic potential is also a factor when considering a cell type. Autografts are non-immunogenic, but allografts but may be used when adequate numbers cannot be harvested. Also, using allografts reduces the number of procedures required for the patient, and potentially decreases the time from diagnoses to surgical treatment.

The immunoregulatory role of MSCs is a potential advantage, although this use is controversial and likely dependent on the microenvironment.[55,91] There is in vitro evidence that MSCs inhibit proliferation of immune cells and cytokine excretion; however, they may also serve as antigen presenting cells stimulating T and B cell immunity.[91] Inflammatory cytokines may also enhance the immunomodulatory function of MSCs. In vivo, autologous MSCs help attenuate induced inflammatory reactions like experimental autoimmune encephalitis, but in contrast, fails to assuage collagen induced arthritis.[91]

Although chondrocytes are not immunosuppressive, they may be less antigenic than other cell types.[92,93] When chondrocytes were isolated from pathogen free mice, major histocompatibility complex (MHC) I and II were not expressed and only occasionally expressed in non-isolated mice.[92] These proteins play a large role in immune recognition of foreign cells. However, inflammatory stimulation by TNFα produced expression of both MHC I and II.[92] Cells could also present antigens for T cell stimulation although not as efficiently as antigen presenting cells.[92] Transplantation of chondrocytes in vivo has given mixed results. Several studies in rabbits with chondrocyte allograft transplantation show no response or transient inflammation that recedes.[93] Theoretically, this protection afforded allografts is due to a physical barrier to

immunologic surveillance by extracellular matrix or scaffold.[93] Additionally, it is proposed that exposed allografted chondrocytes would require only temporary pharmacologic immunosuppression while extracellular matrix was formed.[93]

Scaffold

The scaffold of a tissue engineered implant is nearly as important as the cellular component. In general terms, the ideal characteristics for a scaffold for osteochondral tissue engineering include allowing cell attachment, porous, allow diffusion of nutrients and bioactive molecules, allow integration with native tissue, provide appropriate cell signaling, and have appropriate mechanical characteristics for the site of implantation.[94] There are many scaffolds used in tissue engineering research. These range in strength, texture, porosity, and biointeraction. It is well documented that the interaction between cells and the extracellular matrix influences proliferation, differentiation, attachment and migration, and the effect of growth factors.[63,95,96] For example, cellular attachment via integrins regulates growth factor receptor activity and growth factors can be bound for later use in the extracellular matrix.[63,97,98]

Tantalum trabecular metal is made from elemental tantalum deposition onto a carbon fiber skeleton. The trabecular component refers to the multiple interconnecting pores which resemble cancellous or trabecular bone. The construct also, has the mechanical characteristics of cancellous bone and provides structural support while bone ingrowth is occurring.[99] Tantalum has been FDA approved as a surgical implant material for osteoconduction. The bulk porosity exceeds 80% which favors boney ingrowth while still maintaining sufficient mechanical strength to avoid collapse.[99] Tantalum trabecular metal has unparalleled mechanical and tissue compatible characteristics with a modulus of elasticity near that of cancellous bone. The modulus of elasticity is important because it is a measure of stiffness of the implant.[99] An implant that is too stiff creates a mismatch of the elastic modulus at the bone-implant interface is prone to failure.[100] Alternatively, other metal implants have a strength and stiffness that is 5-10X that of normal bone and to date chondroconductive polymeric implants are too weak for immediate load bearing.

Many of the polymeric-cell constructs provide excellent short-term healing compared to empty controls; however, long-term implants have proved inadequate, degrading over time.[12,56,101] One hypothesis is that the scaffolds are resorbed too quickly leaving vulnerable neocartilage unprotected from the stressful environment.[102,103] These stresses may be compounded by insufficient polymeric strength to resist cellular contraction in vivo. For instance, collagen is used commonly as a biomaterial and has been heavily researched as a delivery vehicle for chondrocytes.[66,74] However, chondrocytic or MSC contraction can shrink the polymeric implant by 50%.[66] In contrast, tantalum trabecular metal has sufficient strength to maintain its shape under physiologic stress. Additionally the tidemark, or subchondral bone, does not reform leaving the neocartilage without foundation.[102,103] Tantalum is non-resorbable and mechanically similar to bone conceivably increasing the probability of successful support for neocartilage long-term.

Culture Conditions

The ideal culture conditions for cartilage tissue engineering vary based on cell type, source and species.[9,21,57] However, these conditions can be categorized into 3 interrelated features of the total in vitro environment. The biochemical, physical and mechanical aspects of the environment play large roles in the success of tissue generation. Optimizing each in relation to the others is an important step in growing a consistent, quality product.

The biochemical environment appears to be the most researched aspect of the culture conditions relating on culture nutrients and growth factors. This is likely due to the complicated responses to growth factors that may fluctuate due to stage of differentiation, age of cells, passage number in vitro, mechanical and physical environment, and individual animal differences.[21,57] There are many different growth factors with the potential to initiate or enhance chondrogenesis.[60,61,68,69,82] The growth factors emphasized to play a role in differentiating MSCs or redifferentiating chondrocytes are FGF2, TGFβ, and IGF. The effect of growth factors published in the recent literature are summarized in Tables 1 and 2.

TGFβ has been heavily studied with regards to MSC differentiation as well as a tool to prevent dedifferentiation in cultured chondrocytes.[61,63,64,67,69,78,81,82] The value of TGFβ in adult chondrocytes is controversial.[64,67,78] Some studies show a dose dependant toxicity of TGFβ on chondrocytes.[67] Others show an increase in matrix production and maintenance of type II collagen production.[64,78] Additionally, differences likely exist in the induction of differentiation between isotypes of the cytokine. MSCs possess receptors for both TGFβ1 and TGFβ3. There is some evidence to suggest that TGFβ1 may predispose the newly differentiated chondrocytes to become hypertrophic leading to an osteoblastic lineage or apoptosis.[82] In conflict, more consistent levels of TGFβ1 may be protective against differentiating into an osteoblastic lineage.[60,63,82]

FGF has also shown the ability to differentiate MSCs into chondrocytes.[80,82] FGF2 added to the media of replicating MSCs has been shown to predispose them to a chondrocyte lineage once pelleted.[69,80,82] Chondrocytes respond to FGF2 by increasing matrix production in 3 dimensional culture and proliferating in monolayer.[74] In addition, FGF2 has been shown to increase healing in large defects in rabbit stifles.[104,105] In theory, the mechanism includes encouraging mitogenisis and differentiation of endogenous MSCs at the site of injury.[104,105] FGF2 has unique qualities that may benefit the speed or quality of extracellular matrix produced by either chondrocytes or MSCs.

Combinations of cytokines may be the most chondrocytic. IGF has been shown to enhance the effect of FGF2, TGFβ3, and TGFβ1 in MSC culture, although the actions of insulin-like growth factor (IGF) alone are debated in the literature.[59-61,69,77-82] Additionally, FGF2 supplemented medium during the proliferation of MSCs in monolayer has improved the matrix composition of pellets differentiated by TGFβ.[69] FGF2 in monolayer may also increase the time to senescence of adult chondrocytes in vitro as well as enhancing matrix formation after 3-dimensional culture.

Other major factors in the culture conditions that make a difference in response to growth factors include serum, dexamethazone, and ascorbic acid. Serum is a factor that adds a tremendous amount of variability to cell culture.[106-108] Differences between lots of serum are profound and require serum testing with the cells to establish an appropriate lot for culture maintenance.[94] Serum diminishes the ability of chondrocyte production of

extracellular matrix formation both in vivo and in vitro.[107] In monolayer, type I collagen expression is upregulated and collagen type II is downregulated in serum containing cultures.[108] Additionally, serum can enhance the toxicity associated with TGFβ1.[83] Another disadvantage of serum culture conditions includes potential increased immunogenicity of cultures placed in vivo. Chondrocytes cultured in fetal bovine serum can present the xenogenic proteins even after implantation in vivo.[109]

Dexamethasone and ascorbic acid are reported as required elements of extracellular matrix formation in vitro. Dexamethasone promotes redifferentiation and is commonly used in 3-dimensional serum-free culture.[74,107] Sekiya found dexamethasone had a dose dependent increase in sox9 which is a transcription factor that activates collagen type II gene expression.[110] However, Mizaki reports that dexamethasone inhibits the ability of TGFβ1 to upregulate collagen type II.[111] Furthermore, it can also induce apoptosis of chondrocytes even at doses in the range used for chondrocyte culture.[112,113]

Ascorbic acid is also commonly used in 3-dimensional culture. Collagen and aggrecan are increased in chondrocyte culture with ascorbic acid; however, the cells also showed signs of oxidative damage.[114] In monolayer, there is evidence that ascorbic acid increases proliferation rate and maintains phenotype.[115]

The physical environment affects the actions and reactions of cells in vitro. Chondrocytes in monolayer dedifferentiate and proliferate. Three-dimensional culture maintains differentiation and promotes matrix formation. Not all 3-dimensional culture systems are equally effective. Specific scaffolds add an additional element to 3-dimensional culture.[63,97,108] The affect of scaffolds on culture systems were discussed previously.

Cell seeding of a scaffold is important for even distribution of engineered matrix.[116] Typically initial high cell densities are required for cartilage formation in vitro due to vital cell to cell interactions.[116] Although not fully elucidated, paracrine signaling is a very important mechanism for signaling matrix production.

Oxygen tension and pH are also controlled to affect cell biosynthetic activity. In normal development, low oxygen tension promotes chondrocytic cell differentiation and

maintenance of cartilage tissue preventing mineralization. In vitro, both MSCs and chondrocytes reach senescence earlier with 21% oxygen versus 5% oxygen.[117] The benefits of low oxygen conditions may be due to a reduction in oxidative stress.[117] Although debated, there is also evidence that matrix formation is enhanced by an acidic environment. In vivo, cartilage does not have a direct blood supply which creates a low oxygen environment and pushes the pH toward acidity.

The mechanical stimulation of cells supports extracellular matrix formation from chondrocytes and differentiation of MSCs toward a chondrocytic lineage. Several different bioreactors are used to provide a dynamic stimulation in the field of cartilage tissue engineering including traditional stir flasks, direct perfusion, and rotating wall bioreactors.[117-119] Dynamic environments improve cell distribution and therefore matrix distribution over scaffolds.[106-117] As matrix increases, the diffusivity of the tissue decreases.[118] Providing a dynamic environment facilitates increased nutrient diffusion.[118] Additionally, chondrocytes and MSCs form matrix with improved biomechanical characteristics when cultured in a dynamic versus static environment.[119,120] More specifically, there is evidence that shear and compressive forces enhance the functional quality the neocartilage grown in vitro.[106,120,121]

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Table 1. The effect of growth factors used in monolayer culture for cartilage tissue engineering. [1]

Cell type	Species	Serum +/-	Cytokine	Dose (respective if >1 cytokine)	Positive Indicators	Negative Properties	Effect on 3D
Chondrocyte	Bovine (73), human (84)	+	FGF2	5ng/ml	+ proliferation (84)		+ wet weight (73), induced response to BMP2 (73)
Chondrocyte	Caprine (62), rat (76), ovine (57), equine (57), bovine (57)	+	FGF2	10ng/ml	+ proliferation (62,57)	+ coll III mRNA (76), coll II mRNA (76), - chondrocyte morphology (57)	become hypertrophic (57)
Chondrocyte	Caprine (62), chick (79)	+	FGF2	100ng/ml	+ proliferation (62,79)		
Chondrocyte	Human (71)	-	FGF2	100ng/ml	+ proliferation (71), + coll II mRNA (71)		
Chondrocyte	Human	-	FGF2, PDGF, EGF (27)	5ng/ml, 5ng/ml, 5 ng/ml	+ proliferation (27)	+ coll I production (27)	
Chondrocyte	Equine (57), bovine (57), ovine (57)	+	FGF2, TGFβ1	10ng/ml, 5ng/ml	+ proliferation (57)		
Chondrocyte	Caprine (62)	+	IGF1	10ng/ml		- proliferation (62)	
Chondrocyte	Caprine (62), chick (79)	+	IGF1	100ng/ml	+ proliferation (62,79) + coll II (62)	+ coll I (62)	
Chondrocyte	Human (70), canine (74)	+	PDGF, FGF2, TGFβ1	10ng/ml, 5ng/ml, 1ng/ml	+ proliferation (70,74)		
Chondrocyte	Equine (60,67,83), Caprine (62)	+	TGFβ1	5ng/ml	+ proliferation (62), + coll II (62), +GAG (83)	- proliferation (60), + coll I mRNA (67), less GAG if serum-free used (83)	sensitized to IGF (70)
Chondrocyte	Caprine (62)	+	TGFβ1	30ng/ml	+ proliferation (62), + coll II (62), + GAG (83)	+ coll I (62)	
Chondrocyte	Equine (83)	+/-	TGFβ1	1ng/ml	+ GAG (83)	less GAG than 5ng/ml (83)	
Chondrocyte	Equine (83)	+/-	TGFβ1	10ng/ml	+ GAG (83)	toxic changes with serum (83), less GAG than 5ng/ml (83)	
MSC	human (22), rabbit (22)	+	FGF2	unstated	+ proliferation (22), + time to senesence (22)		
MSC	Human (87)	+	FGF2	5ng/ml	+ immunosuppressive ability (87)	+ osteogenic differentiation (87)	

[1] The plus (+) and minus (-) symbols represent the presence or absence of serum or increasing (+) or decreasing (-) effect compared to controls. References appear in parenthesis. The following abbreviations are used: Collagen (coll), glycosaminoglycan (GAG), proteoglycans (PG), platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), transforming growth factor (TGF), collagen oligomatrix protein (COMP), and 3-dimensional (3D).

Table 1. (continued)

Cell type	Species	Serum +/-	Cytokine	Dose (respective if >1 cytokine)	Positive Indicators	Negative Properties	Effect on 3D
MSC	Human (68,69,87), chick (79)	+	FGF2	1ng/ml	+ proliferation (68,69,79,87) + sensitized to TGFβ1 (68,69)		
MSC	Human (69, 87)	+	FGF2	10ng/ml	+ proliferation over other doses (69,84), + immunosuppressive ability (87)	+ osteogenic differentiation (87)	
MSC	Human (68)	+	FGF2, EGF	1ng/ml, 1ng/ml	+ proliferation (68)	no reaction to EGF alone (68)	+sensitized to TGFβ1 (68)
MSC	Human (68)	+	FGF2, PDGF	1ng/ml, 1ng/ml	+ proliferation (68)	no reaction to PDGF alone (68)	+sensitized to TGFβ1 (68)
MSC	Equine (60,67), Human (69)	+	TGFβ1	5ng/ml	+ coll II mRNA (67), + proliferation (69)	- proliferation (60), + coll I mRNA (67)	sensitized to IGF1 (60)

Table 2. The effect of growth factors used in 3-dimensional culture for cartilage tissue engineering.[1]

Cell type	Species	Culture type	Serum +/-	Cytokine	Dose (respective if >1 cytokine)	Positive Impact	Negative Impact
Chondrocytes	Rabbit (64)	Agarose (64)	+	BMP	100ug/ml		- repair in vivo (64), no tidemark in vivo (64)
Chondrocytes	Bovine (73)	Polyglycolic acid (73)	+	BMP2	10ng/ml		- no effect unless pretreated with FGF2 (73)
Chondrocytes	Human (73)	Alginate (73)	+	BMP2	100ng/ml	+ coll II mRNA (73)	
Chondrocytes	Bovine (75)	Alginate (75)	-	FGF	2.5ng/ml	+ coll II (75)	- mechanical properties (75)
Chondrocytes	Rabbit (64)	Agarose (64)	+	FGF2	5ng/ml	+ healing in vivo (64)	+ coll I (64), - tidemark in vivo (64)
Chondrocytes	Canine (74)	Coll II (74)	-	FGF2	5ng/ml	+ protein production (74)	
Chondrocytes	Canine (74), bovine (75)	Coll II (74), alginate (75)	-	FGF2	25ng/ml	+ protein production (74), + DNA (75)	- coll II mRNA (75), - mechanical properties (75)
Chondrocytes	Bovine (72)	Polyglycolic acid (72)	+	FGF2	10ng/ml		- wet weights (72), - GAG (72), - FGF2 (72)
Chondrocytes	Chick (79)	Pellet (79)	-	FGF2	100ng/ml		become hypertrophic (79)
Chondrocytes	Canine (74)	Coll II (74)	-	FGF2, IGF1	5ng/ml, 100ng/ml	+ protein production but not over FGF2 alone (74)	
Chondrocytes	Equine (58)	Fibrin (58)	+	IGF1	25ug/ml	+ coll II mRNA (58), + GAG (58), + PG (58), +DNA (58)	
Chondrocytes	Bovine (75)	Alginate (75)	-	IGF1	25ng/ml	+ coll II mRNA (75), + PG (75)	
Chondrocytes	Equine (61)	Fibrin disk (61)	-	IGF1	200ng/ml	+ coll II mRNA (61), + PG (61),+ GAG (61)	
Chondrocytes	Bovine (75)	Alginate (75)	-	IGF1	2.5ng/ml	+ coll II mRNA (75), + PG (75)	
Chondrocytes	Canine (74)	Coll II (74)	-	IGF1	100ng/ml		no effect (74)
Chondrocytes	Bovine (78)	Collagen (78)	-	TGFβ1	unstated	+ PG without serum (78)	- PG with >3%serum (78)
Chondrocytes	Bovine (66)	Hydrogel (66)	+	TGFβ1	30ng/ml	+ cell number (66), + GAG (66)	
Chondrocytes	Bovine (75)	Alginate (75)	-	TGFβ1	25ng/ml		- crosslinks (75), - coll II mRNA (75), - PG (75)

[1] The plus (+) and minus (-) symbols represent the presence or absence of serum or increasing (+) or decreasing (-) effect compared to controls. References appear in parenthesis. The following abbreviations are used: Collagen (coll), glycosaminoglycan (GAG), proteoglycans (PG), platelet derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), transforming growth factor (TGF), collagen oligomatrix protein (COMP), and 3-dimensional (3D)

Table 2. (continued)

Cell type	Species	Culture type	Serum +/-	Cytokine	Dose (respective if >1 cytokine)	Positive Impact	Negative Impact
Chondrocytes	Bovine (75)	Alginate (75)	-	TGFβ1	2.5ng/ml		- crosslinks (75), - coll II mRNA (75), - PG (75)
Chondrocytes	Rabbit (64), human (65,85)	Agarose (64), hyaluronic acid nonwoven mesh (65), pellet (85)	+	TGFβ1	10ng/ml	+ healing in vivo w/ coll II (654,655), + biomechanical properties (655), + COMP (85), + GAG (85)	- tidemark in vivo (64)
Chondrocytes	Bovine (72)	Polyglycolic acid (72)	+	TGFβ1, FGF2	1ng/ml, 10ng/ml	+ wet weights (72), + GAG (72)	
Chondrocytes	Human (65)	Hyaluronic acid nonwoven mesh (65)	+	TGFβ1, FGF2, PDGF	1ng/ml, 5ng/ml, 10ng/ml		- coll II (65)
MSC	Human (82)	pellet (82)		BMP2	50ng/ml	+ coll II (82), - coll I (82)	- COMP (82)
MSC	Equine (61)	Fibrin disk (61)	-	IGF1	200ng/ml	+ coll II mRNA (61), + PG (61),+ GAG (61)	
MSC	Human (60), rabbit (63)	Pellet (60), chitosin/collagen (63)	- (60) + (63)	TGFβ1	Transfected active TGF detected was 5ng/ml	- hypertrophic differentiation (60), + GAG (63), + dry weight (63)	
MSC	Human (68,80), rabbit (80), bovine (86)	pellet (68,80,86)	-	TGFβ1	10ng/ml	+ differentiation pretreated with FGF (29,80)	no differentiation alone (68), - coll II (86), + coll I (86)
MSC	Human (81,82)	Pellet (81,82)	-	TGFβ3	10ng/ml	+ coll II mRNA (81), + coll II (82) + aggrecan mRNA (81)	+ coll I mRNA (81)
MSC	Human (81,82)	Pellet (81,82)	-	TGFβ3 followed by IGF1	10ng/ml, 100ng/ml	+ aggrecan mRNA (81), + COMP (82)	no increase in coll II mRNA (81), + coll I mRNA (81)
MSC	Human (82)	pellet (82)	-	TGFβ3, BMP2	10ng/ml, 50ng/ml	+ coll II (82), - coll I (82), + COMP (82)	

CHAPTER 2. THE RELATIONSHIP BETWEEN LIMB FUNCTION AND RADIOGRAPHIC OSTEOARTHROSIS IN DOGS WITH STIFLE OSTEOARTHROSIS

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ABSTRACT

Objective -To evaluate the relationship between limb function and radiographic evidence of stifle osteoarthrosis (OA) in dogs.

Study Design - The relationship between force platform data and radiographic OA score was evaluated on 2 separate days using regression analysis. Interday variation was also assessed.

Animals - Forty-one dogs with visible lameness and radiographic evidence of stifle OA.

Methods - Force platform data was collected at a velocity of 1.7 – 2.0 m/s for 5 trials on day 1 and day 8. Radiographs taken on day 1 were scored using a previously reported OA scoring system.

Results - No significant relationship was found between force platform data and OA score. No significant differences were found between any day 1 and day 8 force platform values.

Conclusions - Although radiographic evidence of stifle OA provides evidence of pathology, it does a poor job of representing limb function. In addition, the absence of significant differences between day 1 and day 8 values in this population of dogs supports use of only a single force platform evaluation before measuring a treatment effect.

Clinical Relevance - The presence of OA in the stifle joint does not correlate with clinical function; radiographic outcome should be used cautiously as a predictor of clinical outcome.

INTRODUCTION

Several techniques are available for evaluating outcome after treatment of orthopedic disease. These include subjective evaluation of pain or lameness, force platform gait analysis, and radiographic scoring.[1-8] Lameness grade is a common rating system for evaluation of pain in orthopedic patients; however, this is subjective and may be confounded by evaluator bias. Force platform gait analysis has been commonly used to compare treatment modalities and assess long-term outcome.[1,2,6] It has been established as a reliable, objective method of quantifying lameness. Radiographs have been used both clinically and experimentally as a determination of outcome.[1,3,4-7] The presence and severity of osteoarthrosis (OA) is the primary focus of most scoring systems.

Intuitively one would expect that as the severity of OA increases, the capacity for the patient to use the affected limb would decrease. Empirically, however, many authors have suggested that there is no relationship between these two varaibles.[1,10-14] Several clinical studies have reported that the progression of radiographic score is in contrast to the clinical improvement or lack of progression of clinical signs.[3,7-9] Furthermore, Roy et al. found that lameness score did not correlate with radiographic OA score in stifles with medial patellar luxation.[8] All of these reports, however, used subjective scoring systems to evaluate patient limb function. Using force platform gait analysis as an objective measure of outcome may be more sensitive than a subjective lameness score.

Our objective was to evaluate the relationship between limb function, as measured by force platform gait analysis, and radiographic evidence of OA in dogs with stifle osteoarthrosis. We hypothesized that there would be a strong correlation between limb function and radiographic OA score.

MATERIALS AND METHODS

Owners of dogs with history and physical examination findings consistent with unilateral lameness caused by stifle OA were recruited for this study. Criteria for inclusion were as follows: history and physical examination findings consistent with

lameness from a single joint, no administration of non-steroidal anti-inflammatory medications for 2 weeks or steroidal medications for 4 weeks, no surgery on the affected limb in the previous 6 months, and the animal patient's gait was adequate for appropriate force platform trial acquisition.

Force Platform Measurement

All dogs had force platform and radiographic examinations on day 1. Force platform examinations were repeated on day 8. Dogs were trotted across a force platform (AMTI OR-6, Watertown, MA) at a velocity between 1.70 and 2.00 m/s and an acceleration of ± 0.5 m/s^2. The first 5 acceptable trials of the affected leg were used to generate mean force platform values for velocity, acceleration, peak vertical force (PVF), vertical impulse (VI), breaking, and propulsion for the affected limb. Trials where one front limb struck the center of the plate with no other limb present followed by the solitary ipsilateral hind limb were considered acceptable. All data were expressed as a percent of bodyweight.

Radiographic Examination

A board-certified radiologist, unaware of the limb function, scored cranial-caudal and lateral radiographs of the affected stifle. A modification of an OA scoring system reported by Vasseur,[7] was slightly modified for use in this study. Eighteen separate categories were scored on each set of radiographs using a numeric scoring system from 0-3 for none, mild, moderate, or severe signs of OA respectively. The mean OA score was calculated from the categorical scores and used for statistical comparison.

Data Analysis

Regression analyses were used to evaluate the relationship between the mean radiographic OA score and the force platform values for each of the 2 examinations. Paired *t* tests were used to compare the variation between days 1 and 8 in the force platform data. The level of significance for each set of statistical tests (regression: $P<$

.008, *t* test: $P < .006$) was adjusted using the Bonferroni method to decrease the chance of falsely concluding a relationship exists.[15] As multiple tests were performed on related data, the chances of a type I error increase by 0.05% for each test .[15]

RESULTS

Forty-one dogs that fit the inclusion criteria were studied. The affected stifles were evenly distributed between the left and right limbs. Seventeen were male and 24 female, and although multiple breeds were represented, 13 dogs were Labrador Retrievers.

The mean velocity (± SEM) on both day 1 and day 8 was 1.81 (± 0.01) m/s. The mean acceleration on day 1 was 0.06 (± 0.03) m/s^2 and day 2 was 0.03 (± 0.02) m/s^2. Mean (± SEM) values for each of the force platform variables on both days are listed in Table 1. The mean radiographic OA score was 0.91 (range, 0.17 - 1.50). No statistically significant relationship was found between OA score and PVF (day 1: $P = .95$; day 8: $P = .81$), VI (day 1: $P = .01$; day 8: $P = .09$), breaking force (day 1: $P = .16$; day 8: $P = .32$), breaking impulse (day 1: $P = .55$; day 8: $P = .09$), propelling force (day 1: $P = .19$; day 8: $P = .60$), or propelling impulse (day 1: $P = .99$; day 8: $P = .80$). No significant difference was found between any day 1 and day 8 force platform values ($P > .006$).

DISCUSSION

This data strongly suggests that there is no relationship between limb function and the severity of radiographic signs of stifle OA. Conventionally, the relationship between OA score and VI on day 1 would be considered significant; however our level of significance was reduced. When multiple individual statistical tests are performed each at the 0.05 level of significance on related data, the chances of making an incorrect conclusion increase with each test by 5%.[15] Consistent with this ideology, the regression coefficient of day 1 VI and OA score is positive, which suggests that limb function improves as OA increased. This conclusion would not be logical.

The results from this study corroborate those of previous studies. Radiographic OA scores did not correlate with the force platform values in a retrospective study of

humeral condylar fracture repair,[16] and Roy et al, [8] found that there was no correlation in a subjective limb use score and radiographic score for stifle OA after medial patellar luxation. Studies in humans correlating radiographic pathology and pain have also reported results consistent with our results.[10,11] Cobb and others found that only 30% of people with radiographic signs of OA in the knee, had pain at relevant sites, and no association was found between radiographic OA and stiffness as perceived by the patient.[10] Although direct correlations between radiographic OA and limb function are limited, various authors have published statements that compliment our results based on empiric observation.[1,10-14]

In contrast, Evers and others published a follow-up study on coxofemoral luxation showing physical examination score and radiographic OA score correlated well (r=0.63).[9] This was attributed to the accuracy of the subjective scoring system. Although it is possible that the relationship between limb function and OA score is different for the hip and stifle joints, we feel that the objective nature of force platform gait analysis supports our conclusions.

The population of dogs in our study all had histories and clinical signs consistent with OA. By excluding normal dogs from our study population, we eliminated the influence that those dogs would have had on the association of radiographic score to limb function. Although we cannot generalize about the entire population of dogs, we are able to draw conclusions relating to the smaller population of dogs with OA.

Additionally, by using the mean OA score for the 18 individual locations for radiographic changes, continuous, more precise data was generated when compared to scoring a radiograph based on an overall impression. When an overall impression is used to classify a radiograph (eg, mild, moderate, severe) a range of severities are present within each classification. For example, within the radiographs scored as moderate, there are those that almost qualify for the severe classification and those that are barely more severe than mild. The advantage of using the mean score of several anatomic categories is that the continuous data allows for a more specific OA score. This combined with scoring all radiographs at one time by a board-certified radiologist we feel produced a more accurate score for each radiograph. However, the scoring system may still be

improved by weighting certain categorical locations associated with increased pain over others. Unfortunately, since pain is difficult to pinpoint and quantify in veterinary patients, data used to weight the categories would need to be extrapolated from human studies. To investigate this, multivariate regression was performed on the individual categories for day 1 to assess the effect of the individual category on the PVF or VI, but similar to the overall score, none were significantly related.

As imaging modalities continue to improve and become more common, it is possible that quantification of pathologic changes will correlate better with clinical signs. Nuclear scintigraphy has shown a positive association with clinical signs and may be a more sensitive indicator of the loss of function associated with pain and inflammation.[17] Increased detail and 3 dimensional imaging may improve the sensitivity of the evaluation to small changes. Magnetic resonance imaging can detect OA up to 4 weeks earlier than radiographs.[14] Soft tissue changes are also more readily identified by this method, and pain is often caused by changes in the periarticular tissues.[11] However, because pain remains an individual experience, disparities in degree of lameness when compared to a range of lesions will continue to be unpredictable.

Although significant variation in data collected between days has been reported,[18] we found no significant differences between day 1 and 8 values in this population of dogs. In the previous report, dogs with interday variation were evaluated using the force platform on 3 consecutive days.[18] The dogs may have become habituated to the routine, or although they were normal on orthopedic exam, trotting may have caused discomfort, which affected the gait analysis on subsequent days.[11,19] In our study, the 7 day period between gait analyses may have precluded the same level of adaptation and given dogs ample time to recover from overuse. Other sources of variation found in normal dogs using force platform gait analysis include subject morphology and handler.[20,21] In our study, more than one handler was used, but all handlers were experienced, and although morphology may play a role in variation, using the same dogs on both days eliminated this as a source of error.

Although the presence of stifle OA provides evidence of pathology, it does a poor job of representing animal patient limb function. Studies evaluating the patient's

prognosis after treatment of a condition of the stifle in the dog should not focus exclusively on radiographic outcome. Additionally, we found no difference in limb function between day 1 and day 8 of the dogs in this study providing evidence that only a single force platform evaluation before measuring a treatment effect is necessary.

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Table 1. Mean and Standard Error of the Mean Values for Force Platform Variables Presented as a Percent of Bodyweight.

Force Platform Variable	Day 1		Day 8	
	Mean %	SEM %	Mean %	SEM %
Peak Vertical Force	51.10	1.78	51.38	1.72
Vertical Impulse	8.31	0.31	8.34	0.29
Breaking Force	4.75	0.35	4.82	0.30
Braking Impulse	0.43	0.15	0.29	0.03
Propelling Force	-6.04	0.31	-6.22	0.29
Propelling Impulse	-0.75	0.17	-0.60	0.03

CHAPTER 3. THE CHONDROCONDUCTIVE POTENTIAL OF TANTALUM TRABECULAR METAL

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ABSTRACT

Mesenchymal stem cells or chondrocytes have been implanted into joints in biodegradable matrices in order to improve the quality of healing cartilage defects; however, insufficient biomechanical strength of the construct at implantation is a limiting factor for clinical application. Logically, a construct with better biomechanical characteristics would provide better results. Tantalum trabecular metal (TTM) is osteoconductive and mechanically similar to subchondral bone. The objective of this pilot study was to determine if TTM is also chondroconductive. Small sections of TTM were cultured with emu and canine chondrocytes in static and dynamic culture environments. The sections cultured in dynamic bioreactors were diffusely covered with a cartilaginous matrix. Sections cultured in static conditions had no growth. Histologic evaluation from emu and canine dynamic cultures showed tissue that was heavily populated with mesenchymal cells that resembled chondrocytes, and glycosaminoglycan staining that was distributed throughout the matrix. Type II collagen content in the canine dynamic culture was 84% by SDS-PAGE. Tantalum trabecular metal is chondroconductive in vitro in a dynamic environment when cultured with adult canine or emu chondrocytes. This technology could be expanded to determine if cartilaginous-metallic constructs may be used for joint resurfacing of osteoarthritic joints.

INTRODUCTION

It is well known that articular cartilage has a limited capacity for repair. Injuries to articular cartilage that do not penetrate the subchondral bone generally do not heal and progress to degeneration of the articular surface.[1-3] Injuries that do penetrate the subchondral bone undergo repair through the formation of tissues usually characterized as fibrous, fibrocartilagenous, or hyaline-like cartilage, depending upon the species, age of the animal, and the location and size of the injury.[4-8] However, these reparative tissues, including those that resemble hyaline cartilage histologically, differ from normal hyaline cartilage both biochemically and biomechanically. By 6 months, extensive fibrillation and fissuring occur.[9-11] Once articular cartilage is damaged and osteoarthritis (OA) becomes clinically severe, surgical reconstruction is necessary to restore function. Historically, osteotomy or transplantation of whole joints, partial joints, osteochondral segments and cartilage-shells have been used.[12-16] The success of each of these techniques, however, is limited by a frequent end result of progressive OA that requires salvage surgery such as arthrodesis or joint replacement.[12-14,16] Recently, more refined resurfacing techniques using isolated chondrocytes, mesenchymal stem cells, and periosteal membranes have been investigated experimentally and clinically.[10,17-22] Mesenchymal stem cells or chondrocytes have often been implanted in joints in a biodegradable matrix.[10,23-26] Unfortunately, these methods have limited clinical application for the repair of large or multiple articular defects because of restricted availability of donor material when autografting chondrocytes and decreased biomechanical strength of the construct at implantation.[10,19,26] Logically, a construct with better biomechanical characteristics would provide better results.

Tantalum trabecular metal (TTM) (Hedrocel® Implex Corp., Allendale, NJ) is an open-pore biomaterial with a bulk porosity exceeding 80% and a mean pore size ranging from 547 – 710 μm.[27,28] Chemical vapor deposition/ infiltration creates a 1 – 5 μm grain size and a distinct surface microtexture.[28] The implant is U.S. Food and Drug Administration (FDA) approved for its osteoconductive properties, has the appearance and mechanical characteristics of subchondral bone and provides structural support while

bone ingrowth is occurring.[27,28] Finally, its ductility is far superior to many alternative naturally occurring materials or ceramics.[27]

The term chondroconductive has not been used in the literature to the authors' knowledge. The definition of osteoconductive is to provide a scaffold for the ingrowth of bone.[29-32] Using osteoconductive as a guide, chondroconductive should be defined as providing a scaffold for the growth of cartilage and supporting structures.

We hypothesized that cartilage could be grown directly on TTM because of its high porosity and similar mechanical characteristics to subchondral bone. The purpose of this pilot study was to determine the in vitro chondroconductive properties of TTM. If successful, this technology could lead to further investigation into new resurfacing and arthroplasty techniques.

MATERIALS AND METHODS

Articular cartilage was aseptically harvested from the humeral head of 4 adult dogs and from the knee of 1 adult emu undergoing terminal surgeries for other research. Species were chosen based on availability and potential for modeling human disease. Both species were treated separately using the same experimental protocol.

Cartilage was finely chopped and digested overnight at 37°C in a mixture of collagenase type II (Sigma, St Louis, MO) and Hank's solution. Chondrocytes were washed with Roswell Park Memorial Institute 1640 with L-glutamine (RPMI) (Sigma), plated, and expanded for 3 weeks in monolayer culture to obtain sufficient number of cells for three dimensional cultures. Chondrocyte medium containing RPMI, 10% fetal calf serum, penicillin (100IU/ml), and streptomycin (100µg/ml) (Sigma) was changed three times per week. The canine cells from the 4 dogs were pooled for the static and dynamic cultures.

Sixteen segments of TTM, 5 mm^3, were cut from a commercially available TTM acetabular component, sterilized, and placed into a 24-well culture plate (static, canine n=4; emu n=4) or a magnetic stir bioreactor (dynamic, canine n=4; emu n=4). Static cultures were prepared by suspending aliquots of 1 $X10^7$ cells in 0.5 ml of medium which was placed in 4 separate wells of a 24 well plate. A section of TTM was added to each

well followed by 0.5 ml of a solution of Dulbecco's phosphate buffered saline solution (Sigma) and double strength RPMI containing equal proportions 20% fetal bovine serum, and 2% low-melting agarose (Sigma).[23] The static cultures were then covered with chondrocyte medium plus ascorbic acid.

Dynamic cultures were performed in a bioreactor consisting of a 300 ml magnetic spinner flask modified with 3 inch, 18 gauge needles to suspend the TTM pieces. In the bioreactor, implants were individually fixed in place on the needles, and then immersed in a solution combining $1x10^7$ chondrocytes and chondrocyte medium plus ascorbic acid. The bioreactor rotated at 50 rpm. Medium was changed every other day in the static and magnetic stir bioreactor environments. All cultures were incubated at 37ºC with 7% carbon dioxide.

Implants were maintained in their culture environments until a tissue film was visibly covering the metal or after 4 weeks of culture. For canine samples, one sample from each environment was placed into a vial and frozen (-20ºC) for collagen typing; the remaining 3 samples were fixed in 10% buffered formalin for histologic analysis. The percentage of Type II collagen in the matrix was determined in the frozen tissue using a previously reported SDS-PAGE technique.[19] Briefly, the engineered tissue was digested with cyanogen bromide prior to electrophoresis. Bands corresponding to degradation products were stained with Phast-blue and quantified with a laser densitometer. The percent of Type II collagen was then calculated. All emu samples were fixed in formalin. Quantification of Type II collagen in the emu tissue-implant construct was not determined due to the differences in distribution of collagen type reported in birds.[33] The constructs were embedded in glycol methacrylate, and 50 μm sections were prepared using the EXAKT system (Skeletech, Bothwell, WA). Safranin-O staining was used to determine the presence and distribution of glycosaminoglycan. Toluidine blue staining technique was used to detect proteoglycan and cellular distribution.

RESULTS

Tissue growth into and onto the TTM was histologically equivalent for both the canine and emu chondrocytes. After 4 weeks, no tissue growth was present on or in the

TTM cultured in static conditions. However, each implant cultured in the magnetic stir bioreactor had a white, translucent tissue present on its exterior. (Figure 1) Tissue from the samples cultured in a static environment showed no evidence of matrix development. In contrast, histologic samples from the rotating bioreactor were heavily populated with cells that resembled chondrocytes with glycosaminoglycan staining throughout the matrix. (Figure 2, 3) Finally, agarose harvested from the TTM cultured in static conditions contained no detectable collagen by SDS-PAGE. Alternatively, surface matrix harvested from the TTM cultured in the bioreactor contained 84% Type II collagen. (Figure 4)

DISCUSSION

Our findings indicate that TTM is chondroconductive in the dynamic conditions presented in this study. The tissue grown on the tantalum trabecular metal constructs after a period of 4 weeks resembles hyaline-type cartilage in cellularity and in the content and distribution of matrix. In addition, the 84% Type II collagen we found in the tissue grown in this study approaches that of normal cartilage.[34,35] However, additional biochemical testing including collagen quantification, glycosaminoglycan concentration, and proteoglycan concentration was not performed.

We elected to test the chondroconductive properties of TTM because its mechanical properties resemble that of subchondral bone, it is commercially available, currently used for its osteoconductive properties and it can be manufactured in many different shapes and sizes. In addition, the implant is biologically inert, and it has an open pore structure that seemed inviting for the cartilage growth. Similar materials may behave in a like manner. Biodegradable polymers have demonstrated chondroconductive properties, but to the best of our knowledge none have both the structural properties for cartilage ingrowth and the mechanical characteristics for immediate postoperative weight bearing.[10,23,25, 26] Polylactic acid, poly(glycolic acid), poly(L-lactic acid), collagen based sponges, and hyaluronan-based scaffolds have all demonstrated to be useful materials for cellular delivery with varying degrees of successful hyaline-like cartilage tissue production.[10,23,25,26,36,37]

Although TTM has many beneficial qualities that favor chondroconduction, we cannot comment on the reaction of engineered collagen-TTM constructs *in vivo*. Shear forces at the collagen - TTM interface may prevent clinical improvement or even become detrimental.

Recent evidence has shown that chondrogenesis is superior when the culture environment utilizes three-dimensional matrices or is dynamic in nature.[23,38,39] Our findings are consistent with these reports, where no cartilage formed in a static culture environment and tissue resembling normal hyaline cartilage formed in a dynamic environment. Utilization of a magnetic spinner flask to create a dynamic environment is simple in concept, inexpensive and provides the apparent benefits of dynamic culture. Both hydrodynamic laminar flow (rotating bioreactor) and cyclic hydrostatic pressure environments improve the overall growth and maintenance of collagen producing chondrocytes in vitro.[23,38,39] While not used in this study, intermittent positive pressure may also improve biomechanical strength of the growing tissue in response to the force applied.[38]

Mechanical testing would have provided complimentary information regarding the ability of the construct grown in vitro to behave like hyaline cartilage *in vivo*. The biomechanical performance of hyaline cartilage sets it apart from inferior repair tissue or fibrocartilage. Since biomechanical characteristics are dependent on molecular structure, it is promising that the majority of collagen in our study is Type II.[40] However, collagen type is not the only factor in biomechanical stability. Fibril orientation also plays a role during compression tests in normal cartilage.[40] In fact, all of the matrix components including the glycosaminoglycans and their organization are important in this regard. Engineered cartilage is typically tested *in vivo* by implantation instead of by direct mechanical means *ex vivo*.[5,10,12,16-18,25,26] Such testing will ultimately be necessary to validate the concepts derived from the present study.

Emu and canine cells were chosen based on availability and the potential for modeling human disease. Articular disease is commonly studied in the canine.[6,23] More recently, emus have been used to model avascular necrosis of the femoral head which leads to articular collapse.[41]

Our findings, though preliminary, indicate that TTM is chondroconductive in dynamic conditions. Due to the favorable biomechanical and osteoconductive characteristics of TTM, further investigation is warranted to determine the suitability of the integration of TTM into joint resurfacing technology.

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Figure 1. Picture of gross appearance of tantalum trabecular metal-emu cartilage construct.

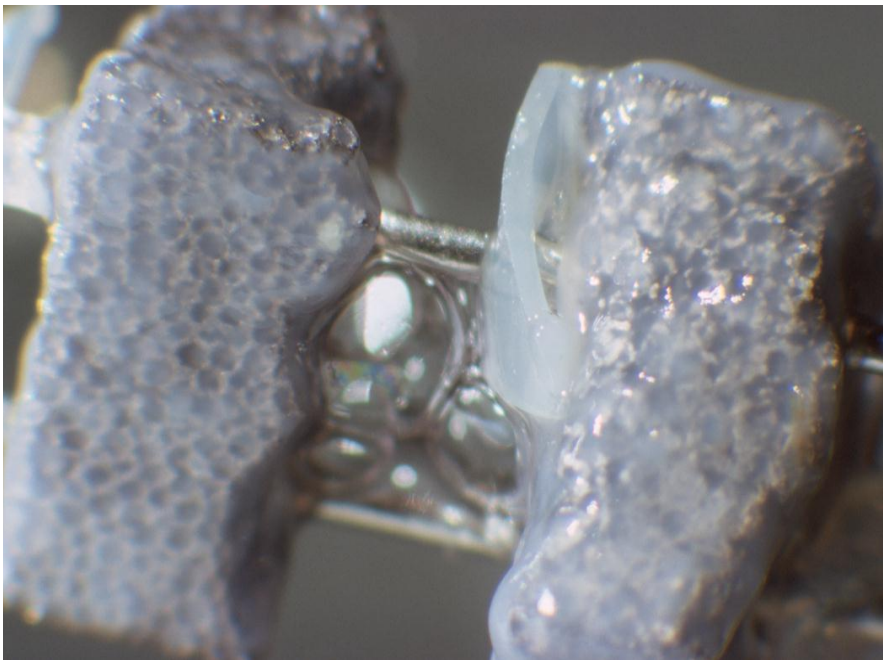


Figure 2. A) Safranin-O staining of emu construct determined that glycosaminoglycan (GAG) was present throughout the intercellular matrix. (500x) White bar equals 200µm. B) Normal emu cartilage (500X)

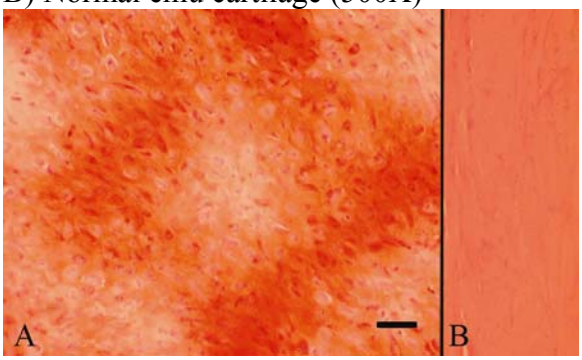


Figure 3. Toluidine blue staining of emu construct from the dynamic condition showing distribution of cells and extracellular matrix dark purple on the TTM (black) (200X) White bar equals 50µm.

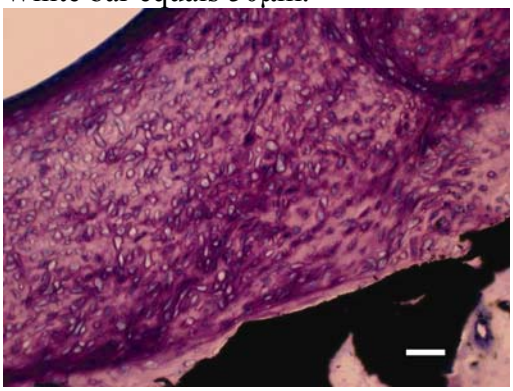
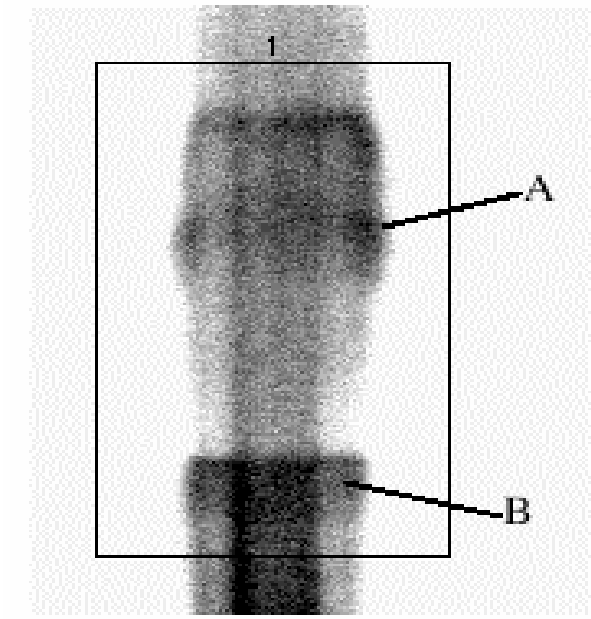


Figure 4. Results from semi-automated SDS-PAGE of the canine tissue grown demonstrating that 85% of the collagen present was Type-II collagen. Bands A and B are used to determine the ratio of Type II collagen.



CHAPTER 4. SEQUENTIAL USE OF FIBROBLASTIC GROWTH FACTOR 2 AND TRANSFORMING GROWTH FACTOR β1 FOR CARTILAGE TISSUE ENGINEERING USING CANINE CHONDROCYTES

A paper to be submitted to the *Journal of Regenerative Medicine*

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ABSTRACT

Defining the ideal culture requirements for production of consistent high quality tissue is important for developing a commercially available implant. This study investigates the use of fibroblast growth factor 2 (FGF2) in monolayer followed by transforming growth factor β1 (TGFβ1) in 3-dimensional pellet culture of canine chondrocytes. Cells in monolayer were divided into 2 groups. One group received FGF2. Following proliferation, each monolayer group was pelleted using serum free medium with TGFβ1, FGF2, serum, or no growth factors. Both monolayer groups receiving TGFβ1 in 3-dimensions were grossly larger than the other groups. The group receiving TGFβ1 subsequent to FGF2 stained less intensely for glycosaminoglycans, and chondrocytes were less mature than the group without FGF2 pretreatment. Additionally, the group receiving TGFβ1 without pretreatment with FGF2 showed more consistent collagen deposition; however, type I collagen was predominant. Under the culture conditions presented, FGF2 is mitogenic but not chondrogenic. In addition, TGFβ1 stimulation produced a predominance of type I collagen.

INTRODUCTION

Osteoarthritis (OA) is the second leading cause of disability in the United States, and it has been estimated that the disease will affect 18.2% of the population in the United States by 2020.[1] Eighty percent of those diagnosed with OA have limited mobility and 25% cannot perform major daily activities.[2] Recent efforts to improve the

quality of life of those afflicted have focused on joint resurfacing technologies including tissue engineered constructs.[3-5]

Growth factors are used commonly in an effort to improve the amount and quality of engineered tissue. However, many growth factors may produce dramatically different results. More specifically, Transfoming Growth Factor β1 (TGFβ1) and Fibroblastic Growth Factor 2 (FGF2) have produced variable results depending on the culture conditions, species and type of cell used.[6-17] Additionally, the biosynthetic activity may be influenced by sequential or concomitant use of growth factors.[15]

FGF2 has been reported to be mitogenic, decrease dedifferentiation of chondrocytes in monolayer, increase the time to senescence, and prime the cells for collagen production in 3-dimensional culture.[18-21] In contrast, FGF2 can also decrease the production of type II collagen, increase type I collagen production, and decrease the time to hypertrophic differentiation.[8,13,22,23]

The effect of TGFβ1 is also controversial and dependent on culture conditions.[24] TGFβ1 has demonstrated a negative effect on proteoglycan production when added to medium containing serum with conflicting results when serum-free medium is used.[25,26] Additionally, TGFβ1 may increase or decrease type II collagen deposition affecting the biomechanical properties of the engineered cartilage.[8,9,22,24] The species, cell density, and dose ranges present in the literature likely contribute to the variation in results.[24]

Determination of the ideal culture environment for promoting the appropriate extracellular matrix production is important for subsequent in vivo joint resurfacing studies using the canine model. Production on a clinical scale requires predictable, repeatable matrix grown in a relatively short period of time.

The purpose of this study was to determine the effect of FGF2 as a pretreatment in monolayer on chondrocytes as well as the effect of TGF β1 on 3-dimensional pellets in culture. The hypothesis was that the chondrocytes pretreated the FGF2 and cultured 3 dimensionally with TGF β1, would have the fastest growth with the largest amount and highest quality of matrix produced.

MATERIALS AND METHODS

Cytokines were purchased from R&D Systems, Minneapolis, MN. All other components were purchased from Sigma-Aldrich, St. Lois, MO unless otherwise stated.

Chondrocyte Harvest and Proliferation

Articular cartilage was taken from the humeral head and the femoral condyle of a small mixed breed adult dog estimated to be between 1 and 3 years of age that was euthanized for reasons unrelated to this study. Harvested cartilage was immediately placed in DMEM with 1% penicillin/streptomycin. The cartilage was diced into 1-2mm pieces and placed into type II collagenase in DMEM (0.5mg/ml concentration). The pieces were incubated at 37ºC and 5% CO_2 for 24 hours. The released cells were pelleted, washed with DMEM, and counted. Cells were plated at 0.8 x 10^5 cells per 25cm^2 culture flask. Culture medium used consisted of DMEM, 10% FBS, 1% penicillin/streptomycin. After the first passage, the pellets were replated in 75cm^2 flasks with 300,000 cells and randomized to receive FGF2 at 5ng/ml or no additional cytokines. Media was changed 3 times per week. When cells reached about 90% confluent, they were trypsinized washed and replated within the consistent group.

Pellet Cultures

Passage 3 chondrocytes were used in 3-dimensional pellet culture. The cells were trypsinized and counted. Cells were diluted to 1 x 10^6 cells/ ml of serum free medium. Each 15ml tube was aliquoted 0.5ml of the cell solution. The tubes were centrifuged at 500g for 5 minutes and incubated at 37ºC and 5% CO_2. The serum free medium consisted of DMEM with final concentration of 0.1mM ascorbic acid, 1.25mg/ml BSA, 10^{-7} M dexamethazone, 1% penicillin/ streptomycin, and 1ml/ml ITS+1 premix (10mg/ml insulin, 5.5 mg/L transferrin, and 5 µg/ml selenium).

After 2 days in 3-dimensional culture, cytokines were added to the pellets in the appropriate group with 10 pellets per group. Four groups were created for each of the monolayer groups for a total of 8 groups: 1. FGF2 at 5ng/ml, 2. TGF β1 at 10ng/ml, 3.

No growth factors 4. 10% FBS. Appropriate medium and growth factor was changed every 3 days for 3 weeks.

Histopathology

Three of the 10 pellets from each group were processed for histopathology. The pellets were preserved in 10% formalin for 24 hours. To facilitate handling, the pellets were grossly stained with 50% hematoxylin, and imbedded in agar base. Agar was heated to 38-40ºC and was pored over the pellet. The agar-pellet construct was refrigerated for 10 minutes at which time the agar was solid and it was returned to formalin for processing.

The agar-pellet construct was routinely embedded with paraffin and 5 micron slices were prepared. The slides were evaluated qualitatively using hematoxylin and eosin stains for cell morphology and safranin O with fast green counterstain for glycosaminoglycan (GAG) visualization.

In addition to subjective evaluation, the slides were also evaluated using imaging software to determine the percent of tissue stained by Safranin O and the density of viable cells. The cell density was determined by hand marking the cells with clearly defined nuclei and cell borders. Those cells with undefined nuclei and/or marked vacuolization were considered degenerate. The number of cells is then expressed as a ratio to the area of the tissue.

The quantitative analysis was performed using image analysis software (IPLab from Scanalytics, Inc). The area of GAG staining was quantified and expressed as a percent of the total area. The number of viable cells were counted and presented as a ratio to the area of the H and E slides.

Capillary Electrophoresis

The remaining pellets were digested in cyanogen bromide (CNBr) and collagen types I and II were quantitated by capillary electrophoresis using methods similar to those previously described.[27-28] Pellets were preserved in RNAlater (Ambion) and frozen at -20°C until digestion.

At the time of digestion, the pellets were thawed and soaked in PBS overnight at 4°C to leach out the preservative. The pellets were then heated in 70% formic acid at 60°C for 1 hour followed by digestion with 5% CNBr for 48 hours. Samples were diluted with distilled water to halt the reaction, and freeze dried. The samples were then rehydrated in 1 mL 1% (v/v) acetic acid and heated in a water bath for 60 minutes at 60°C. Samples were then filtered through MacroSpin SEC G-10 columns (The Nest Group, Inc.). After filtration the sample was transferred to a 2 mL CE vial. 1 µl bradykinin (Sigma) at 1mg/mL and 999 µl distilled water were added to the sample vial. Bradykinin was used as an internal control with a final concentration of 0.5 µg/ml.

Samples were injected (20 seconds at 1 psi, 177 nl) into a Beckman Coulter P/ACE MDQ Capillary Electrophoresis System coupled to a selectable wavelength UV/VIS detector set at 214 nm. A neutral, coated capillary (MicroSolvTech 04675-ZF) was used with a capillary temperature of 20°C and sample storage at 4°C. The capillary was washed with buffer prior to sample injection. Separation was carried out at 25kv for 60 minutes with normal polarity. The buffer used was 100mM Sodium Phosphate at pH 6.0. Type I and II collagen were quantitated using a standard curve. The standard curve was derived using purified type I and type II bovine collagen (Chondrex). The R^2 value for the type I and II collagen curves was 0.95 with a mean square error of 0.009 and 0.0001, respectively. Although the minimum detection limit was not sought, the lowest known amount of collagen used for the standard curve was 0.89 ng.

RESULTS

The cells receiving FGF2 in monolayer took an average 3 days after passage to grow to confluency whereas it took 6 days until the serum only group was confluent. Therefore the cells that were supplemented with FGF2 in monolayer (32 days) allowed completion of the experiment 14 days prior to the group that did not receive the cytokine during monolayer culture (46 days).

In pellet culture, TGFβ1 had a grossly visible effect on both the pellets in both monolayer groups. (Figure 1) This also was apparent in wet weight. The pellets in both monolayer groups that received FGF2, serum, or no cytokines in pellet culture weighed

less than 0.1mg. The group that received serum only in monolayer, but TGFβ1 in pellet culture had a mean weight (± SE) of 2.7mg (± 0.05). Similarly, the group that received FGF2 in monolayer and TGFβ1 in pellet culture had a mean weight of 2.5mg (± 0.16).

Histopathology

Histopathologic evaluation revealed large differences between groups. (Figures 2 and 3) H and E stained sections showed mature chondrocytes in lacunae sparsely distributed across a dense matrix in the pellets treated with TGFβ1 without FGF2 pretreatment. This group was the most similar to articular cartilage. The FGF2 pretreated pellets that were also treated with TGFβ1 showed healthy mature cells in a matrix, but lacunae were not well formed and cells were subjectively denser than the group without treatment in monolayer. (Figure 2) Image analysis confirmed this observation as the FGF2 pretreated group had a cell density of 1084 cells/mm^2 compared to 495 cells/mm^2 for the group that did not receive FGF2. The remaining groups did not show the matrix formation or the cellular maturity of the TGFβ1 treated pellets.

The group treated with FGF2 in both monolayer and 3-dimensional culture showed dense cellularity with marked necrocellular debris, degenerate cells evidenced by pyknotic nuclei, and little to no matrix present. The group provided FGF2 in 3 dimensional culture, but not in monolayer showed immature cells on the periphery with necrocellular debris centrally. Immature cells were defined by multiple nucleoli, dispersed chromatin and large nuclei. There was not a large difference in viable cell population between the groups receiving FGF2 in 3-dimensional culture (3715 cells/mm^2 pretreated and 3070 cells/mm^2 no pretreatment); however this group contained the highest number of cells by a factor of 3 over the other groups.

The pellets cultured 3-dimensionally in serum-free media without growth factors showed marked necrosis centrally with a ring of immature cells in the periphery. The group receiving FGF2 in monolayer but no growth factor in 3-dimensional culture showed cells with slightly less basophilic staining. The groups receiving serum supplementation while in 3-dimensional culture, showed less debris; however, signs of cell death were prominent. Irregular nuclear and cellular margins and the presence of

large vacuoles were considered signs of cell death. The peripheral cells appeared healthy, but immature. Basophilic matrix was more prominent in the group receiving serum in 3-dimensional culture without FGF2 pretreatment than the serum-free group. For both the serum and serum-free groups that were pretreated with FGF2 in monolayer (683 and 1176 cells/mm^2, respectively), there were more viable cells in the pretreated group than the non- pretreated group (451 and 207 cells/mm^2, respectively).

Safranin O staining of proteoglycans in the matrix was present in only 3 groups. (Figure 3) The TGFβ1 group without FGF2 pretreatment showed uniform, intense orange staining and 100% of the total tissue area stained with the safranin O. In contrast, the pretreated TGFβ1 group showed more diffuse, mottled staining of the extracellular matrix with only 51.8% of the tissue area showing staining for GAG. The pellet treated with serum without FGF2 pretreatment showed a small thin ring of safranin O staining (3.0% of the total area) in the periphery corresponding to matrix seen on H and E stained sections.

Capillary Electrophoresis

Capillary electrophoresis showed type I collagen present consistently in the group receiving TGFβ1 without FGF2 pretreatment (mean 3.57 ug; range 1.02 – 6.00 ug). Additionally, this group also had one pellet with detectable type II collagen (4.5 ug). The group pretreated with FGF2 followed by TGFβ1 had detectible type I collagen in only 2 pellets 2.33 and 4.37ug. No other group had any detectable type I or type II collagen.

DISCUSSION

As expected, the overall size of the pellets treated with TGFβ1were grossly larger than the other groups, and pretreatment with FGF2 prior to 3-dimensional culture decreased the time to produce the cartilage. This is a desirable effect for clinical scale production of engineered cartilage. However, the groups pretreated with FGF2 had decreased quality and quantity of matrix histologically. This was evident in the pellets treated with serum in addition to those treated with TGFβ1.

The larger cell number likely contributed to the size of the TGFβ1 group pretreated with FGF2. This is supported by the 2-fold higher cell count/unit area compared to the TGFβ1 group without FGF2 pretreatment. Consistently, the extracellular matrix was less prominent and stained less intensely for glycosaminoglycans compared to the non-pretreated TGFβ1 group despite overall equivalent size and weight. Although this is in contrast to some reports,[19-21] FGF2 has been reported to decrease large proteoglycan deposition and collagen II production in 3-dimensional culture.[13,22,29]

This is also apparent in the FGF2 pretreated groups treated with serum or no growth factors in 3-dimensional culture; whereas, the group receiving FGF2 only in pellet culture did not have a large difference in cell count/unit area in the pretreated verses non-treated monolayer culture. Additionally, both groups treated with FGF2 in 3-dimensional cultures had a 3-15 fold higher cell count/ unit area than the other groups.

The higher cell numbers in the FGF2 treated groups may be due to inhibited apoptosis, increased proliferation, or a combination. A similar study found that DNA content decreased over time in pellet culture suggesting cell apoptosis.[30] This supports the supposition that FGF pretreatment inhibits apoptosis instead of promoting proliferation in the pellet cultures. However, the cells were less mature which is consistent with proliferating chondrocytes. Increased proliferation rates are consistent with previous in vitro studies of chondrocytes in monolayer and 3-dimensional studies.[13,29-31] Ideally, whole pellet cell counts, DNA quantification, or proliferation assays would be needed to confirm this deduction.

Unexpectedly, FGF2 pretreatment in monolayer showed lower matrix production when redifferentiation was induced by TGFβ1. This is consistent with studies in 3-dimensional cultures which show negative effects of FGF2 on GAG and collagen type II production when used alone.[13,22] However, other studies found using FGF2 in monolayer enhanced the activity of TGFβ.[20,30] It is possible that matrix formation may have surpassed the group without FGF2 treatment in monolayer if cell cultures were maintained for a longer period of time. Additionally, the combination of more than 1 growth factor may enhance the redifferentiation and matrix formation of the chondrocytes over individual growth factors.[30,31]

Other studies may have shown enhanced results by using a matrix instead of pellet culture.[15,21] The presence of a 3-dimensional matrix has a significant impact on the effect of the growth factor.[33,34] One limitation of this study is that it cannot be directly applied to in vitro engineering on a scaffold.

Fibroblastic growth factor 2 also negatively influenced collagen deposition. Only 2 pellets out of 6 showed any collagen using capillary electrophoresis; whereas, TGFβ1 without FGF2 pretreatment consistently produced detectable amounts of collagen type I. There may also be a dose dependent negative effect of FGF2 at doses of 5ng/ml or greater.[15] In this study, 5ng/ml of FGF2 was chosen to based on a previous report addressing the amount optimal for mitogenic activity.[15] Potentially, a lower dose would preserve the mitogenic activity without negatively effecting 3-dimensional culture.

Unfortunately, only one pellet in the TGFβ1 group produced detectable type II collagen. This was in the group without pretreatment. Typically, TGFβ1 is thought to increase type II and decrease type I collagen production.[8,9,35] However, fibrocartilage, the main reparative tissue of cartilage injury in vivo is predominantly type I collagne despite increases in TGFβ1 with injury in vivo.[24] A study of the phenotypic maintenance of chondrocytes over time found that at 10ng/ml of TGFβ1, type II collagen was upregulated initially but then suppressed after 12 days of treatment.[36] Additionally, TGFβ1 has also shown decreased levels of collagen type II mRNA in bovine chondrocytes exposed to 25 ng/ml of TGFβ1.[22,36] One study using dedifferentiated chondrocytes showed TGFβ1 did not increase type II collagen unless combined with insulin-like growth factor 1, but production of type I collagen was consistent.[32] Combining growth factors or establishing the optimal concentration of TGFβ1 to use may improve the consistency and quality of the engineered tissue.

Other aspects of the medium may also contribute to the resulting tissue characterization. Despite wide use of dexamethasone in similar studies, there is evidence that it inhibits the upregulation of collagen type II caused by TGFβ use in vitro.[24] In this study, dexamethasone was used in the serum-free media and may have contributed to the low to absent type II collagen deposition noted. However, the overall size of the pellets

were grossly larger than those not receiving TGFβ1 suggesting that GAG and type I collagen production was minimally affected.

Many of the studies evaluating the effect of TGFβ1 on collagen production have utilized mRNA techniques or semi-quantification based on immunohistochemistry.[8,9,22,35,37] In this study we used a biochemical assay to quantify the amount of type I and II produced. It is possible that small quantities of type II collagen were present beneath the detectable threshold. Limitations of the technique can cause underestimation of type II collagen including incomplete cyanogen bromide digestion and decreased relative sensitivity of type II compared to type I collagen.

Although these results are not entirely consistent with the literature, studies are extremely difficult to compare. Similarities between papers include cell type, harvest method, basic techniques such as pipetting and cell passage, and the physical monolayer environment. Additionally, the use of pellet culture for 3-dimensional studies is common. However, medium additives, individual characteristics, and species differences cause variation in outcome. In addition, there is large variation between individuals.[15] Although comparing the effects of growth factors within a species would arguably be more consistent, studies using canine chondrocyte cultures are uncommon.

Under the culture conditions described here, TGFβ1 increases the size and GAG content of matrix produced by chondrocytes. Additionally, FGF2 in monolayer is mitogenic, but negatively influences matrix formation in 3-dimensions. However, FGF2 and TGFβ1, individually or in succession, did not promote collagen type II deposition.

ACKNOWLEDGEMENTS

Capillary electrophoresis run by The Protein Facility of the Iowa State University Office of Biotechnology. Image analysis was performed with the aid of the Image Analysis Facility of the Iowa State University Office of Biotechnology.

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Figure 1. Gross appearance of pellets after 3 weeks in culture with pellets receiving FGF2 in monolayer A and without pretreatment B. Marks represent 1mm spaces. Pellets labeled by 3-dimensional treatment.

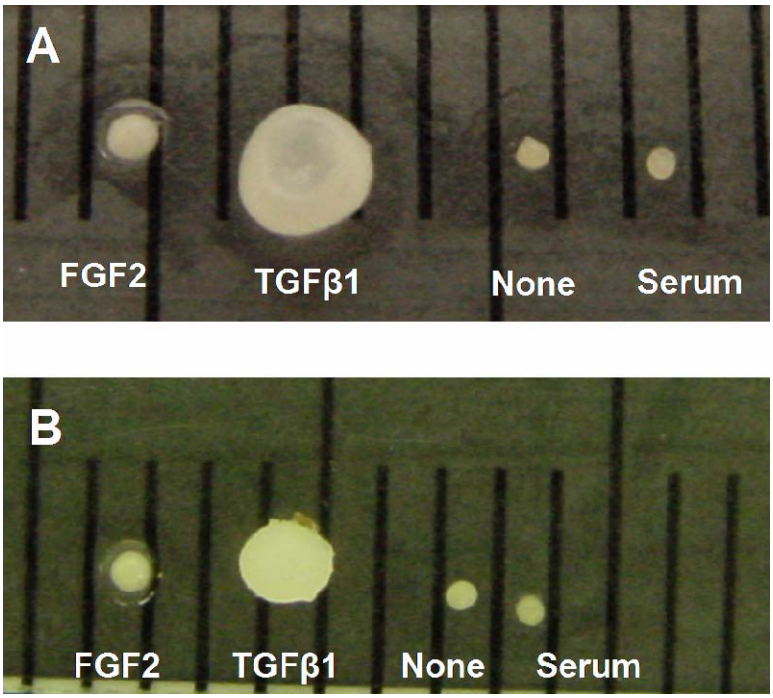


Figure 2. Chondrocyte pellet histopathology stained with hematoxylin and eosin after 3 weeks in culture. All pictures are at 40X magnification. Yellow bar represents 200 μm.

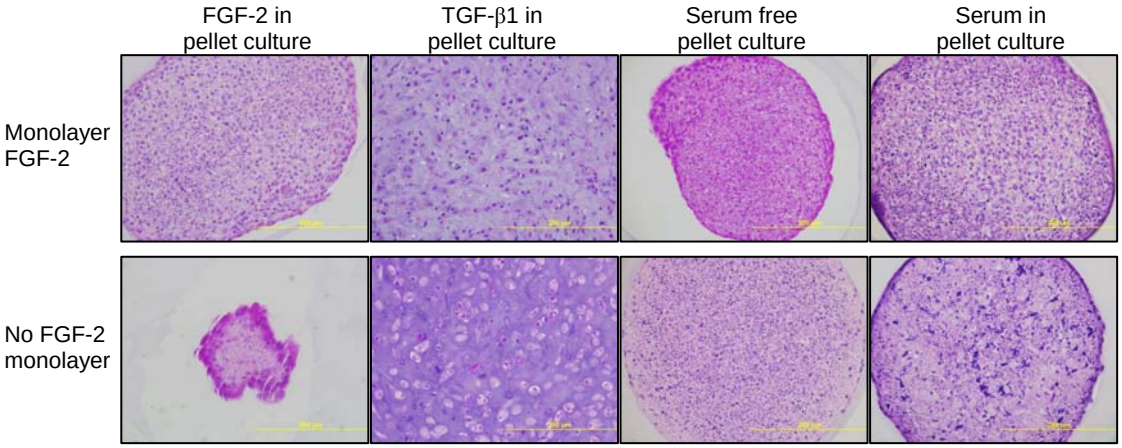
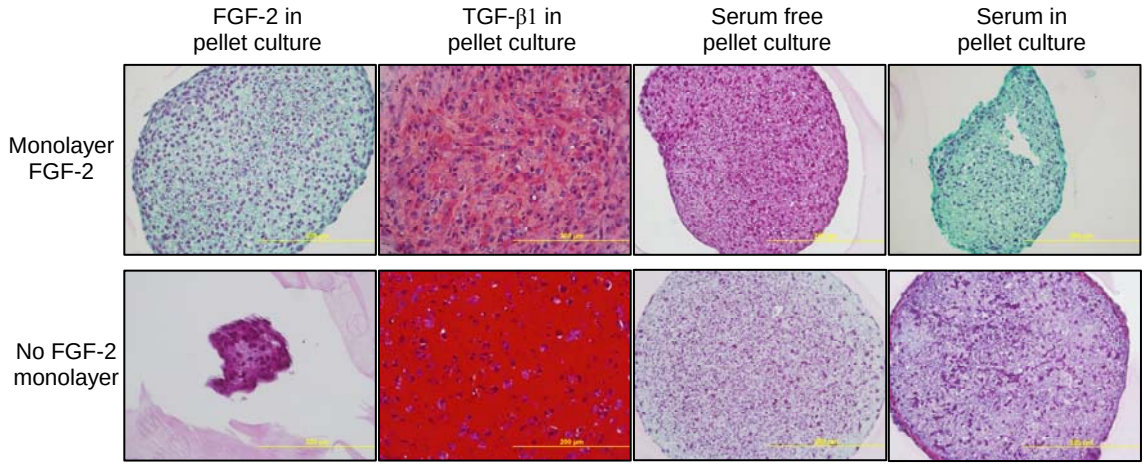


Figure 3. Chondrocyte pellet histopathology stained with safranin O and fast green after 3 weeks in culture. All pictures are at 40X magnification. Yellow bar represents 200 μm.



CHAPTER 5. CONCLUSION

SUMMARY

This dissertation focuses on clinical outcome measures and articular cartilage tissue engineering for the treatment of OA. In the first study, radiographic evidence of OA was compared to limb function. The results show no correlation between the degree of radiographic OA and ground reaction forces produced by dogs diagnosed with OA. Additionally, there was no difference from day to day in ground reaction forces. Despite wide spread use, radiographic OA is not correlated with clinical outcome based on limb function.

Osteoarthritis treatment research is focused on a regenerative medicine paradigm. Tissue engineering is one aspect of this philosophy. The 2nd study in this dissertation is focused on using a novel material as a scaffold for cartilage growth. Using chondrocytes, tantalum trabecular metal was a chondroconductive scaffold supporting matrix that was 84% collagen type II.

In order to improve the efficiency and quality of tissue engineered cartilage, growth factors were tested on canine chondrocytes in monolayer followed by 3-dimensional pellet cultures. Fibroblastic growth factor 2 was mitogenic as predicted; however, after 3 weeks of pellet culture, those cells that were treated in monolayer with FGF2 contained less collagen and overall matrix than those not pretreated. Both monolayer groups treated with TGFβ1 in pellet culture contained more matrix than any other group. Although disappointing, collagen type I and II quantification showed a predominance of type I collagen when using TGFβ1 in pellet culture.

DISCUSSION

Clearly, the medium used to grow cartilage in vitro has not been optimized. Differences are abundant between species, cell type, and individuals.[1,2] This is accentuated in cells from older patients or those with OA.[2] If this technology is to be used on a large scale for a clinical treatment, a predictable and repeatable result is necessary. There are several research directions feasible to overcome this problem

including banking select donor cells used for allograft development or optimizing conditions for the specific cells harvested.

Additionally, the presence and type of scaffold is important. Scaffold mechanical properties effect short and long term matrix properties.[3] Interactions between the scaffold and chondrocytes influence matrix production and response to growth factors.[4] Although the pellet culture model used in the medium optimization study is widely used, it may not reflect the chondrocytic response to the same growth factors when cultured with tantalum in a dynamic environment.

In chapter 2, chondrocytes were cultured with tantalum producing matrix with concentrations of type II collagen that approach normal articular cartilage. The pellet cultures did not have detectible amounts of type II collagen. However, the 2 studies cannot be directly compared because differences in culture environment are abundant. Besides the dynamic environment and presence of a scaffold, the chondrocytes seeded on the tantalum trabecular metal were exposed to serum throughout the culture process. Multiple unknown signaling molecules were present in the serum affecting the cells. Defining an efficient serum-free mitogenic and matrix forming medium for optimal cartilage growth is necessary for a commercial implant production.

An alternative to defining matrix growth medium is to use the patient as an in vivo bioreactor. A scaffold is loaded with cells and implanted into the defect with limited time in vitro. There has been some short-term success with this approach in animals.[5] One potential benefit of using tantalum trabecular metal is the biomechanical properties are similar to cancellous bone and can withstand immediate load bearing in vivo. Tantalum as a scaffold may protect neocartilage through maturation promoting cartilage formation with appropriate biomechanical properties.

After defining the appropriate culture conditions, evaluation of the neocartilage under load bearing conditions is necessary. In vivo trials in tissue engineering have traditionally looked at the histopathologic, biochemical, and to a lesser extent biomechanical properties of repair tissues. Although valuable, traditional measures of therapeutic success do not address the main goals of treatment: decreased pain and

increased function. In future studies, clinical measures of success like gait analysis should be used to determine outcome in addition to the traditional measures.

This dissertation addresses both clinical and basic science aspects of research into OA. The future of OA research lies in integrating the knowledge of several fields into clinically applicable treatment options. Although a cure for OA may seam unfeasible, the current tissue engineering research paradigm shows promise.

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