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The Proliferative Response of Equine Chondrocytes to Bovine Lymph Node Proteins *In Vitro*

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THE PROLIFERATIVE RESPONSE OF EQUINE CHONDROCYTES TO BOVINE LYMPH NODE PROTEINS IN VITRO

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ABSTRACT

Lameness poses a substantial challenge to the horse industry, costing millions of dollars per year in treatments, labor, and loss of use of performance horses. Osteoarthritis is a leading cause of chronic lameness. Treatments that stimulate cartilage development may promote cartilage healing. Many current treatment options for osteoarthritis have limited effectiveness and are expensive. Bovine supramammary lymph nodes are widely available at no cost and contain bioactive growth factors that could promote cartilage healing. The purpose of this study was to determine the proliferative effect of bovine lymph node extract on equine chondrocytes in *vitro*.

Equine chondrocytes were harvested from the scapular cartilage of adult horses. Lymph node extract (LNE) was prepared by mixing freeze-dried lymph node powder with PBS followed by heat inactivation. Chondrocytes were cultured *in vitro*, first in DMEM with 10% bovine growth serum (BGS) and antibiotics, then with treatment media containing either BGS or LNE on equal and unequal protein bases. Additionally, LNE fractions from ammonium sulfate precipitation were examined as BGS replacements. The response of the cells to IGF-I and TGF-β was also examined. DNA accumulation was measured using the CyQUANT DNA assay as a means to quantify cell stimulation, i.e. growth.

When BGS and LNE were compared on equal and unequal protein basis over a range of protein concentrations, there were no statistically significant differences

observed in proliferation between BGS– and LNE– treated cells. However, in a separate experiment, cells treated with LNE had significantly higher cell proliferation than cells treated with BGS or different LNE fractions. IGF-I and TGF-β had no significant effects on cell proliferation.

Overall, the results indicate LNE is capable of stimulating chondrocyte proliferation to an equal or greater degree than BGS, suggesting LNE could promote cartilage healing. Further studies are needed to determine if LNE can be used to develop new therapies for osteoarthritis in horses.

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INTRODUCTION

Lameness poses a substantial challenge to the horse industry. A recent national study showed that the economic cost due to lameness in America per year ranged from $678 million to $1 billion (APHIS 2001). Two-thirds of incurred cost comes solely from loss of use of horses, with the rest attributable to veterinary bills and additional treatment costs. A separate study showed that approximately 67% of lame horses are chronically lame, that is, lame for 4 months or longer (NAHMS 2000). Ongoing treatment costs, loss of use of affected horses, and time spent treating chronically lame horses can lead to a considerable investment in time and money. Lameness can be considered the most significant factor contributing to loss of performance, with over 67% of days lost being due to lameness (Rossdale *et al.*, 1985). Although lameness can be attributed to many factors, osteoarthritis can be considered one of the most economically important because of its prevalence and variety of causes (Auer and Fackelman, 1981). Todhunter and Lust (1990) estimate that 42% of lameness is directly related to joint disease.

Osteoarthritis is a degenerative joint disease characterized by degeneration of articular cartilage. Osteoarthritis can cause a tremendous amount of pain and restricted motion in the affected joints, severely limiting the horse's athletic potential and negatively impacting welfare. Although cartilage degeneration occurs naturally with age, it occurs much faster when the joints are subject to high levels of stress. In performance horses, osteoarthritis commonly occurs in knees, hocks, and fetlocks – all joints that undergo substantial wear and tear during

strenuous exercise. The articular cartilage is gradually destroyed via chondrocyte apoptosis and subsequent matrix degradation, and it cannot be regenerated quickly in adult horses. Osteoarthritis is becoming more common in younger, hard-working horses and can severely limit the horses' useful working careers.

Currently, there is no cure for osteoarthritis. There are, however, a variety of treatments that have proven to be fairly effective. These include non-steroidal anti-inflammatory drugs (NSAIDs) such as phenylbutazone (bute) and corticosteroids, which both serve to reduce inflammation and relieve pain. Other treatments include polysulfated glycosaminoglycans (PSGAGs) and nutraceuticals, such as glucosamine and chondroitin sulfate. The treatments attempt to prevent further cartilage degradation. Hyaluronic acid is also commonly used as a lubricant to reduce the effects of depleted cartilage and to prevent further damage.

Unfortunately, none of the above options can provide a cure for osteoarthritis. Most of these treatments are not used until the cartilage is already degraded and the horse is in pain. Many treatments have negative side effects which accumulate with long-term use. For example, NSAIDs such as phenylbutazone can lead to the development of gastric ulcers, if given in high doses for an extended period of time, and may also cause decreased proteoglycan synthesis in articular cartilage. Numerous studies have suggested that prolonged use of corticosteroids, while effective for reducing inflammation and pain, may cause detrimental effects on the biochemical composition and morphological features of cartilage. NSAIDs, corticosteroids, PSGAGs, and nutraceuticals must all be used either continuously

or on a regular basis if the effects are to be maintained and these treatments can be quite expensive. New options such as gene transfer and the use of autologous cell transplantation are being explored. However, these therapies could potentially be very expensive and not readily available to the average horse owner even if the techniques were possible, thereby necessitating the development of better and more cost-effective treatments.

The focus of this research is to examine the proliferative response of equine chondrocytes to bovine mammary lymph node proteins. Bovine supramammary lymph nodes contain cytokines and growth factors and are available in large quantities at very low cost. If nodal proteins are capable of stimulating equine chondrocyte proliferation and matrix synthesis, they could potentially be used as the basis for an effective treatment for osteoarthritis that could be made widely available at relatively low cost.

Rationale

Bovine serum is commonly used as a supplement in cell culture media. Previous research has indicated that bovine serum is capable of supporting chondrocyte proliferation and maintenance of phenotype *in vitro*. Kolettas *et al*. (1995) showed that chondrocytes cultured long-term in medium supplemented with 10% fetal bovine serum (FBS) were able to retain their differentiated phenotype. Chaipinyo *et al*. (2002) found that chondrocytes cultured in medium containing 20% FBS showed equal proliferation and similar protein synthesis to chondrocytes receiving serum-free medium containing IGF-I, TGF-β, and bFGF.

Finally, chondrocyte viability, cell density, and proteoglycan synthesis after long-term storage was found to be much greater if the chondrocytes were stored in medium containing 10% FBS rather than in serum-free medium (Pennock *et al*., 2006). An alternative source of bovine proteins may be able to support cell growth and metabolism to the same degree as serum. Bovine lymph nodes may be able to serve as an adequate alternative source of bovine proteins.

LITERATURE REVIEW

Characteristics of articular cartilage

Articular cartilage is found covering the surfaces of diarthroidial joints in the body, providing cushioning and preventing excessive wear and tear of joint surfaces. It is made up exclusively of hyaline cartilage, which is the most common type of cartilage found in the body. Hyaline cartilage usually is covered by a layer of dense connective tissue called the perichondrium, which plays an important role in cartilage growth and maintenance; however, the hyaline cartilage found in articular cartilage lacks perichondrium. Instead, it is supported by nutrients brought in by the synovial fluid circulating between joint surfaces. Articular cartilage is avascular and thus undergoes primarily anaerobic metabolism.

Articular cartilage is not homogenous; instead it contains three distinct zones – superficial, intermediate, and deep. The cells and matrix found in each zone are homogenous within a zone and heterogeneous between zones. Differences in morphology, metabolic activity, and gene expression may be observed (Chubinskaya *et al.*, 2003).

Cells

The cellular unit of articular cartilage is the chondrocyte. Chondrocytes are differentiated cells of mesenchymal origin. They make up approximately 5-10 % of the total cartilage volume. Chondrocytes tend to exist in groups of up to eight

cells, called isogenous groups. Isogenous groups are separated from each other in matrix cavities called lacunae. As a result, chondrocytes must communicate with each other via the extracellular matrix. Chondrocytes produce and maintain the extracellular matrix, as well as producing growth factors involved in normal anabolic and catabolic reactions in the cartilage. The action of chondrocytes is influenced by existing matrix composition, mechanical load, hormones, growth factors, cytokines, aging, and injury (Ulrich-Vinther *et al.*, 2003).

Fiber

Articular cartilage is built on an extensive scaffold of collagen fibrils. Fibrils, containing many glycine, proline, and hydroxyproline residues, are formed by three amino acid chains wrapped together to form a left-hand helix. Articular cartilage contains predominantly collagens type II, IX, and XI (Mendler *et al.*, 1989). Type II collagen, discovered by Miller and Matukas in 1969, is the primary collagen type found in articular cartilage, making up 90-95% of the total collagen and at least 50% of the dry weight of the cartilage. Type II collagen is found almost exclusively in articular cartilage and thus is often used as a marker for the differentiated chondrocyte phenotype. The presence of type I collagen has often been considered a marker of chondrocyte dedifferentiation; however, studies have shown that differentiated chondrocytes do produce small amounts of collagen I (Kolettas *et al.*, 1995). Collagen types IX and XI are present in much smaller amounts but are essential for collagen cross-linking, which stabilizes the fibrils and provides structural integrity. Collagen XI may also regulate the fibril diameter

of type II collagen (Ulrich-Vinther *et al.*, 2003). Collagen fibril arrangement differs in the three cartilage zones. Fibrils are aligned parallel to the cartilage surface in the superficial layer and perpendicular to the cartilage surface in the deep layer, with the fibrils forming arches in the intermediate layer. Benninghoff (1925) described this arrangement as a Gothic arch (Zambrano *et al.*, 1982).

Collagen primarily provides structural support but may play an important role in the composition of the extracellular matrix produced by chondrocytes (Qi and Scully, 2003). Collagen fibrils undergo slow metabolism and have a very long lifespan. However, in disease states, turnover may increase rapidly and exceed the chondrocyte's ability to produce matrix, leading to matrix deterioration and failure (Ulrich-Vinter *et al.*, 2003). As a result, and also because the structural support provided by collagen is necessary for proper cartilage function, collagen fibrils are a major target of cartilage degeneration (Aigner and Stove, 2003).

Matrix

The extracellular matrix consists of the above mentioned collagen fibers and aggregating proteoglycans. Aggrecan is the primary proteoglycan found in articular cartilage. It is a large molecule made of chondroitin sulfate and keratan sulfate chains bound to a protein core. Aggrecan is capable of binding non-covalently to hyaluronic acid and forming aggregates up to several hundred million Daltons in size. Link protein stabilizes the bond between aggrecan and hyaluronic acid by binding both simultaneously and decreasing the dissociation constant, making the bond much less likely to dissociate under normal

physiological conditions (Todhunter, 1996). Proteoglycan aggregates have a negative charge and thus attract cations. The increased osmolality in the tissue attracts water, which accounts for approximately 75% of the weight of articular and also gives cartilage unique biochemical properties. The water causes a high tissue pressure but collagen fibers prevent swelling and provide rigidity. One of the earliest changes in osteoarthritis involves loss of integrity of the collagen fibrils, which leads to swelling and loss of proteoglycans from the matrix (Ulrich-Vinther *et al.*, 2003). Cartilage also contains many smaller proteoglycans such as decorin, fibromodulin, and others which bind to other molecules such as collagen II and help stabilize the matrix.

Interactions between chondrocytes and the extracellular matrix are vital for biological processes such as growth, differentiation, and matrix synthesis and degradation. An understanding of the interface through which the chondrocyte and surrounding matrix interact may provide insight into the pathogenicity of osteoarthritis (Lapadula and Iannone, 2005). Integrins are the primary cell surface receptors responsible for chondrocyte-matrix attachment complexes that allow communication between the chondrocyte and its surrounding matrix (Svoboda, 1998). In particular, the β1 family of integrins is thought to mediate chondrocyte interaction with collagen types I and II and fibronectin (Motomi *et al.*, 1993).

Synovium

The synovium lines the joint capsule and includes a layer of connective tissue and a layer of cells, synoviocytes, with no basement membrane. Type A

synoviocytes have phagocytic and immune function; type B synoviocytes are secretory, synthesizing the hyaluronan and lubricin found in synovial fluid. Synovial fluid acts as the medium of exchange between blood and the avascular articular cartilage, and also helps provide lubrication for the joint (Junqueira and Carneiro, 2005). There are two types of joint lubrication (Todhunter 1996). The first, often referred to as boundary lubrication, is provided by hyaluronan and lubricin in synovial fluid, which are highly viscous and move over the surface of the joints, causing a low coefficient of friction. The second type of lubrication is known as weeping lubrication, which is exudation of fluid from the cartilage itself when under compression. This fluid also decreases the coefficient of friction due to the incompressible nature of fluids, which provides a small bit of space between articulating surfaces.

Articular cartilage growth and development

Articular cartilage develops from mesenchymal stem cells. Mesenchymal stem cells (MSCs) are capable of differentiating into a variety of cell types such as chondrocytes, osteoblasts, and adipocytes, as well as muscle, tendons, ligaments, dermis, etc. Each cell type follows a unique pathway with specific conditions for initial differentiation and its own set of local cues required for lineage progression. Manipulation of the cells or the culture conditions can alter the pathway, leading to differentiation of different cell types (Caplan, 1991). Articular cartilage is thought to grow appositionally, i.e. from the articular surface. Archer *et al.* (1994) used antibodies to detect proliferating chondrocytes

and chondrocytes synthesizing IGF-1 and -2 and IGF binding protein in young marsupials and found that as the animals aged, these chondrocyte populations became more restricted to the articular surface, providing supporting evidence for an appositional growth mechanism. In a similar study by Hayes *et al.* (2001), antibodies against TGF-β isoforms were used and the results supported those obtained in Archer's study. In addition, Dowthwaite *et al.* (2004) was able to identify a progenitor cell population on the surface of articular cartilage, lending further support to the appositional growth mechanism theory. Differentiated chondrocytes have been shown to have the ability to dedifferentiate and act as progenitor cells themselves. Cells of chondrogenic, osteogenic, adipogenic, myogenic, and neurogenic lineages have all been produced from dedifferentiated human articular chondrocytes in vitro (Tallheden *et al.*, 2003; Fuente *et al.*, 2004).

In addition to the multipotent cells found in cartilage itself, multipotent mesenchymal cells from other nearby locations can also differentiate and undergo chondrogenesis. Bone marrow-derived mesenchymal cells have been used successfully for chondrogenesis both in vitro and in vivo. Chen *et al.* (2005) were able to remove MSCs from sheep, culture them in a 3-D scaffold, and then implant them back into the joint cavity where they were able to express collagen II and synthesize sulfated proteoglycans. In addition, MSCs cultured in synovial fluid in vitro were also able to undergo chondrocytic differentiation, suggesting the synovial fluid is important in promoting the chondrogenic lineage.

Cells harvested from the synovial membrane have also been shown to possess properties of multipotent mesenchymal cells. These cells are able to proliferate

extensively and maintain their multilineage differentiation potential in vitro. Cultured cells from the human synovial membrane were able to differentiate into chondrocytes, osteocytes, and adipocytes under appropriate culture conditions (De Bari *et al.*, 2001). Some studies have also suggested that cells from the periosteum may exhibit chondrogenic potential (O'Driscoll *et al.*, 1994). However, in a study by Brittberg *et al.* (2005) in which chondrocytes were co-cultured with periosteal tissue, the periosteum was found to play an important role in stimulating chondrocyte growth and differentiation but periosteal tissue itself did not exhibit chondrogenic potential.

Growth factors involved in articular cartilage metabolism

Bone morphogenetic proteins

Bone morphogenetic proteins are members of the transforming growth factor β superfamily. BMPs 1, 2, 4-6, and 11 are present in fetal, adult, and osteoarthritic cartilage, while BMPs 7 and 8 are only present in fetal cartilage and BMPs 9 and 10 only present in osteoarthritic cartilage (Chen *et al.*, 2004). BMPs play an important role in commitment to the chondrogenic lineage and maintenance of chondrocyte phenotype. Enomoto-Iwamoto (1998) transfected cultured chick embryo chondrocytes with dominant negative BMP receptors, which bind BMPs but do not transmit further signals, and found increased cell proliferation, a more fibroblastic morphology, and decreased production of collagen II and aggrecan, suggesting that BMPs are required for maintenance of chondrocyte phenotype and control of cell proliferation. Human multipotent mesenchymal cells cultured in

the presence of BMPs-2 and -9 express collagen II and increased levels of aggrecan, suggesting chondrocytic differentiation. These chondrocytes showed increased expression of Sox-9 transcription factor, which regulates collagen II and aggrecan expression (Majumdar *et al.*, 2001). Park *et al.* (2005) found similar results when bovine synovial progenitor cells were cultured with supplemental BMP-2. Bovine chondrocytes cultured long-term in the presence of BMP-2 maintained increased production of collagen II and aggrecan compared to controls and did not undergo hypertrophy (Sailor *et al.*, 1996). Treatment with BMP-7 (osteogenic protein-1) has also been shown to enhance production of aggrecan and hyaluronan in bovine full-thickness cartilage slices (Nishida *et al.*, 2000) and, when placed in a collagen carrier and implanted into full-thickness cartilage defects in dogs, was able to induce cartilage repair (Cook *et al.*, 2003). Hills *et al.* (2005) showed that BMP-9 was able to induce increased collagen and proteoglycan synthesis in cultured juvenile bovine chondrocytes but did not seem to have a significant effect on adult chondrocytes.

Insulin-like growth factor I

Insulin-like growth factor I (IGF-1) is another growth factor influencing articular cartilage metabolism. IGF-1 affects almost every cell in the body, playing a vital role in normal physiology and growth and development. In articular cartilage, IGF-1 promotes matrix synthesis and cell proliferation, providing a counterbalance against cytokines such as interleukin I-β, which are responsible for cartilage breakdown.

IGF-1 has been shown to help maintain the chondrocyte phenotype in cultured bovine chondrocytes in a 3D matrix (Chaipinyo *et al.*, 2002) and is able to increase collagen II and aggrecan gene expression in the growth zone of goat chondrocytes cultured in monolayer (Darling and Athanasiou, 2005). Along with TGF-β, IGF-1 is also able to stimulate chondrogenesis from bone marrow (Worster *et al.*, 2001) and periosteal mesenchymal stem cells (Fukumoto *et al.*, 2003), with the cultured MSCs exhibiting increased proliferation and production of collagen II and aggrecan, although not to the degree of fully differentiated chondrocytes. IGF-1 is also useful in expansion and redifferentiation of chondrocytes used in tissue engineering. Especially when using autologous chondrocyte transplantation, chondrocytes must first undergo proliferation, during which they often lose their phenotype, and then must redifferentiate before being transplanted into the joint. Pei *et al.* (2002) found that after chondrocytes were expanded in monolayer, subsequent culture with supplemental IGF-1 yielded constructs with high levels of glycosaminoglycans and collagen II. Additionally, IGF-1 plays a role in preventing articular cartilage damage caused by mechanical injury and cytokines. Bovine and human cartilage explants cultured with supplemental IGF-1 after exposure to mechanical injury showed a significant decrease in apoptosis compared to explants cultured without IGF-1 (D'Lima *et al.*, 2001). IGF-1 was also able to increase net proteoglycan synthesis in cultured porcine chondrocytes, even in the presence of interleukin 1β and other cytokines (Tyler, 1989).

Transforming growth factor β

Transforming growth factor β plays a significant role in promoting cartilage anabolism both in vitro and in vivo. TGF-β is most often used in concert with other growth factors such as insulin-like growth factor 1 or basic fibroblast growth factor. Rabbit articular chondrocytes cultured with supplemental TGF-β show marked increases in collagen and glycosaminoglycan production but little change in cell proliferation (Redini *et al.*, 1988). Numerous other studies, reviewed by Grimaud *et al.* (2002), have found the effects of TGF-β in vitro and in vivo to vary depending on environmental conditions. In addition, cell response to TGF-β supplementation in vivo seems to depend on length of exposure, with short-term exposure having the positive benefits mentioned above but long-term exposure leading to disruption of cartilage homeostasis and ultimately cartilage destruction.

When used in combination with other growth factors, TGF-β contributes significantly to the overall stimulation of cartilage matrix synthesis. Darling and Athanasiou (2005) found that when cultured goat chondrocytes were supplemented with IGF-1, bFGF, and TGF-β, TGF-β had the greatest effect on cell proliferation and was able to stimulate protein expression, collagen II production, and glycosaminoglycan secretion in both the superficial and growth zones. Also, cultured bovine chondrocytes exposed to TGF-β, IGF-1, and bFGF exhibited similar proliferation rates and amount of protein synthesized as chondrocytes supplemented with 20% fetal calf serum; however, proteoglycan production was much lower in the serum-free group (Chaipinyo *et al.*, 2002).

In addition to exerting positive effects on chondrocyte proliferation and matrix production, TGF-β is able to affect chondrocyte differentiation and promote chondrogenesis of mesenchymal stem cells. Studies by Jakob *et al.* (2001) and Pei *et al.* (2002) showed articular chondrocytes cultured in monolayer with supplemental TGF-β and FGF-2 had enhanced proliferation rates and increased dedifferentiation. However, these chondrocytes were easily induced to redifferentiate when exposed to IGF-1 or when transferred to a 3D matrix with supplemental growth factors. Chondrocyte dedifferentiation induced by TGF-β/FGF-2 showed by far the greatest amount of redifferentiation when exposed to these same growth factors rather than other growth factors once cultured in a 3D matrix (Jakob *et al.*, 2001). Worster *et al.* (2001) also found that cultured equine MSCs from bone marrow supplemented with TGF-β exhibited increased proliferation, development of a rounded morphology, and somewhat increased proteoglycan content; however, MSC differentiation was not complete when compared to mature chondrocytes and TGF-β had less effect on the MSCs than did IGF-1.

Interleukin-1

Articular function relies on a balance of matrix synthesis and destruction. While the above-mentioned and many other growth factors are responsible for cartilage anabolism, cytokines such as interleukin-1 (IL-1) are responsible for matrix degeneration and are often involved in the pathogenesis of osteoarthritis. Chondrocytes from osteoarthritic patients have increased levels of cytokines, including IL-1 (Shinmei *et al.*, 1991). Chondrocytes cultured in the presence of

IL-1α show increased production of matrix metalloproteinases and release of proteoglycans from the matrix (Shinmei *et al.*, 1989). IL-1 may also play a role in linking mechanical trauma to decreased proteoglycan synthesis. Murata *et al.* (2003) showed when compressed cartilage explants were cultured in the presence of IL-1 receptor antagonist, the decrease in proteoglycan synthesis was minimized, suggesting that IL-1 is in some way responsible for the decrease in proteoglycan synthesis that occurs following mechanical compression.

Impact of mechanical force on articular cartilage

Mechanical factors play a very important role in the development, maintenance, and degeneration of articular cartilage. Articular cartilage requires intermittent hydrostatic pressure to maintain its phenotype and prevent continuing endochondral ossification leading to osteoarthritis (Carter *et al.*, 2004). Low amplitude dynamic compression of bovine cartilage explants increases protein and proteoglycan synthesis and accelerates the biosynthetic response to IGF-1, with the combined anabolic result being much greater than either compression or IGF-1 alone (Bonassar *et al.*, 2001). However, excessive trauma to the joints can lead to the breakdown of articular cartilage and allow the early onset of osteoarthritis.

Osteoarthritis is caused by cartilage degeneration, which occurs when the balance of matrix synthesis and degradation is disrupted. Mechanical trauma can disrupt this balance by causing cell death, loss of proteoglycans, or damage to collagen integrity. Numerous studies utilizing full-thickness cartilage explants

have shown that mechanical compression leads to chondrocyte apoptosis which increases in a dose-dependent manner with increasing peak stress levels (Loening *et al.*, 2000; D'Lima *et al.*, 2001; D'Lima *et al.*, 2001; Patwari *et al.*, 2004). Although there is some thought that chondrocyte apoptosis may be the cause of loss of matrix integrity following injury, Loening *et al.* (2000) found apoptosis to occur at stresses lower than that required to cause matrix degradation, measured by glycosaminoglycan (GAG) release into the media, suggesting apoptosis may be simply one of the earliest responses to injury without actually causing any subsequent matrix degeneration or altered biochemical response. However, a high degree of apoptosis is obviously not going to promote cartilage integrity. One particular study exposed the 2mm center of 4mm cultured cartilage discs to cyclic impact and found that when chondrocytes in impacted regions undergo apoptosis, they send a signal, perhaps nitric oxide or a cytokine such as IL-Iβ, throughout the unimpacted regions nearby, causing formerly unaffected chondrocytes to undergo apoptosis as well (Levin *et al.*, 2001). The induced apoptosis of unimpacted cells could have potentially much more damaging effect on the cartilage than just the effect from mechanical trauma. In addition to cell death, repeated mechanical trauma has been shown to disrupt the collagen framework of bovine articular cartilage explants (Broom, 1986) and strenuous exercise in young horses has resulted in damage and loosening of the collagen framework in the fetlock joint (Brama *et al.*, 2000). Since the collagen fibrils provide the structure that entraps proteoglycans and prevents swelling due to water content, a disruption in the collagen network leads to loss of proteoglycans from the matrix and increased

water content of the cartilage. Torzilli *et al.* (1999) showed that subjecting bovine articular cartilage explants to nominal impact stresses resulted in decreased proteoglycan synthesis and increased water content, and at high enough stress, cell death and rupture of the collagen fibril matrix. Proteoglycan turnover is fairly fast and depends a great deal on environmental conditions; however, collagen turnover is very slow, thus damage to the collagen framework can pose a more serious problem.

The effects of mechanical trauma depend on several factors such as peak stress, rate of loading, and duration of loading. Bovine and canine articular cartilage explants exposed to greater peak stress levels and higher rates of loading or stress rates showed increase cell death and matrix damage, as indicated by altered proteoglycan synthesis, release of GAGs into the media, and cracks in the tissue (Chen *et al.*, 1999; Ewers *et al.*, 2001; Quinn *et al.*, 2001). These experiments indicated high rates of loading can lead to visible cracks in the superficial zone and increased cell death near the cracks, although lesser rates of loading can still cause cell death even if unaccompanied by visible surface damage. Longer duration of loading can also cause increased damage that persists longer. Overall, the extent of injury and patterns of damage vary depending on the nature of the load applied.

The influence of aging on articular cartilage

Aging and osteoarthritis are often considered to go hand in hand. Cartilage degradation naturally occurs with aging due to the additive effects of trauma and

also due to endochondral ossification which dramatically slows down at skeletal maturity but never ceases completely. Loeser *et al.* (2000) examined chondrocytes from joints of primates of various ages and found that osteoarthritis does not always accompany aging; however, there are several factors caused by aging that can encourage the development of osteoarthritis. One of the most noticeable responses due to aging is a decreased response to IGF-1. Cartilage explants from steers were found to synthesize less protein and proteoglycans than cartilage explants from immature calves when stimulated with the same concentrations of IGF-1 (Barone-Varelas *et al.*, 1991). Because of the importance of IGF-1 in maintaining the chondrocyte phenotype and synthesis of matrix components, a decrease in response to IGF-1 disrupts the balance between matrix synthesis and degradation, which ultimately can lead to osteoarthritis. Why this age-related decrease in IGF-1 sensitivity occurs is still under debate. Martin *et al.* (1997) observed that cultured rat chondrocytes from older rats exhibited decreased proteoglycan synthesis and increased expression of IGF binding protein, suggesting decreased response to IGF-1 with aging may be linked to increasing levels of IGF binding protein. However, Loeser *et al.* (2000) showed that cultured rat chondrocytes showed decreased response with aging to both IGF-1 and des(1-3) IGF-1, with the latter having the same ability to stimulate the IGF receptor but decreased affinity for IGF binding protein. These results suggest the IGF receptor may play a larger role than IGF binding protein, perhaps by age-related changes in the IGF receptor or alterations in the intracellular signaling pathway by which IGF exerts its effects. Messai *et al.* (2000) showed that age-related decrease in

response of cultured rat chondrocytes to IGF-1 was correlated with decrease in IGF-1-induced cAMP production and protein kinase C activation, further supporting the idea of decreased ability of the chondrocyte to properly transduce downstream signaling.

Chondrocyte senescence has also been proposed as a factor linking aging and osteoarthritis. After a lifetime of being exposed to mechanical stress, chondrocytes may accumulate oxidative damage that causes them to senesce (Martin *et al.*, 2004). The number of senescent chondrocytes increases with age and the senescent cells may not be able to adequately maintain and repair the articular cartilage (Martin and Buckwalter, 2001; Martin and Buckwalter, 2003). However, it has been shown that cultured chondrocytes taken from older or osteoarthritic patients can begin proliferating and synthesizing matrix components under the right environmental conditions, even if these activities had ceased in vivo.

Current therapies for osteoarthritis

Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin, ibuprofen, indomethacin, diclofenac, naproxen, dexamethasone, and phenylbutazone are widely used to treat the symptoms of osteoarthritis, primarily because they provide pain relief and many can be easily obtained over the counter. However, there is evidence to suggest the use of NSAIDS, especially long-term use, may inhibit cartilage repair processes. Dingle (1991) showed that adding NSAIDS to

cultured porcine and human cartilage samples inhibited proteoglycan synthesis and reduced the ability of the cartilage to recover from treatment with IL-1. Phenylbutazone, the most commonly used treatment for osteoarthritis pain in horses, has been shown to inhibit proteoglycan synthesis almost to the same degree as exposure to IL-1β in articular samples taken from healthy horses (Beluche *et al.*, 2001). In vivo injections of various NSAIDS have been shown to introduce morphological changes leading to degeneration in articular cartilage and subchondral bone in knee joints of rabbits and hens (Kalbhen, 1989). Thus, while these drugs may be effective in reducing inflammation associated with osteoarthritis, their use may actually exacerbate the condition. In addition, NSAIDs may have other undesirable side effects such as gastric ulcers. Many studies, including MacKay *et al.* (1981) and Monreal *et al.* (2004), show that high doses of phenylbutazone administered orally to horses for as little as two weeks caused gastric ulcers and other serious side effects. However, these studies all used much higher doses of phenylbutazone than is recommended for daily use in the treatment of osteoarthritis.

Corticosteroids

Corticosteroids are commonly used in the treatment of osteoarthritis because of their potent anti-inflammatory properties, although there is still much debate concerning their use. Human osteoarthritic patients given intra-articular injections of corticosteroids reported significant decrease in pain lasting up to 8 weeks post-injection. However, by 4 weeks post-injection, there was no significant difference

between treated and placebo groups (Friedman and Moore, 1980). Numerous studies have also indicated that intra-articular steroid injections can damage the integrity of articular cartilage. In 1976, Gibson *et al.* studied the effects of intra-articular steroid injections on primate cartilage. The results showed no difference in the joint structure between treated and control groups, which provided striking contrast to many earlier studies showing deleterious effects of steroid injections. Nevertheless, studies continue to support the idea that intra-articular steroid injections may damage articular cartilage. Fubini *et al.* (2001) showed that chondrocytes taken from young healthy horses and cultured in the presence of corticosteroids had decreased synthesis of collagen II and the fibronectin $(V+C)^-$ isoform, both of which are markers of the chondrocyte phenotype. Intra-articular steroid injections also led to decreased strength, permeability, and thickness in articular cartilage of exercised horses (Murray *et al.*, 1998). There is some thought that the effects of corticosteroids on articular cartilage may vary depending on whether the joints tested are normal or osteoarthritic. Todhunter *et al.* (1998) induced acute synovitis in ponies and then treated half with corticosteroids. There was no difference in protein or proteoglycan synthesis between treated, untreated, and control groups. However, when normal joints were injected with corticosteroids, there was a significant decrease in proteoglycan synthesis and increase in protein synthesis when compared to the three aforementioned groups.

Glucosamine and chondroitin sulfate

Glucosamine and chondroitin sulfate are both important components of articular cartilage proteoglycans, and as such, are often used to treat the symptoms of osteoarthritis. Numerous studies, reviewed by Brief *et al.* (2001), have shown that the use of glucosamine and chondroitin sulfate leads to decreased pain and tenderness, increased mobility, and sustained improvements after drug withdrawal in patients suffering from osteoarthritis. These compounds are thought to have chondroprotective properties, making them more useful than simple anti-inflammatory agents in the treatment of osteoarthritis. Bassler *et al.* (1998) found that articular chondrocytes taken from osteoarthritic patients and cultured in the presence of glucosamine sulfate showed increased production of proteoglycans but no change in DNA synthesis or collagen II production. A more recent study by Dodge and Jimenez (2003) showed that glucosamine sulfate stimulates the production of aggrecan mRNA and inhibits the production and activity of matrix metalloproteinase-3 in cultured chondrocytes taken from osteoarthritic patients, although chondrocytes taken from some patients did not respond at all to glucosamine sulfate. Glucosamine HCl was also able to reduce induced cartilage degradation in equine cartilage explants cultured with lipopolysaccharide or recombinant human IL-1 (Fenton *et al.*, 2000). In vivo, oral glucosamine supplements given to rabbits were able to partially reduce articular degradation caused by anterior cruciate ligament transection (ACLT), as evidenced by decreased loss of proteoglycans and less histologic evidence of disease in the treatment group (Tiraloche *et al.*, 2005). Additionally, human osteoarthritic

patients receiving oral glucosamine supplements showed no continued lessening of joint space width after beginning treatment (Reginster *et al.*, 2001).

Hyaluronic acid

Hyaluronic acid is a major component of synovial fluid and, along with lubricin, helps provide lubrication for the joints. Additionally, each hyaluronic acid chain is capable of binding many molecules of aggrecan and thus is essential for maintaining the large proteoglycan aggregates that remain entrapped in the collagen network and provide cartilage with its unique properties. Because of its vital role in articular cartilage function, hyaluronic acid is commonly used as a treatment for osteoarthritis.

Auer *et al.* (1980) found that intra-articular injections of hyaluronic acid in horses with experimentally induced and naturally occurring osteoarthritis resulted in decreased lameness and improved performance. Lameness was measured using force plates to determine the amount of weight the horses were willing to bear on each leg before and after treatment. Using the similar methods, Gingerich *et al.* (1981) found that a single injection of 20 or 40 mg of hyaluronic acid into the intercarpal joints of horses with experimentally induced osteoarthritis was able to restore normal joint function, with functional improvement evident within one week after treatment. Hyaluronic acid has also been shown to stimulate proteoglycan synthesis in equine cartilage explants (Frean *et al.*, 1999) and significantly reduce chondrocyte apoptosis and nitric oxide production in rabbits with osteoarthritis induced by anterior cruciate ligament transection (Diaz-

Gallego *et al.*, 2005). Numerous other clinical studies in animals and humans, reviewed by Goldberg and Buckwalter (2005), have shown that injections of hyaluronic acid prevent cartilage destruction following experimental induction of osteoarthritis, help promote tissue repair and cartilage metabolism, and reduce joint space narrowing caused by osteoarthritis.

Gene therapy

Another treatment option currently being explored is gene therapy. Because osteoarthritis typically occurs when the balance between matrix production and breakdown is disrupted, controlling the cellular factors that are responsible for these changes may prove beneficial. Frisbie and McIlwraith (2000) used adenoviral mediated gene transfer to cause over-expression of interleukin-1 receptor antagonist in the intercarpal joints of horses. Results showed improvement in clinical parameters of pain and disease activity, less cartilage erosion, and decreased loss of proteoglycans in treated horses.

Inducing cells to overexpress BMP-7 has also shown successful results. Hidaka *et al.* (2001) used an adenovirus vector encoding for human BMP-7 to induce cultured bovine chondrocytes to express BMP-7. The results showed increased proteoglycan and protein synthesis and increased expression of the cartilage-specific genes for collagen II and aggrecan. When transplanted into cultured cartilage explants, these chondrocytes were able to form tissue 1.9 times thicker than that produced by control chondrocytes. In a later study also by Hidaka *et al.* (2003) chondrocytes over-expressing BMP-7 were transplanted into

full-thickness cartilage defects in horses and were able to cause accelerated healing and a more hyaline-like morphology when compared to controls.

Mesenchymal stem cell transplantation

As described above, mesenchymal stem cells from the bone marrow, synovium, and periosteum have all been able to undergo chondrocytic differentiation in culture and in some cases, in vivo. These findings suggest transplantation of mesenchymal stem cells may be used to repair cartilage defects. Wakitani *et al.* (1994) cultured stem cells taken from bone marrow and periosteum and transplanted them into full-thickness cartilage defects in the knees of rabbits. The stem cells were able to undergo chondrocytic differentiation and completely repair the defects in the cartilage and subchondral bone. In a similar experiment, Grande *et al.* (1995) transplanted MSCs from muscle into rabbit knees and achieved comparable results.

Autologous chondrocyte transplantation

Autologous chondrocyte transplantation (ACT) is the process of using a patient's own chondrocytes to treat articular cartilage defects. A cartilage biopsy is taken from a non-weight bearing region in a joint, chondrocytes are isolated from the cartilage and expanded in monolayer, and the chondrocytes are then transplanted back into the cartilage defect and sealed with a flap of some sort, usually periosteal. One of the earliest experiments utilizing this procedure was performed in rabbits with successful results (Peterson *et al.*, 1984). Brittberg *et al.*

(1994) used the same procedure to treat full-thickness cartilage defects in the knee joints of humans. Patients reported reduced pain and swelling and arthroscopy showed the transplants grew level with the surrounding cartilage and exhibited properties of normal hyaline cartilage. Litzke *et al.* (2004) used Brittberg's ACT procedure to treat extensive cartilage defects in horses, demonstrating for the first time the efficacy of ACT in a large animal model. All sites into which chondrocytes were transplanted showed filling of cartilage defects level and fully integrated with the surrounding cartilage and the newly grown cartilage produced collagen II and other specific markers for the articular cartilage phenotype.

ACT continues to evolve, with different materials being considered for use as a flap to seal the defect and tissue engineered scaffolds made from biomaterials gaining use as a means of holding the chondrocytes in the defect area and enhancing proliferation and differentiation (Marlovits *et al.*, 2006).

Summary

Articular cartilage is a unique tissue that enables the joints to withstand a large amount of loading while operating in a smooth fashion. The maintenance of articular cartilage is extremely important for proper joint function and is controlled by a balance between matrix synthesis and degradation. Often as patients age or subject their joints to extreme mechanical stress, such as during heavy exercise, the cartilage loses its ability to maintain the extracellular matrix and cartilage degradation ensues, leading to osteoarthritis. While there are many available treatments for osteoarthritis, many have limited effectiveness. Because

lameness in general and osteoarthritis in particular represent a tremendous loss in profitability due to veterinary costs, loss of usefulness, and additional labor, it is vital for the equine industry to find improved ways of treating osteoarthritis that can be widely available and cost-effective.

MATERIALS AND METHODS

Lymph Node Preparation

Supramammary lymph nodes were harvested from cows at slaughter (Brown's Packing, Gaffney, SC), and fat and other connective tissues were removed. Nodes were ground with a tabletop meat grinder (Hobart) and frozen at -80ºC. Frozen node grind was broken into chunks under liquid nitrogen and freeze-dried. The dried chunks were ground into fine powder using a small food processor and stored at -80ºC in vacuum-sealed bags.

Working Stock Preparation

A 10%-by weight solution of lymph node extract was prepared by resuspending lymph node powder in phosphate buffered saline (anhydrous Na_2HPO_4, 795 mg/L; KH_2PO_4, 114 mg/L; NaCl, 9000 mg/L). The solution was allowed to incubate for 10-20 minutes , followed by centrifugation at 1000 x *g* for 15 minutes. The top fat layer and bottom precipitate layer were discarded. The middle fluid layer was saved and heat-inactivated at 60ºC for 60 minutes. Heat-inactivated lymph node extract (LNE) was centrifuged at 9000 x *g* for 30 minutes. The supernatant was removed and stored at -20ºC. The LNE was filter sterilized using 0.2 μm Acrodisc syringe filters (Pall Corporation, Ann Arbor, MI) prior to use in cell culture.

Ammonium Sulfate Fractionation

Ammonium sulfate (AS) solutions were prepared by adding AS powder to 10 ml distilled, deionized water and stirring until AS was dissolved. Ten ml of lymph node working stock was added to the flasks, which were then placed in beakers, covered with ice, and stirred for 30 minutes. The solutions were transferred to thick-walled 15 ml conical tubes and centrifuged at 9000 x *g* for 30 minutes. Each solution was dialyzed against phosphate buffered saline (PBS) using SnakeSkin® Pleated Dialysis Tubing (Pierce, Rockford, IL; 7,000 MWCO) until the osmolarity reached 280-320 mOsm/kg. The osmolarity was measured using a vapor pressure osmometer.

Protein Assay

The protein concentration of each sample was determined by measuring absorbance at 260 and 280 with an Eppendorf® Biophotometer. The protein concentration was calculated by the following formula:

$$\text{Concentration (mg/ml)} = (1.55A_{280} - 0.76A_{260}) \times \text{dilution factor}$$

(Warburg and Christian, 1942)

Each sample was diluted 1:10 – 1:200 with PBS to ensure the concentrations remained constant across the different dilutions. In addition, the concentrations were confirmed using a handheld refractometer.

Chondrocyte Harvest

Scapular cartilage was harvested from euthanized adult horses (n=3). Muscle and connective tissue were removed and cartilage was washed with 70% ethanol.

Cartilage was minced and transported on ice in medium containing DMEM (with 4.5g/L glucose; without L-glutamine and sodium pyruvate) and 5x antibiotics (penicillin, 200 units/ml; streptomycin, 200 μg/ml; amphotericin, 0.5 μg/ml), followed by overnight digestion with collagenase (1.85 mg/ml), DNase (50 μg/ml), and hyaluronidase (50 μg/ml) at 37ºC under stirred conditions. Following digestion, the medium was poured through a cell strainer and centrifuged at 1000 x *g* for 20 minutes at 23ºC. The cell pellet was resuspended in medium containing DMEM, 10% bovine growth serum (HyClone, Logan, UT), and 2x antibiotics. Cells were placed in a 75 cm^2 tissue culture flask and incubated at 37ºC (5% CO_2). At confluency, medium was removed, and cells were washed with PBS and trpsinized. The cell suspension was centrifuged for 20 minutes at 1000 x *g*. Cells were counted using a hemacytometer and resuspended to a concentration of 1 x 10^6 cells/ml in medium containing 50% bovine growth serum (BGS) and 5% DMSO. Cells were frozen overnight at -80ºC, then stored in liquid nitrogen.

Cell Culture

Cells were thawed and cultured in DMEM, 10% BGS, and 2x antibiotics as described above. At confluency, cells were washed, trypsinized for 10-15 minutes at 37ºC, and passaged into 96 well plates at a density of 5000 cells per well (Day 0). On Day 3, medium was removed, cells were washed with PBS, and treatment medium was added. For Experiment 1, the medium was supplemented with either 0-10% BGS or 0-10% LNE. For Experiment 2, medium was supplemented with BGS or LNE on an equal protein basis (0.5 mg/ml to 5.0 mg/ml). Treatments for

Experiment 3 included BGS, LNE, and LNE fractions obtained from 20% or 30% AS solution precipitation. All media contained an equal protein load (1.5mg/ml). Finally for Experiment 4, treatments included BGS, LNE, insulin-like growth factor-1 (IGF-1), and transforming growth factor-β (TGF-β). Bovine serum albumin (BSA) was added to medium containing IGF-1 and TGF-β to maintain a protein concentration of 1.0 mg/ml. Concentrations of IGF-1 ranged from 10 to 500 ng/ml and concentrations of TGF-β ranged from 0.25 to 20 ng/ml. Treatment media containing equal amounts of protein were prepared by determining the desired amount of protein in milligrams and freeze-drying the appropriate volume of each supplement. Just prior to cell culture, the freeze-dried supplement was resuspended in DMEM with antibiotics to ensure the volume of DMEM also remained the same for every treatment. The control medium for all experiments contained only DMEM and antibiotics. In addition, some wells received no medium at all on Day 3 so that the cells would rapidly desiccate, die, and serve as a reference value for the DNA accumulated between Day 0 and Day 3. On Day 7, treatment medium was removed and plates were stored at -20ºC. Each experiment was performed with each of the three primary chondrocyte isolates, with multiple replicates for each treatment.

Measurement of Cell Proliferation

Cell proliferation was measured using the CyQUANT® Cell Proliferation Assay Kit (Invitrogen, Carlsbad, CA). Cell proliferation results are reported as a stimulation index calculated by dividing the DNA content of each well at the end

of culture by the DNA content of the wells that received no medium on Day 3. A standard curve using known cell numbers was generated each time the assay was performed to ensure that consistent results were obtained for each assay.

Microscopy

Photographs of cultured cells were taken with a SPOT camera on an Olympus IMT-2 inverted phase contrast microscope using the 10X and 20X objectives.

Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was used to visualize components of each protein sample. Both 15 % and 4-20% gradient gels were used (Bio-Rad Laboratories, Hercules, CA). Samples containing equal amounts of protein were diluted 1:2 with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) containing 5% β-mercaptoethanol (2-ME) and boiled for 3 minutes at 100ºC. Proteins were separated at 170V for 30-40 minutes. Following electrophoresis, the gels were washed 3 times for 15 minutes each with nanopure water then stained with Gel Code Blue Stain Reagent (Pierce, Rockford, IL).

Statistical Analysis

Results were analyzed using SAS (SAS Institute, Cary, NC) PROC MIXED ANOVA to evaluate effects of treatment, concentration, and the interaction between treatment and concentration for each experiment. P values were adjusted

using the Sidak procedure to control experimentwise error rate. The Sidak adjustment utilizes the following equation:

> Adjusted p-value = $1 - (1 - \text{unadjusted p-value})^{k}$ where k = number of comparisons
>
> (Sidak, 1967)

Additionally, effects of date and horse were analyzed to ensure no significant differences existed between experiments conducted on different days or with chondrocytes isolated from difference horses. α was set at 0.05.

RESULTS

Protein Assay

The protein concentration of each supplement was measured so that treatment media could be normalized for protein load. The protein concentration of BGS was 68.0 mg/ml ± 4.2 (SEM), and the protein concentration of LNE was 23.6 mg/ml ± 0.9 (SEM). The protein concentrations of ammonium sulfate fractions were 2.9 mg/ml ± 0.2 (SEM) and 2.2 mg/ml ± 0.3 (SEM), for 20% AS and 30% AS, respectively.

Experiment One: BGS vs. LNE - Unequal Protein Basis

Cell proliferation in response to treatment with media containing 0-10% (v/v) BGS and LNE was investigated. Cells cultured in medium containing greater than 5% BGS did exhibit greater proliferation than those receiving control medium, but cell proliferation in response to treatment with LNE was not different from the control at any concentration. Additionally, there was no significant difference in cell proliferation between cells cultured with BGS and cells cultured with LNE at any concentration. (Fig. 1)

Cultured chondrocytes had an elongated cobblestone-like appearance and exhibited expanded nuclei and prominent nucleoli. Cell morphology remained consistent over all concentrations of BGS and most concentrations of LNE (Fig. 2A-C). Cells receiving the highest LNE concentrations (>9%) showed a dramatic

change in morphology (Fig. 2D). They became more rounded with less prominent nuclei and a darker, somewhat grainy appearance.

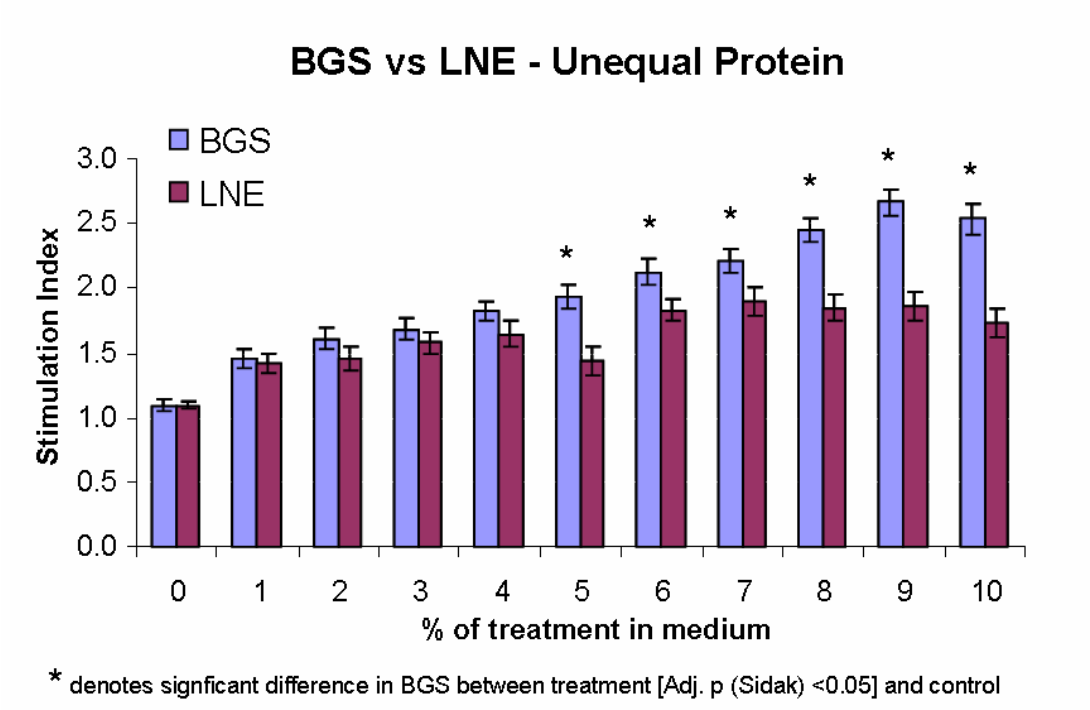


Figure 1: Bovine Growth Serum vs. Lymph Node Extract – Unequal Protein Basis

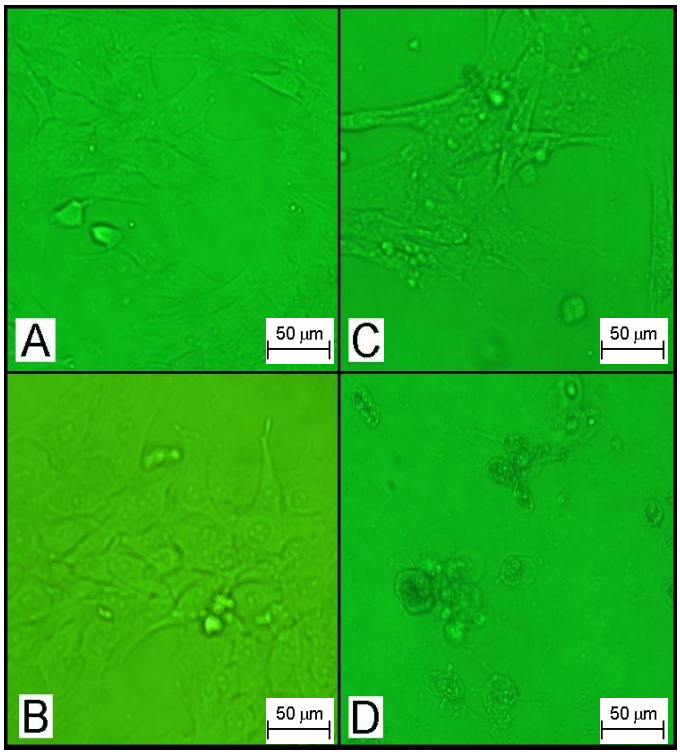


Figure 2: Morphology of cells receiving BGS or LNE. Panels A and B depict cells receiving medium containing 5% BGS and 10% BGS.Panels C and D depict cells receving medium containing 5% LNE and 10% LNE. (10X objective)

Experiment Two: BGS vs. LNE – Equal Protein Basis

In the previous experiment, cell proliferation in response to treatment media containing BGS and LNE on a volume/volume basis was investigated. Because of the substantial difference in protein concentration between BGS and LNE, the next experiment compared cell proliferation in response to treatment media containing BGS and LNE on an equal protein basis. No significant difference in proliferation was found between cells receiving BGS and those receiving LNE at any concentration (Figure 3). Additionally, there was no difference in

proliferation between cells cultured in the control medium and cells cultured in treatment media at any concentration.

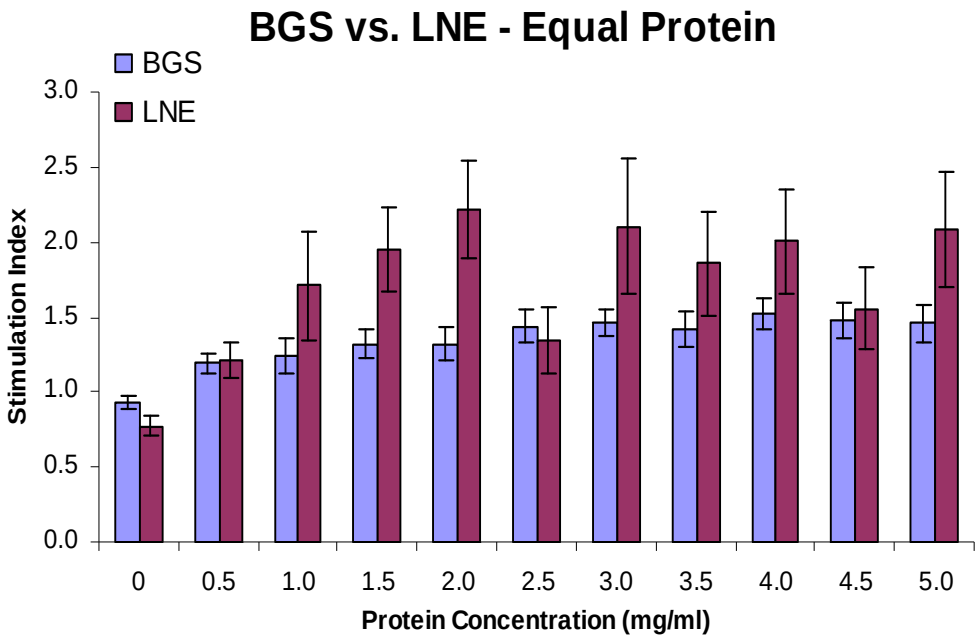


Figure 3: Bovine Growth Serum vs. Lymph Node Extract – Equal Protein Basis

Experiment 3: BGS and LNE vs. LNE fractions from precipitation with 20% and 30% ammonium sulfate – equal protein basis

Cells cultured in treatment media containing LNE fractions from ammonium sulfate precipitation were compared to cells cultured in media containing BGS and LNE to determine if certain LNE fractions possessed factors more likely to stimulate or inhibit cell proliferation. Cells receiving LNE at 1.5 mg/ml exhibited greater proliferation than cells receiving BGS or either AS fraction on an equal protein basis [Adj. p (Sidak) < 0.05] (Fig. 4) When comparing just the AS

fractions over a range of protein concentrations (0.5 mg/ml – 1.5 mg/ml), there was no difference in proliferation between the 20% fraction and the 30% fraction at any concentration (Fig. 5). However, cells cultured in media containing the 20% AS fraction at 0.5 mg/ml and 1.0 mg/ml and the 30% AS fraction at 0.5 mg/ml showed a significant increase in proliferation over cells cultured in the control medium.

Cells receiving BGS or AS fractions exhibited the same elongated, cobblestone-like morphology described previously. (Fig. 6A, C, D) Cells receiving LNE were more rounded and less spread out than the others. (Fig. 6B)

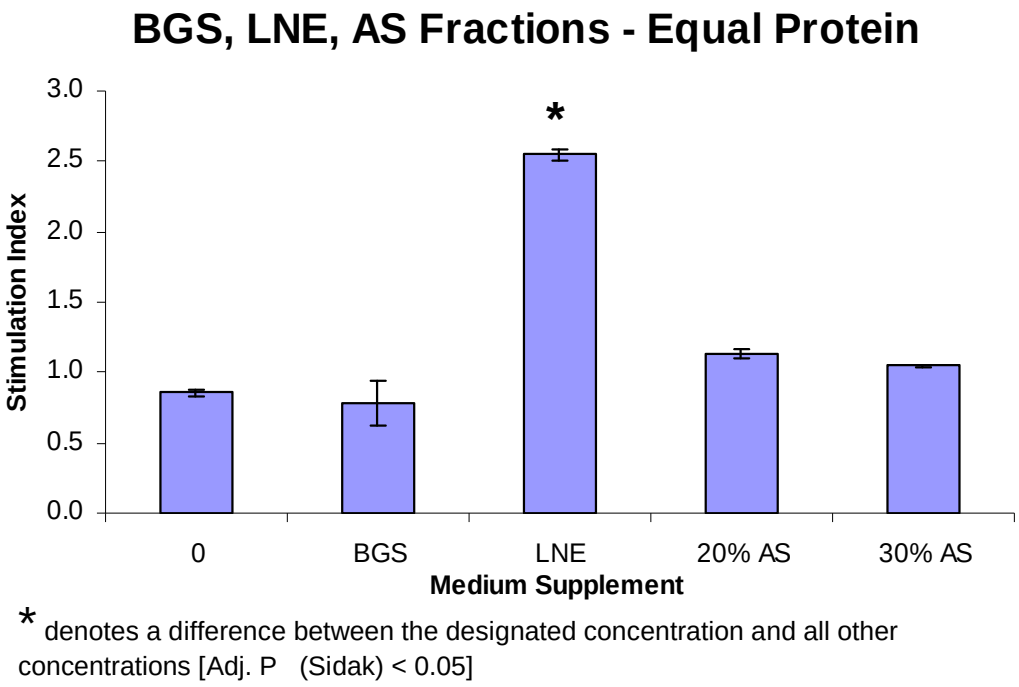
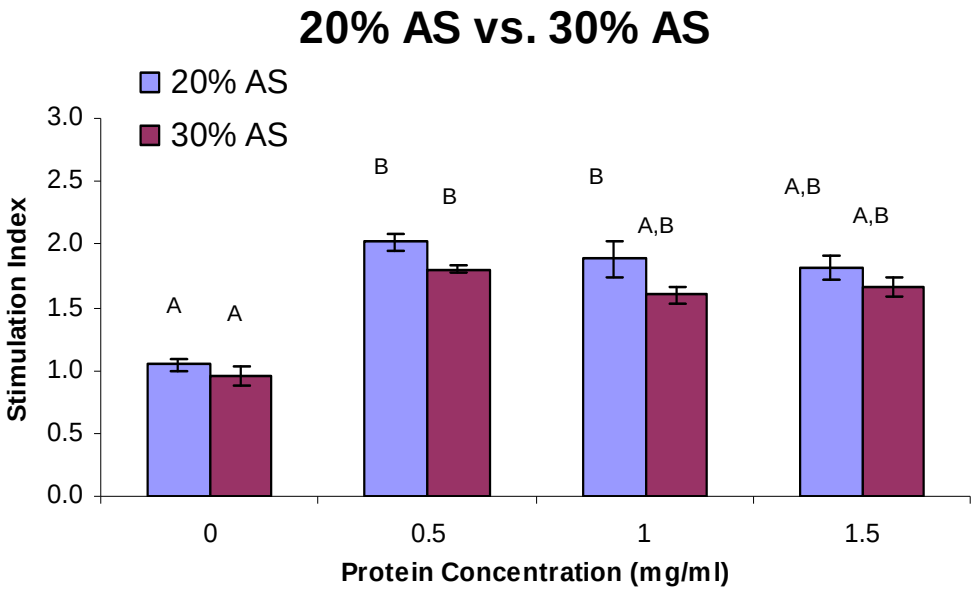


Figure 4: BGS and LNE vs. LNE fractions from precipitation with 20% or 30% ammonium sulfate. Equal protein basis (1.5 mg/ml).



Different letters indicate differences between treatments [Adj. p (Sidak) < 0.05]

Figure 5: LNE fractions from precipitation with 20% or 30% ammonium sulfate over a range of protein concentrations. Equal protein basis (0 – 1.5 mg/ml).

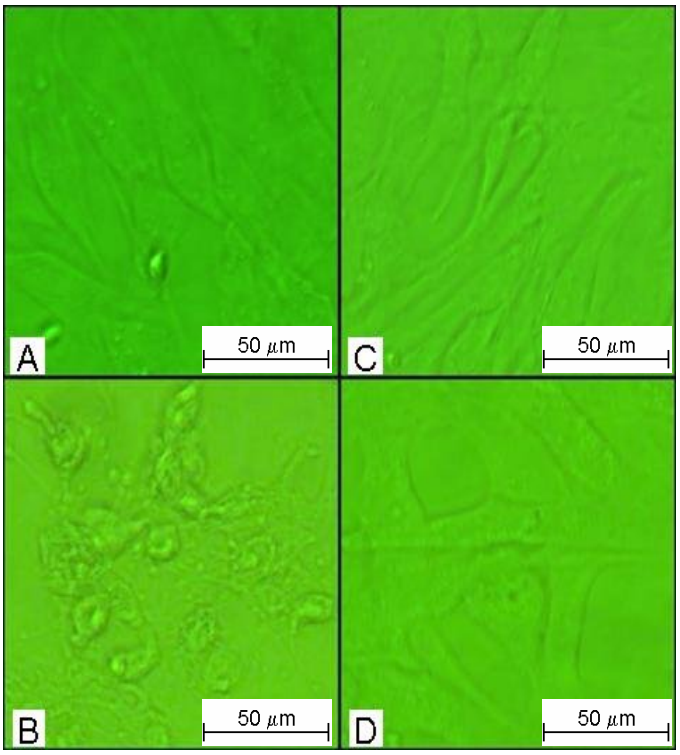


Figure 6: Cultured cell morphology. Panel A depicts cells receiving BGS, Panel B depicts cells receiving LNE, and Panels C and C depict cells receiving LNE fractions from precipitation with 20% ammonium sulfate and 30% ammonium sulfate, respectively. (20X objective)

Experiment 4 – Supplementation with IGF-1 and TGF-β

IGF-I and TGF-β are often added to chondrocyte culture media. Cell proliferation in response to culture in media containing IGF-I and TGF-β was investigated. There was no difference in cell proliferation between any of the IGF-1 concentrations (Fig.7). Additionally there was no difference in proliferation between cells receiving IGF-1 and those receiving control medium, or between cells receiving IGF-1 and those receiving medium supplemented only with BSA.

Cell proliferation was also compared to that from cells receiving 1 mg/ml BGS or LNE, since IGF-1 treatment medium also contained 1 mg/ml BSA. There was no difference in proliferation between cells receiving any concentration of IGF-1 and those receiving 1 mg/ml of either BGS or LNE.

There was no difference in cell proliferation between any concentrations of TGF-β and no overall effect of supplementation with TGF-β (; Fig.8) Additionally, there was no difference in proliferation between cells receiving TGF-β and those receiving control medium or medium supplemented only with BSA, nor was there any difference in proliferation between cells receiving TGF-β and those receiving 1 mg/ml of either BGS or LNE.

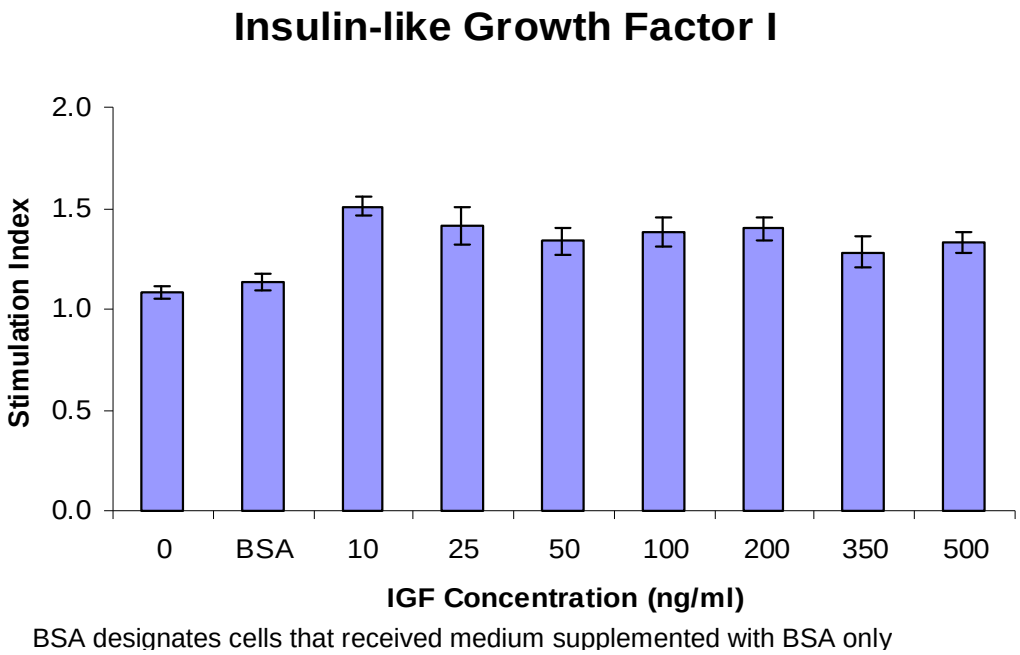


Figure 7: Cell proliferation in response to IGF-1

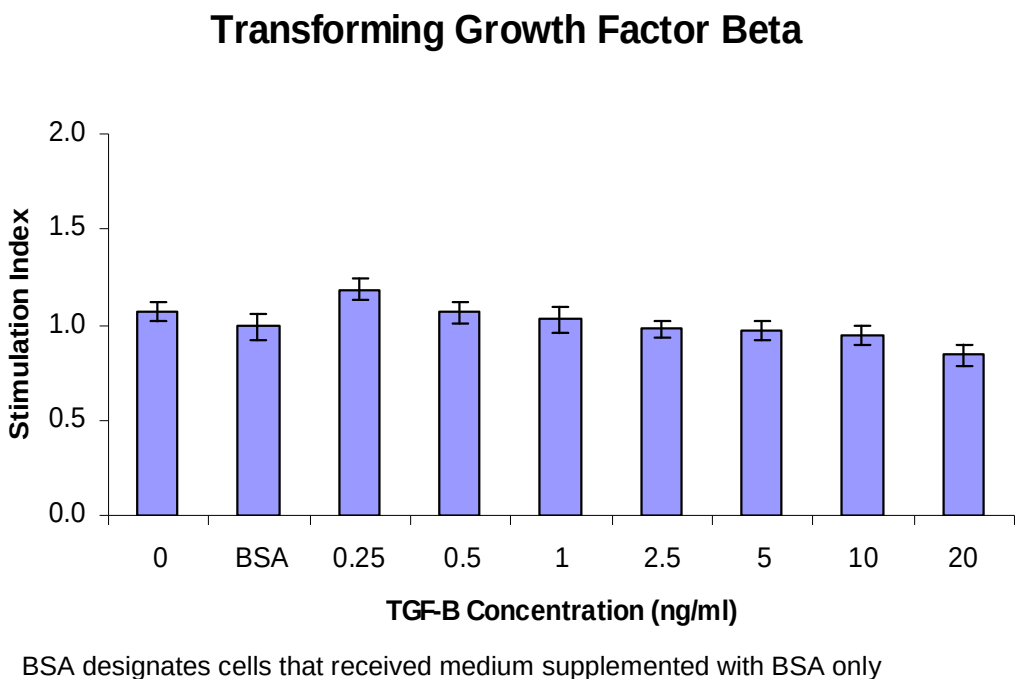


Figure 8: Cell proliferation in response to TGF-β.

Gel Electrophoresis

There did not appear to be bands present in either BGS or LNE that disappeared after precipitation with 20% or 30% AS. All four samples showed heavy dark bands between 50 and 75 kDa and others at 25 and 10-15 kDa. However, the 20% and 30% AS fractions had darker bands present in the lower molecular weight range (< 50 kDa) than either BGS or LNE (Fig. 9) Bands less than 25 kDa were almost indistinguishable for BGS and were very light for LNE. Gradient gels showed many dark bands in the 10 – 15 kDa region rather than one band darker than the others as seen in the 15% gel (Fig. 10). Protein load was approximately the same in each lane.

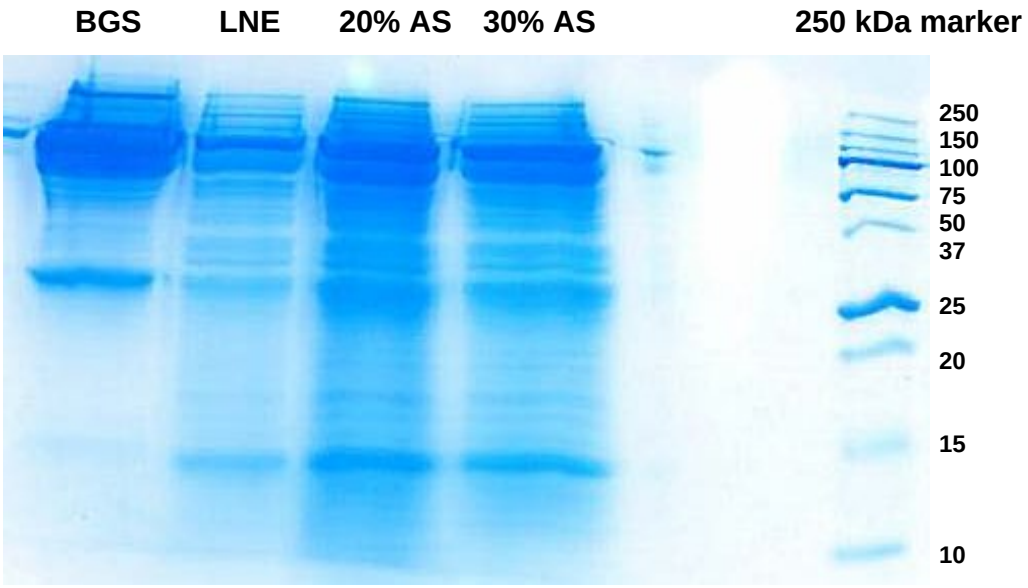


Figure 9: 15% polyacrylamide gel of BGS, LNE, and LNE fractions

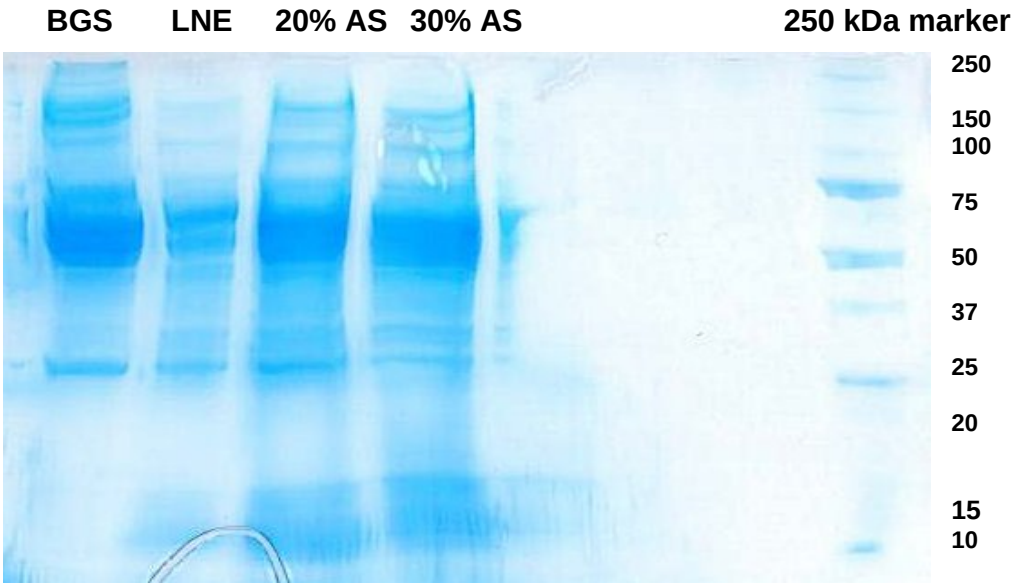


Figure 10: 4-20% polyacrylamide gradient gel of BGS, LNE, and LNE

DISCUSSION

The results suggest LNE is capable of stimulating cell proliferation to an equal or greater degree than BGS. For Experiment 1, which compared BGS and LNE on a volume/volume basis, results indicate no difference in cell proliferation between cells receiving medium supplemented with BGS and medium supplemented with LNE. Considering the respective protein values of BGS and LNE (68.0 mg/ml ± 4.2 and 23.6 mg/ml ± 0.9), a greater increase in proliferation in response to BGS compared to that of LNE would have been expected. The fact that LNE managed to perform reasonably well against BGS, even at a much lower protein concentration, suggests there may be growth factors present in LNE capable of stimulating cell proliferation to a greater degree than those present in BGS. However, the change in cell morphology which occurred at higher levels of LNE supplementation may also indicate the presence of factors which are detrimental to the cells at higher concentrations. A similar change in morphology occurred in preliminary experiments utilizing non-heat inactivated LNE and was accompanied by decreased proliferation and cell death.

Experiment 2 compared BGS and LNE on an equal protein basis to correct the large difference in protein concentration occurring when the culture medium was supplemented with BGS or LNE on a volume/volume basis. Results from Experiment 1 suggested that LNE may be capable of stimulating increased proliferation than BGS when protein concentrations of both were equal. However, the results from Experiment 2 did not support this hypothesis. When BGS and

LNE were compared across a range of protein concentrations, there was no difference between treatments at any concentration. There also was no significant effect of increasing protein concentrations within either treatment. However, these results may be inconclusive. Figure 3 indicates that the average proliferative response to LNE was much higher at some concentrations than BGS, but there was also a great deal of variation, causing the differences to be insignificant. Experiment 2 was conducted on one day only and there was much more variation within treatments than had occurred for any other experiment on any other day. Cells from only two horses were used, and there were fewer replicates within each treatment. Cell culture, media preparation, and proliferation assays were conducted in the same manner as for every other experiment. If the experiment was conducted again with more replications and included cells from the third horse, more consistent results could be obtained.

Experiment 3 compared the response to BGS and LNE with the response to LNE fractions from precipitation with 20% and 30% AS. Precipitation was not carried out with greater than 30% AS because preliminary experiments showed no difference between 30% AS and other subsequent fractions. 1.5 mg/ml was arbitrarily chosen as the protein concentration used because it was the highest common dilution that could be achieved with all four samples based on the protein quantity recovered that day from the precipitation procedure. Results from Experiment 3 showed significantly higher cell proliferation in response to treatment with LNE [Adj. p (Sidak) < 0.05]. Cell proliferation was not different among cells receiving BGS or either AS fraction, and none of the these three

treatments resulted in proliferation different from the control. The difference between BGS and LNE supports the conclusion from Experiment 1 that LNE may induce greater proliferation than BGS on an equal protein basis. The increased response to LNE was also expected in Experiment 2. Experiment 3 was conducted on two different days with chondrocytes from all three horses and fourteen replicates per treatment. The results obtained in Experiment 3 suggest that LNE may be capable of inducing increased proliferation, despite the results of Experiment 2. However, cells receiving LNE did exhibit a change in morphology. While not as pronounced as the change in morphology occurring in cells receiving high concentrations of LNE in Experiment 1, this change in morphology indicates that, in addition to increased proliferation, the cells respond differently to LNE than to the other treatments.

The difference in proliferation between LNE and the two AS fractions could indicate that proteins involved in stimulating cell proliferation were lost during the fractionation procedure. It could also indicate that the precipitation procedure resulted in an increased concentration of suppressive factors in the AS fractions. The response could also come from the combined effect of proteins lost, interactions between different proteins, and increased concentrations of proteins left in the fractions. Gel electrophoresis showed no obvious disappearance of bands after LNE fractionation but did show darker bands in the lower molecular weight region, indicating increased concentrations of those proteins. Since many growth factors tend to have lower molecular weights, there may be increased concentration of growth factors in the fractions. However, since there was no

increase in proliferation in response to either fraction, this may not be the case. There may also be a greater concentration of suppressive factors.

When comparing just the two AS fractions over a range of concentrations, there was no difference in proliferation between cells receiving either fraction. However, lower concentrations of both fractions resulted in increased proliferation over the controls, indicating that there are still factors present in the fractions capable of stimulating cell proliferation. However, as the protein concentration of both fractions increased, cell proliferation decreased, although not significantly so. Nevertheless, decreased proliferation could indicate an increased concentration of suppressive factors in the AS fractions.

Experiment 3 would have been carried out over a greater range of protein concentrations, 0.5 – 5.0 mg/ml rather than just 0.5 to 1.5 mg/ml, but media contamination was a problem. All treatments were filter-sterilized just prior to cell culture, but cells receiving medium supplemented with AS fractions became contaminated with bacteria nearly every time the experiment was carried out. Cells receiving other treatments were never affected, and the source of contamination was never identified.

Experiment 4 tested the effects of supplementing the culture medium with IGF-1 or TGF-β. These two growth factors were selected because they have often been used to stimulate chondrocyte proliferation, differentiation, and cartilage matrix synthesis (Chaipinyo *et al.*, 2002; Darling and Athanasiou, 2005; Jakob *et al.* 2001; Pei *et al.* 2002; Worster *et al.*, 2001) Results showed no effect on proliferation in response to either growth factor. No concentration of either factor

resulted in proliferation different from the control, although proliferation did appear to decrease slightly with increased concentration, but not significantly so. The concentrations of IGF-1 and TGF-β were selected based on published concentrations of each factor used to stimulate chondrocytes in *vitro*. A range of concentrations from much lower to much higher than the published concentrations was used for each treatment. Additionally, treatment media contained 1 mg/ml BSA to act as a protein source since all other treatments contained protein from BGS or LNE. There was no difference in proliferation among cells receiving either growth factor, medium containing only BSA, and control medium. There also was no difference in proliferation between cells receiving any of the latter and those receiving 1 mg/ml BGS or LNE. These results indicate that none of these treatments are capable of stimulating cell proliferation.

Just because treatments are not stimulating proliferation does not mean they do not have any effect on the cells at all. In many instances, IGF-1 and TGF-β stimulate production of collagen and aggrecan rather than stimulating proliferation. Differentiated chondrocytes that are producing matrix components do not tend to proliferate. In many cases increased proliferation is seen as a sign of dedifferentiation (Aulthouse et al., 1989; Archer et al., 1990; Schnabel *et al.*, 2002). It is possible that cells not proliferating in response to these different treatments may be differentiated and trying to produce matrix components. However, chondrocytes cultured in monolayer often become dedifferentiated (Aulthouse et al., 1989; Archer et al., 1990; Schnabel *et al.*, 2002) so this may not be the case. As a point of interest, cells exposed to LNE (>1.5 mg/ml) developed a

more rounded morphology which is often associated with the differentiated phenotype (Kolettas et al., 1998) The production of collagen II and aggrecan by these cells would confirm that they have remained differentiated, while the absence of these would indicate a return to a dedifferentiated, fibroblastic state.

Conclusion

Overall, LNE was capable of stimulating chondrocyte proliferation to an equal or greater degree than BGS, as shown in three experiments. Further studies are needed to determine if certain LNE fractions possess greater mitogenic activity than the whole nodal extract. Gene expression studies would show whether or not chondrocytes treated with LNE retain their phenotype. Additional experiments investigating proliferation of chondrocytes cultured in a three dimensional matrix versus monolayer and proliferation of chondrocytes passaged directly into medium containing LNE without the three-day adjustment period could provide more insight as well. However, bovine serum is the most tested and tried, commonly used supplement for cell culture medium, and LNE was able to stimulate cell proliferation to the same or greater extent. These results alone suggest LNE could promote cartilage healing and has the potential to be used in developing new therapies for osteoarthritis in horses.

APPENDIX

CyQUANT Standard Curve

A standard curve, created from known chondrocyte numbers, was generated each time the CyQUANT assay was performed. Figure 1 shows the standard curve generated for each of 45 separate assays. The trend line shown is based on the average fluorescence output seen for each known quantity of cells.

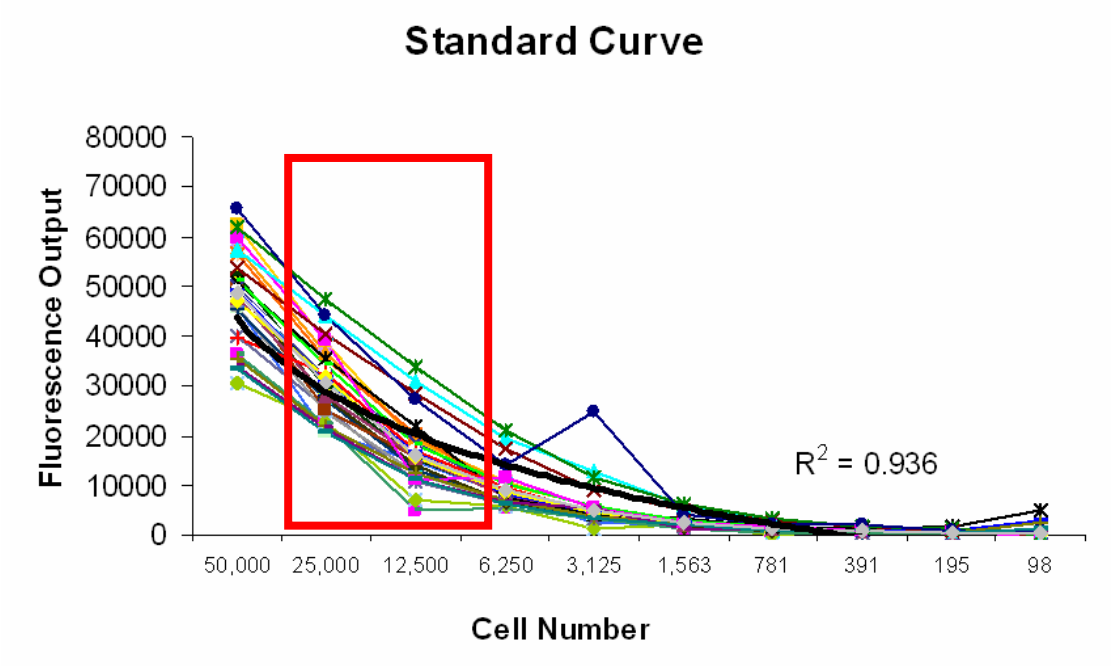


Figure A-1: CyQUANT assay standard curve based on known chondrocyte numbers. The vast majority of observations recorded for cultured cell samples fell within the boxed portion of the curve, where linearity was most consistent.

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