

**Insulin-like growth factor-I in growing horses and RNA isolation from
small articular cartilage samples**

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State
University
in partial fulfillment of the requirements for the degree of

Master of Science
In
Animal and Poultry Sciences

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August 10th 2007
Blacksburg, VA

Keywords: IGF-I, synovial fluid, plasma, skeletal development, equine growth, RNA
isolation, articular cartilage

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ABSTRACT

A longitudinal study was designed to characterize developmental patterns of plasma (**PL**) and synovial fluid (**SF**) total insulin-like growth factor –I (**IGF-I**) concentrations, as well as their association with measurements of skeletal growth in Thoroughbred horses. Horses were randomly assigned to one of two dietary treatment groups and fed diets with either a high or low starch content to examine the effects of dietary energy source on PL and SF IGF-I. At 3, 6, 9, 12 and 15 mo of age, PL and carpal SF samples were collected for analysis of total IGF-I. Body weight gain, wither height gain and forearm length gain were calculated for the 90 day periods between SF and PL sampling. No influence of diet on PL or SF IGF-I was detected ($P > 0.05$). Average SF IGF-I concentrations were $30.1 \pm 1.8\%$ of that found in PL, and PL and SF IGF-I were positively correlated ($r = 0.48$, $P = 0.0003$) There was an effect of month of age on both PL and SF IGF-I concentrations ($P < 0.05$). There was a positive correlation between all measures of gain except forearm length gain with PL and SF IGF-I ($r = 0.41$ to 0.55 , $P < 0.05$). In our second study, we evaluated the use of a liquid-nitrogen cooled mortar and pestle, motorized freezer mill and rotor-stator homogenizer for homogenization of small (<50mg) articular cartilage samples. The rotor-stator homogenizer produced quantifiable RNA yields, and was used to evaluate three different RNA isolation protocols. Two of the protocols were commercially available RNA extraction kits, with the third a modified guanidinium isothiocyanate/acid-phenol extraction procedure. The combined average yield for all protocols was 91.9 ng RNA/mg of cartilage. All protocols yielded a sufficient quantity of quality RNA suitable for gene expression analysis.

ACKNOWLEDGEMENTS

The completion of this thesis would not have been possible without the help of the many faculty, students, staff, family members, and friends that have supported me through my graduate coursework and research. First, I would like to thank the members of my committee. I am grateful that they have allowed me to complete this thesis, and for their support and encouragement throughout the process. Special thanks to my advisor, Dr. Burt Staniar for accepting me into this program and encouraging my growth as a scientist over the past two years. His genuine concern for the well-being of his students and ability to challenge my way of thinking have made these past two years an experience I will never forget. Thanks to Dr. Tschetter for putting up with me in the lab, and for all of the help with my revisions.

The moral support I have received from the graduate students has been invaluable. Tania Cubitt, Lindsey George, Bridgett Macintosh and Kibby Trieber helped to show me the ropes and encourage my research. Becky Carter was also largely responsible for collecting the growth data that was vital for this project, and for that I am truly grateful. In Blacksburg, Sarah Denham has been there to help study for classes, share ideas and help me figure out what I really am doing here in the first place. Thank you to Tracy Smith, quite possibly the greatest lab tech in the world. Without her I may not have even been able to locate all of the samples, but she kept everything organized and even stayed late to help me run ELISA's in the lab.

I would like to also extend my gratitude to the students and staff of the MacLeod Laboratory at the University of Kentucky. Jamie MacLeod and Mike Mienaltowski were extremely helpful in helping me to perfect the RNA isolation

protocol. I am looking forward to working with them in the future, even if it means playing lucky Christmas music all year long.

Last but not least, I would like to thank my family for their support and understanding throughout all of my endeavors. With high expectations they have encouraged me to pursue my dreams, and I know that they will stand behind me no matter what I do.

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CHAPTER I

Introduction

Healthy skeletal growth and development is vital to the future performance of the equine athlete. Abnormalities in skeletal development leading to the occurrence of developmental orthopedic diseases (**DOD**) contribute to significant economic losses in the equine industry (Jeffcott, 1996). In order to develop management strategies designed to avoid DOD, it is first important to gain an understanding of the chemical communication network regulating skeletal development. Components of the somatotrophic axis such as growth hormone (**GH**) and insulin-like growth factor-I (**IGF-I**) help direct growth in the young animal. IGF-I is particularly important in skeletal development, acting as a regulatory factor which directs chondrocyte proliferation (Lindahl et al., 1987).

Many factors including age, season and nutrition may influence changes in the somatotrophic axis. Possible links between diet and the occurrence of DOD continue to be investigated. Specifically, it has been suggested that diet induced changes in circulating IGF-I may play a role in the etiology of DOD (Henson et al., 1997b, Ralston, 1996). A more clear understanding how diet influences circulating IGF-I may be useful in creating dietary management strategies designed to avoid DOD. Of particular interest is the relationship between dietary energy source and circulating concentrations of IGF-I (Ropp et al., 2003, Staniar et al., 2007, Treiber, 2005). We designed a longitudinal study in young Thoroughbred horses fed either high or low starch diets in an effort to further characterize this relationship. Examining total circulating IGF-I in these horses is a

necessary step in investigating the potential influence of diet on endocrine actions of IGF-I. However, circulating IGF-I provides little insight into the chemical communication network to which chondrocytes are exposed. Articular cartilage is an avascular tissue, and therefore chondrocytes are reliant upon surrounding synovial fluid for provision of hormones and nutrients. Thus, one of the objectives of our study was to investigate synovial fluid IGF-I concentrations in addition to circulating IGF-I.

Circulating IGF-I is an important, although broadly acting regulatory factor in skeletal development (Yakar et al., 2002). Chondrocyte response to IGF-I has been studied *in vitro*, but information defining the relationship between circulating and synovial fluid IGF-I and skeletal growth in horses is lacking. Circulating IGF-I has been positively correlated with measurements of growth such as average daily gain in bodyweight, cartilage maturation and curves of body growth (Davicco et al., 1994, Fortier et al., 2005, Staniar et al., 2007). In this study, we hoped to further define the link between growth and circulating IGF-I by examining the relationship between direct measurements of skeletal growth, synovial fluid and plasma IGF-I concentrations.

Information regarding molecular mechanisms which direct equine chondrocyte metabolism has been primarily obtained from isolated chondrocytes *in vitro*. Chondrocyte behavior *in vitro* may differ greatly from that within articular cartilage (Cancedda et al., 1995). Collection of articular cartilage samples from horses exposed to different dietary treatments would provide the most accurate assessment of chondrocyte metabolism. However, the acellular nature of articular cartilage makes it difficult to collect sufficient amounts of RNA for gene expression analysis (McKenna et al., 2000). More robust RNA isolation techniques are needed before *in vivo* chondrocyte metabolism

may be accurately assessed. In order to investigate optimal RNA isolation procedures, several tissue homogenization methods were evaluated. Next, three RNA extraction protocols were evaluated for use with small (≤ 50 mg) articular cartilage samples.

Objectives

1. Determine relationship between circulating and synovial fluid concentrations of total IGF-I in growing Thoroughbreds.
2. Investigate influence of dietary energy source on circulating and synovial fluid concentrations of total IGF-I.
3. Determine relationship between measurements of skeletal growth, circulating and synovial fluid concentrations of total IGF-I.
4. Develop a robust method for isolation of high quality total RNA from small (<50mg) equine cartilage samples for use in transcriptional profiling experiments.

Hypothesis

1. Total IGF-I concentrations in synovial fluid will be lower than that in circulation, but changes in concentration will follow a similar pattern throughout growth.
2. Horses fed a high starch diet will display higher circulating and synovial fluid concentrations of total IGF-I than horses fed diets low in starch.
3. The correlation between concentrations of total IGF-I in synovial fluid will be at least as strong, if not stronger than the correlation between circulating IGF-I and measurements of skeletal growth.
4. Modifications to commercial RNA extraction protocols will increase total RNA yield and quality obtained from small (<50mg) equine cartilage samples in order for use in transcriptional profiling experiments.

Literature Review

IGF-I Physiology

Originally known as somatomedin C, IGF-I is a 76 kDa single chain peptide hormone that shares structural homology with pro-insulin. Biological actions of IGF-I are mediated by the IGF-I receptor (**IGF-IR**). The IGF-IR is comprised of hormone binding sites on two extra-cellular alpha-subunits and two membrane-spanning beta-subunits which include a tyrosine kinase domain within the intracellular segment. Binding of IGF-I to the receptor activates the receptor tyrosine kinase domain which results in receptor autophosphorylation and tyrosine phosphorylation of multiple substrates (LeRoith et al., 1995). These initial tyrosine phosphorylation reactions are transduced through a network of serine/threonine kinases that are ultimately responsible for cell proliferation, modulation of tissue differentiation, and protection from apoptosis (Laviola et al., 2007).

Synthesis. Up to 75% of circulating IGF-I is of hepatic origin, with many other tissues also contributing to total circulating IGF-I concentrations (D'Ercole et al., 1984, Yakar et al., 2002). Both endogenous and recombinant GH (Mathews et al., 1988, Ropp et al., 2003) as well as insulin (Russell-Jones et al., 1992) increase hepatic IGF-I production. Increased circulating IGF-I provides negative feedback for GH stimulation of hepatic IGF-I synthesis (Wallenius et al., 2001).

Transport. Less than 1% of total IGF-I circulates in free form, with the remaining percentage bound to 1 of at least 6 known structurally related IGF-I binding proteins (**IGFBP**). The IGFBP may sequester IGF-I receptor binding activity or enhance IGF-I activity at the cellular level. The majority of IGF-I present in circulation (70-80%) is found in ternary complex with IGFBP-3 and the acid-labile subunit, a glycoprotein

produced in the liver (Yakar et al., 2002). IGF-I has a greater binding affinity for this complex than the IGF-IR. Therefore, the IGFBP play a primary role in regulating the bioavailability of IGF-I. Specific proteases may decrease the affinity of IGFBP-3 with the IGF-I/IGFBP-3/acid-labile subunit complex. This protease activity results in increased binding of IGF-I to the IGF-IR (Clemmons et al., 1983, Flanagan et al., 2000, Frystyk et al., 1997, Holt et al., 2003). Activity of IGFBP may also change in response to diet, having an inverse relationship with insulin secretion (Holt et al., 2003). Binding activity of IGFBP also likely follows circadian variation (Holly et al., 1988). Frystyk and colleagues (2003) suggested that increased nocturnal IGFBP activity and resulting decreases in free IGF-I may serve to reduce the overall insulin-like activity at a time when there is no nutritional intake.

Factors influencing IGF-I Secretion

Gender and Age. In horses, circulating concentrations of IGF-I are highest in intact males, with mares and geldings demonstrating lower but similar IGF-I concentrations (Cymbaluk and Laarveld, 1996, Malinowski et al., 1996, Noble et al., 2007). Mammalian species show a general decline in both GH and IGF-I synthesis with age (Sherlock and Toogood, 2007). This was demonstrated in a study where mares and geldings aged 1 to 18 years displayed a gradual decrease (50-70%) in serum IGF-I concentrations with advancing age (Noble et al., 2007). Stallions in this study had higher circulating IGF-I overall and demonstrated a less severe (20-30%) decline in serum IGF-I with increasing age. Another study with young fillies found that plasma concentrations of IGF-I increased from birth through 14 d, remaining relatively constant until 9 mo of

age. Thereafter, plasma IGF-I followed a general decline (Malinowski et al., 1996). Others have suggested that serum IGF-I peaks at a slightly earlier age (225 d), defining the onset of puberty (Fortier et al., 2005).

Seasonal Effects. Horses do not demonstrate a clear circadian rhythm in circulating IGF-I, but may display changes associated with seasonal variation (Noble et al., 2007). Factors such as day length, temperature, and pasture nutrient availability help to define the relationship between the environment and circulating concentrations of IGF-I. Increased average daily temperature has been positively correlated with circulating concentrations of IGF-I in cattle (Sarko et al., 1994), sheep (Lincoln et al., 2001) and horses (Staniar et al., 2007). Burton and colleagues (1992) attributed elevated circulating IGF-I concentrations to increases in tissue IGF-I mRNA synthesis in response to increased temperature (Burton et al., 1992). Longer day length has also been positively correlated with circulating IGF-I in horses and cattle (Dahl et al., 1997, Staniar et al., 2007). In the northern hemisphere, seasonal peaks in circulating IGF-I tend to correspond with spring months. It is notable that this is a time of compensatory growth and associated increases in average daily gain (**ADG**) in young Thoroughbreds (Staniar et al., 2004). Significant but moderate correlations ($P < 0.001$, $r = 0.32$) between ADG and circulating IGF-I have been described in growing horses, further supporting the link between growth and IGF-I (Cymbaluk and Laarveld, 1996, Staniar et al., 2007).

Nutrition. Grazing animals are exposed to seasonal variations in pasture nutrient availability. Changing nutrient composition of the diet may be yet another factor which contributes to seasonal variation in circulating IGF-I. During the spring months in the northern hemisphere pasture dietary energy, crude protein and non-structural

carbohydrate content increase by approximately 5-10% as compared to the winter months (Cubitt, 2004). Nutrition of foals may also have a long-lasting impact on circulating IGF-I concentrations. Foals fed commercial milk replacer displayed lower serum IGF-I concentrations than those that suckled on their dams (Cymbaluk and Laarveld, 1996). At weaning total IGF-I decreased in all foals. Differences in serum IGF-I between mare-nursed and milk-replacer foals persisted to 1 yr of age despite similarities in dietary management and body weight of the foals. The authors suggested that early exposure to growth factors found in mare milk but absent in milk replacer are responsible for these continuing effects.

Nutritionally induced changes in GH and insulin may lead to indirect stimulation and/or inhibition of IGF-I. Short term (< 48h) feed restriction in adult geldings did not cause changes in circulating concentrations of IGF-I (Christensen et al., 1997). In contrast, long term dietary energy and/or protein restriction may lead to decreased IGF-I production despite increased GH production (Sticker et al., 1995). These authors concluded that localized tissue IGF-I synthesis declined drastically in response to nutritional deficiencies, overshadowing any GH induced IGF-I production. Local decreases in GH receptor activity may contribute to these changes (Sticker et al., 1995).

Long term (> 6 weeks) changes in dietary energy source may lead to adaptive changes in components of the somatotropic axis (Pratt et al., 2006), but conflicting data has been presented regarding the influence of dietary energy source on circulating IGF-I. One longitudinal study followed Thoroughbreds fed diets high in either sugar and starch or fat and fiber as energy sources from birth through 16 mo of age (Stanjar et al., 2007). These authors found that circulating concentrations of IGF-I were higher in horses fed

diets high in sugar and starch (non-structural carbohydrates (NSC) = 53%) than those fed a low starch diet (NSC = 24%) at approximately 360 days of age during the months of April and May. Another study utilizing similar dietary treatments sampled Thoroughbred weanlings which were approximately 230 days old and again found higher circulating IGF-I in those fed the diet high in sugar and starch (NSC = 49%) as compared to a diet high in fat and fiber (NSC = 12%) (Treiber et al., 2005). In contrast, a study with weanling Quarter Horses between 151 to 226 d of age found no differences between serum IGF-I in those fed starch-based concentrate (NSC = 34%) or those receiving a fat supplemented diet (NSC = 24%) (Ropp et al., 2003). It should be noted that dietary treatments administered to the Thoroughbred foals were the same as those fed to their dams throughout gestation and lactation. However, Quarter Horse foals were not acclimated to their respective treatment diets until after weaning. As discussed previously, early nutrition of foals may have lasting effects on circulating IGF-I (Cymbaluk and Laarveld, 1996).

The effects of dietary energy source are of particular interest in relation to the etiology of DOD. Previous studies have found a correlation between meals which create high glycemic and insulinemic responses meals and the development of DOD ($r = 0.74$) (Pagan et al., 2001). One *in vitro* study demonstrated that increased exposure to insulin suppressed chondrocyte differentiation and apoptosis in foal neonatal chondrocytes (Henson et al., 1997a). These authors hypothesized that relative hyperinsulinaemia may therefore be a contributory factor to DOD. Staniar and colleagues (2007) found that horses adapted to high glycemic meals displayed higher circulating IGF-I concentrations during a period of rapid growth in the spring months. Rapid growth has previously been

associated with the development of DOD, as has increased IGF-I gene expression in articular chondrocytes (Ruff et al., 1993, Semevolos et al., 2001). The etiology of DOD is multifactorial, and the data suggests diet, growth rate and IGF-I as important contributing factors. Further research investigating how dietary energy source influences IGF-I production and metabolism may help to clarify the relationship between dietary energy source and DOD.

Cartilage Physiology

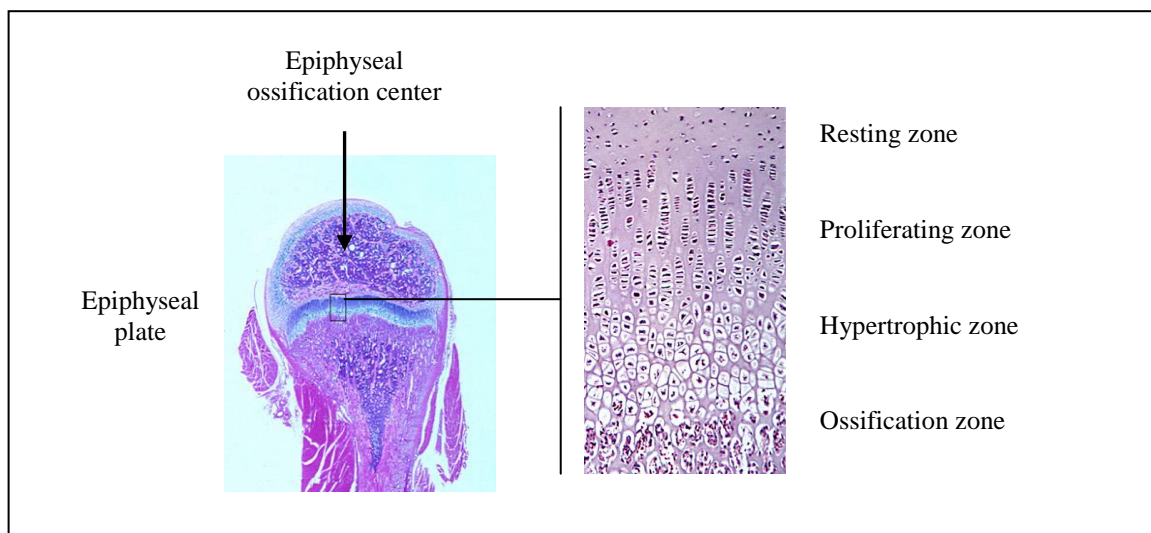


Figure 1. The epiphyseal ossification center and zones of cartilage proliferation.

Adapted from *Skeletal Function and Form*, DR. Carter (Carter, 2001)

Growth plate cartilage. Various nutritional, neuronal, and hormonal mechanisms direct linear bone growth at the epiphyseal growth plate. Located near the end of long bones, the growth plate consists of several distinct, zones of chondrocytes (Figure 1).

Within the growth plate chondrocytes undergo two distinct phases of growth: cell

division, which takes place in the proliferating zone, and cell differentiation, which occurs in the hypertrophic zone (Yakar et al., 2002b). Terminal differentiation of chondrocytes occurs through a process leading to endochondral ossification.

Early *in vitro* research demonstrated that IGF-I increased growth plate chondrocyte DNA (Smith et al., 1989). This led to further work clarifying the role of IGF-I in directing both chondrocyte proliferation and differentiation (Smith et al., 1989, Lupu et al., 2001). IGF-I exerts differential effects on chondrocyte proliferation and differentiation through multiple, partially interacting intracellular signaling pathways (Ciarmatori et al., 2007). Ciarmatori and colleagues (2007) found that suppression of mitogen-activated protein kinase/extracellular signal-regulated kinase (**MAPK/ERK**), protein kinase A (**PKA**) pathway by H-89 or KT5720, and the PKC pathway by bisindolylmaleimide suppressed IGF-I-stimulated cell proliferation. In contrast, IGF-I enhanced early cell differentiation was not affected by inhibition of the MAPK/ERK1/2 pathway. Inhibition of the PI-3 kinase, the PKC and the PKA pathways diminished IGF-I enhanced cell differentiation. These authors proposed that chondrocytes progressively inactivate IGF-I signaling pathways as they progress from proliferating cells to early and terminal differentiating cells.

While GH helps to direct these processes, the exact relationship between GH, IGF-I and chondrocyte proliferation has long been debated. The traditional “somatomedin hypothesis” suggests that the major role of GH in bone growth is through regulation of hepatic IGF-I production (Le Roith et al., 2001). This idea was supported by data suggesting the absence of IGF-I mRNA in growth plate chondrocytes (Shinar et al., 1993, Wang et al., 1995). More recent research using a liver IGF-1-deficient knockout

mouse model has suggested that a threshold concentration of circulating IGF-I is likely necessary for normal skeletal growth (Yakar et al., 2002a). The influence of hepatic IGF-I production in response to GH has also been implicated by data demonstrating that GH does not specifically bind to juvenile chondrocytes or increase IGF-I mRNA levels (Hutchison et al., 2007). Other researchers believe that IGF-I is produced locally by chondrocytes under the influence of GH (Reinecke M 2000, Isaksson OG 1987, Isgaard 1988). As evidence has been provided for both the somatomedin hypothesis and local production of IGF-I by chondrocytes, it is likely that IGF-I may mediate chondrocyte development and thus longitudinal bone growth in an endocrine as well as autocrine/paracrine manner.

Articular Cartilage. Located at the ends of long bones, articular cartilage creates a low friction surface that enables joints to withstand weight bearing through their range of motion. The cellular portion of articular cartilage is composed of chondrocytes which produce and maintain components of the extracellular matrix. Collagen within the extracellular matrix provides its tensile strength, while proteoglycans are responsible for its compressive resistance. As in growth plate cartilage, IGF-I increases articular cartilage chondrocyte metabolism (Trippel et al., 1989). Cultured chondrocytes exposed to IGF-I demonstrate increased collagen and proteoglycan synthesis and deposition. (Jenniskens et al., 2006, Smith et al., 1989). IGF-I may also influence nutrient transport into chondrocytes through upregulation of glucose transporters (Phillips et al., 2005).

An increasing amount of attention has been focused on the role of IGF-I in adult articular cartilage maintenance and repair. Processes which inhibit normal physiological and anabolic actions of IGF-I have been implicated in the etiology of degenerative

diseases such as osteoarthritis (Mobasher et al., 2002). Although less widely studied, limited research had demonstrated that altered IGF-I metabolism may be a contributing factor to several forms of DOD. Young, growing horses affected by Juvenile Digital Degenerative Osteoarthropathy display lower circulating concentrations of IGF-I than clinically normal horses (Lejeune et al., 2007). Foals with osteochondrosis also showed significantly lower serum IGF-I activity than osteochondrosis-negative foals (Sloet van et al., 1999). Further research is needed to examine the specific molecular mechanisms that may lead to these abnormalities.

Chondrocytes are exposed to a limited blood supply for provision of hormones and nutrients during early stages of growth. Articular cartilage becomes increasingly avascular until approximately 6 months of age, when cartilage canals disappear (Fortier et al., 2005, Shingleton et al., 1997). At this time chondrocyte exposure to circulating concentrations of hormones and nutrients becomes contingent upon their diffusion through the synovial membrane (Stockwell, 1979). Single chondrocytes are surrounded by vast areas of matrix through which IGF-I must be transported to reach a responsive cell. This transport is hindered in areas of greater cartilage thickness but facilitated by joint advection in response to loading (Mauck et al., 2000, Stockwell, 1979). To further investigate the effects of joint loading on synovial fluid and chondrocyte metabolism, researchers incubated normal chondrocytes in synovial fluid collected from either exercised or rested horses. Chondrocytes incubated in the synovial fluid from exercised horses demonstrated elevated metabolism as evidenced by increased turnover of the extracellular matrix. These authors concluded increased chondrocyte metabolism could

be attributed to higher concentrations of IGF-I found in the synovial fluid of exercised horses (van de Lest et al., 2000).

In human subjects the concentration of total IGF-I in synovial fluid is an order of magnitude lower than that in serum, while concentrations of free IGF-I are similar in both fluids. (Schneiderman et al., 1995). This difference may be partially explained by the limited permeability of the synovium (Schneiderman et al., 1995). Bioactivity of IGF-I is regulated through a balance of IGF-I, IGFBP, and protease activity (Bhakta et al., 2000, Matsumoto et al., 1996, Zhang et al., 2007). It is likely that the 150 kDa IGF-I/IGFBP-3/ALS ternary complex is too large to pass through the synovial membrane. It has previously been shown that complexes of smaller size than the ternary complex such as 67 kDa IgG are partially excluded from the synovial space (Levick, 1991). The majority of total IGF-I in circulation is found in a “large” IGFBP complex as compared to small complex (82 and 17%, respectively). IGF-I in synovial fluid is bound in nearly equal proportions to large and small IGFBP complexes (47 and 52%, respectively) (Schneiderman et al., 1995). Concentrations of IGF-I in SF may also reflect local production of IGF-I by chondrocytes, which varies in response to ageing as well as nutrient availability (Martin et al., 1997, Richardson et al., 2003). Previous research in this area has been obtained from humans and it is not known if similar relationships between circulating and synovial fluid concentrations exist in horses.

Chondrocyte RNA Isolation

Challenges. Chondrocyte phenotypes *in vitro* differ greatly from that found *in vivo* (Cancedda et al., 1995). Thus, information about chondrocyte metabolism obtained

from *in vitro* research may not provide an accurate assessment of chondrocyte behavior within articular cartilage. There are a number of obstacles to obtaining data from articular cartilage samples including restricted sample size, the acellular nature of the tissue and high levels of proteoglycans within the tissue. Removal of small biopsies allows for subsequent sampling from the same individual, but creates limitations due to small sample size. Another concern is the capacity of articular cartilage to repair itself at the site of harvest. This is particularly an issue in older individuals due to low mitotic activity, the absence of vessel and nerve supply, and the immobility of chondrocytes within articular cartilage (Mandelbaum et al., 1998). Biopsy size that may be harvested without producing significant joint damage has not been established in horses. A study utilizing a canine model found that harvest of samples as small as 250mg from the stifle joint negatively affected cartilage strength and integrity at other sites within the joint (Lee et al., 2000). Yields from current RNA isolation protocols may be as low as 0.001-0.018 $\mu\text{g}/\text{mg}$ of tissue (McKenna et al., 2000). This suggests that sample size must be greater than 100mg in order to obtain several micrograms of RNA for gene expression analysis.

Protocols that are commonly used to isolate RNA from animal tissues are often unsuccessful with articular cartilage samples. Traditional extraction methods are designed to be used with highly cellular tissues and not those of an acellular nature such as articular cartilage. The actual composition of cartilage also presents obstacles for the isolation of pure RNA. The extracellular matrix is composed of a high proportion of large, negatively charged proteoglycans which tend to co-purify with RNA (Carney and Muir, 1988).

Tissue disruption and homogenization. The first and one of the most critical steps in isolating RNA from tissues is to ensure complete tissue disruption and homogenization. Various methods have been developed for this purpose. It is important to avoid RNA degradation by maintaining samples at a very low temperature. Snap freezing of samples in liquid nitrogen is frequently performed for this reason. Many homogenization methods also incorporate the use of liquid nitrogen to maintain sample temperature. Use of a liquid nitrogen-cooled mortar and pestle is often successful but inappropriate for use with small samples due to inherent sample loss during the homogenization process (Fehr et al., 2000). A motorized freezer mill is also effective for homogenization of larger samples but inherent sample loss is problematic for smaller samples (McKenna et al., 2000). The ability of rotor-stator homogenizers to handle small samples has led to their increased use with cartilage samples (Baelde et al., 2001, Smith et al., 2006).

Cell lysis and RNA purification. Inhibition of RNase activity remains a concern throughout tissue disruption and homogenization. In addition to the use of liquid nitrogen to maintain temperature, many lysis buffers contain RNase inhibitors such as beta-mercaptoethanol (Louveau et al., 1991). Placing samples in such lysis buffers and maintaining a low temperature throughout the homogenization process effectively minimizes RNA degradation. Lysis of chondrocytes may be facilitated through the use of mono-phasic solutions of phenol and guanidine isothiocyanate. Several commercial solutions have been formulated for this purpose based upon an acid guanidinium thiocyanate-phenol-chloroform mixture proposed by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Addition of chloroform and subsequent centrifugation

separates the extraction solution into aqueous and organic phases (Louveau et al., 1991). Disturbance of the interphase and lower organic phase creates a risk for proteoglycan contamination of RNA within the aqueous phase. Some isolation protocols used for cartilage suggest isopropanol precipitation of RNA (Flannery et al., 1999, McKenna et al., 2000). Others have found no difference in final RNA quality with the omission of this step (Clements et al., 2006). Silica membrane columns are frequently used for further purification and isolation of chondrocyte RNA (Fehr et al., 2000).

RNA quality assessment. Many methods may be used to assess the quality of isolated RNA. Optical density measured by UV Spectroscopy at 260nm and 280nm can provide insight into RNA purity. A 260nm/280nm ratio greater than 1.8 is indicative of pure RNA (Glasel, 1995). Residual organic contaminants such as proteoglycans may be detected by the presence of a peak at 230 nm (Manchester, 1996). Further evaluation may involve electrophoresis analysis utilizing agarose gel. The proportion of the ribosomal bands (28S:18S) has conventionally been viewed as the primary indicator of RNA integrity, with a ratio of 2.0 considered to be typical of high quality intact RNA (Sambrook, 1989).

Although the above methods are frequently used for tissue RNA analysis they may not be practical for use with cartilage samples. The quantity of RNA required for these methods creates a challenge when small cartilage samples are used. In recent years RNA quality assessment using microfluidic capillary electrophoresis with the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) has become popular (Schroeder et al., 2006a). The electrophoresis trace created by the Bioanalyzer (Figure 2) may be used to assign a RNA integrity number (RIN) to each sample (Mueller et al., 2006). The RIN is

calculated using an algorithm which considers features such as the height of the 18S peak; the ratio of the area of the ribosomal bands compared with the total area of the electropherogram; and the ratio of the fast area of the electropherogram to the total area of the electropherogram. The number assigned by the RIN software ranges from 1-10, with 1 being the most degraded profile and 10 being the most intact. The RIN has been positively correlated with success of downstream reactions (Schroeder et al., 2006b). Samples with an $RIN \geq 7$ are generally considered ideal for downstream reactions, although quality standards may vary between individual laboratories.

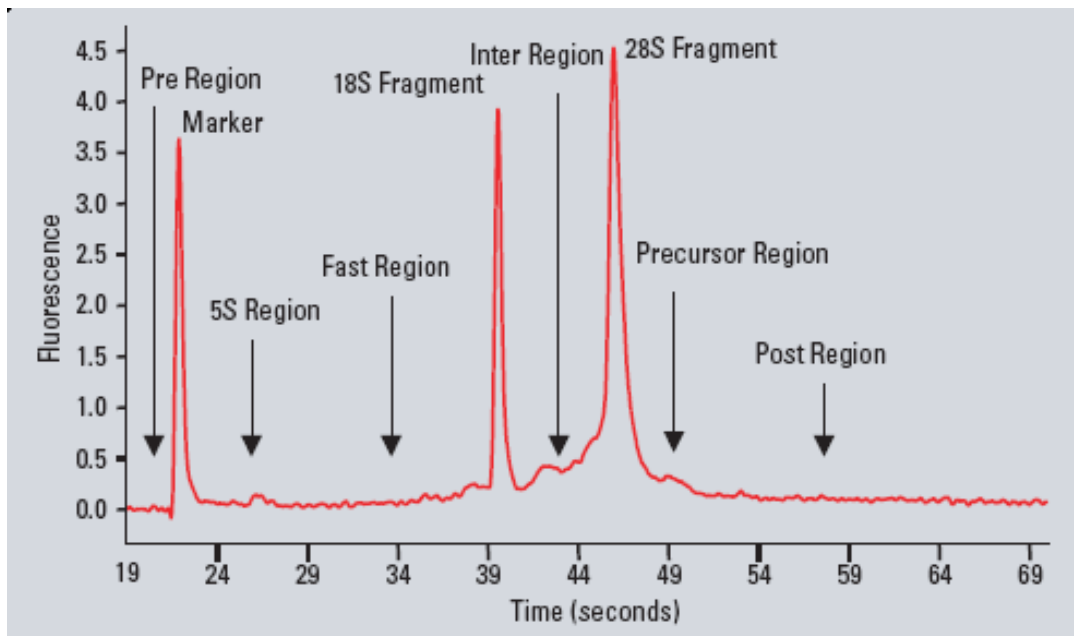


Figure 2. Electropherogram created by the Bioanalyzer (Agilent Technologies, USA) detailing the regions that are indicative of RNA quality. These regions are used for the calculation of an RNA integrity number (RIN), which indicates the quality of purified RNA within a sample.

Calculation of the RIN has proven more reliable than methods previously used to assess RNA integrity (Imbeaud et al., 2005). A study comparing several methods found

that the RIN was more sensitive and specific for determining RNA quality than UV spectrometry (Clements et al., 2006). This technology has the additional benefit of requiring only a small amount (as low as 200 pg) of sample for comprehensive quality analysis.

In summary, the nature of articular cartilage presents many challenges for isolating RNA from small samples. While UV spectroscopy is useful for assessing RNA purity and more specifically proteoglycan contamination, the Bioanalyzer provides an accurate assessment of RNA integrity using a small amount of sample (Clements et al., 2006).

CHAPTER II

Synovial fluid and plasma insulin-like growth factor I in growing Thoroughbred horses

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ABSTRACT

The objective of this longitudinal study was to characterize developmental patterns of plasma (**PL**) and synovial fluid (**SF**) total IGF-I concentrations, as well as their association with measurements of skeletal growth in Thoroughbred horses. In addition, the influence of dietary energy source on PL and SF IGF-I was examined. Broodmares were randomly assigned to one of two dietary treatment groups and fed isocaloric and isonitrogenous ($DE = 3.0 \pm 0.02$ Mcal/kg, $CP = 14.8 \pm 0.01$ %) rations with either a high starch (**HS**, starch = $39 \pm 2\%$, ether extract = $3.8 \pm 0.3\%$, $n = 6$) or low starch content (**LS**, starch = $4.8 \pm 0.7\%$, ether extract = $13.8 \pm 0.4\%$, $n = 6$) throughout gestation and lactation. Foals remained on these diets after weaning through 15 mo of age, and at 3, 6, 9, 12 and 15 mo of age, PL and carpal SF samples were collected for analysis of total IGF-I. Monthly measurements of growth were recorded for calculation

of body weight gain, wither height gain and forearm length gain for 90 day periods between 0-3mo, 3-6 mo, 6-9 mo, 9-12 mo and 12-15 mo of age. No difference was detected between total plasma IGF-I concentrations in fillies (n = 5) and colts (n = 7) ($P > 0.05$). No influence of diet on PL or SF IGF-I was detected ($P > 0.05$). Average SF IGF-I concentrations were $30.1 \pm 1.8\%$ of that found in PL, and PL and SF IGF-I were positively correlated ($r = 0.48$, $P = 0.0003$). There was an effect of month of age on both PL and SF IGF-I concentrations ($P < 0.05$), with peak values obtained at 3 mo of age (462.0 ± 13.7 , and 140.3 ± 8.9 ; respectively). A secondary peak in PL, SF (409.6 ± 15.7 , and 144.9 ± 18.1 ; respectively), and rates of gain occurred at 12 mo of age, corresponding with spring months. There was a positive correlation between all measures of gain except forearm length gain with PL and SF IGF-I ($r = 0.41$ to 0.55 , $P < 0.05$).

Key Words: Synovial Fluid, IGF-I, Skeletal Growth, Dietary Energy

INTRODUCTION

Healthy skeletal growth and development is essential for horses to achieve their full athletic potential. A combination of genetic and management factors may lead to the occurrence of bone disorders such as developmental orthopedic disease (**DOD**), which contributes to considerable economic losses in the equine industry (Jeffcott, 1996). In order to develop management strategies designed to avoid DOD, it is important to better understand the chemical communication network regulating skeletal development. The somatotrophic axis, with principle hormones such as growth hormone (**GH**) and insulin-like growth factor-I (**IGF-I**) is at the heart of this chemical communication network. A principal component of the somatotrophic axis, IGF-I promotes chondrocyte proliferation and differentiation resulting in longitudinal bone growth and articular cartilage development (Lupu et al., 2001). Multiple factors including age, environment and nutrition may influence circulating concentrations of total IGF-I in horses. We hypothesize that these components will influence similar changes in PL and SF IGF-I throughout growth.

In young horses, circulating IGF-I concentrations have been correlated with measurements of growth and skeletal development such as average daily gain in bodyweight, cartilage maturation, and curves of body growth (Davicco et al., 1994, Fortier et al., 2005, Staniar et al., 2007). However, data examining the relationship between circulating IGF-I and chondrocyte metabolism is lacking. Articular cartilage is an avascular tissue, reliant upon surrounding synovial fluid (**SF**) as a source of the hormones and nutrients that regulate and support its growth. It is our hypothesis that SF

IGF-I concentrations will be more strongly correlated with measurements of skeletal growth than PL IGF-I.

Conflicting data has been presented regarding the influence of dietary energy source on circulating IGF-I. Growing Thoroughbreds fed diets high in sugar and starch demonstrated increased circulating IGF-I as compared to those fed diets high in fiber and fat (Staniar et al., 2007, Treiber et al., 2005). In young Quarter Horses fed diets high in fat or high in starch, no differences in circulating IGF-I concentrations were detected (Ropp et al., 2003). In this study, we further examine this relationship with dietary energy source.

Our objectives were to conduct a longitudinal study in growing Thoroughbreds in order to characterize the developmental changes in PL and SF total IGF-I between 3 and 15 mo of age, to investigate the relationship between PL IGF-I and SF IGF-I with quantitative measurements of skeletal growth, and to investigate the influence of dietary energy source on PL and SF IGF-I.

MATERIALS AND METHODS

Animals and Management

Twelve Thoroughbred foals born at the Middleburg Agricultural Research and Extension Center (MAREC) in Northern Virginia were studied from birth through 15 mo of age. Broodmares were paired by weight, expected foaling date and sire, and then randomly assigned to one of two dietary treatment groups. Broodmares were fed their assigned diets throughout gestation and lactation. Prior to weaning foals shared meals with their dams, and were continued on the feeds from weaning through 16 mo of age.

All horses were collectively fed at 07:00 and 14:00 in a 30-m circle of feed pans containing individual portions. Feed was adjusted to maintain a body condition score (BCS) of 4.5–5.5 on a 9 point scale (Henneke et al., 1983) in both mares and foals throughout growth (Table 1). Horses were rotated monthly between two matched 30-acre mixed grass and ladino clover pastures. Water was available *ad libitum*. Colts were gelded at 3-4 wk of age and all foals were weaned between 6 and 9 mo of age.

Feeds

A high starch (HS, starch = $39 \pm 2\%$, ether extract = $3.8 \pm 0.3\%$, n = 6,) and low starch (LS, starch = $4.8 \pm 0.7\%$, ether extract = $13.8 \pm 0.4\%$, n = 6) feed were formulated for this study (Table 2). Feeds were formulated to be isocaloric and isonitrogenous as described previously (Williams et al., 2001). Mineral and vitamin contents of the feeds (DSM Nutritional Products, Inc., Parsippany, NJ, USA) were balanced to complement the pastures in central and north-central Virginia and to meet or exceed current recommendations (Greiwe-Crandell et al., 1995, Hoffman et al., 1999, NRC, 2007). Starch content was determined using a glucoamylase enzyme and measuring dextrose in an automated biochemical analyzer (YSI 2700 SELECT Biochemistry Analyzer, YSI Incorporated, Yellow Springs, Ohio, USA, Application Note Number 319).

Collection of Blood and SF

At 3, 6, 9, 12 and 15 months of age, horses were transported 32 km to an equine hospital for sample collection. Prior to sample collection, horses were sedated with a mixture of xylazine (1mg/kg BW) and butorphanol (0.01 mg/kg BW). Following aseptic

preparation, 2-4 mL carpal SF samples were collected and stored at -20° C in 2mL polypropylene tubes. Blood was collected via jugular venipuncture into heparinized tubes (Becton Dickinson and Company, Franklin Lakes, NJ). Within one hour of collection, PL was separated via centrifugation at 3,000 x g and stored at -20° C for further analysis. Total IGF-I was measured in SF and PL using an enzyme-linked immunosorbent assay (ELISA) including an initial acid ethanol extraction for separation of binding proteins (DSL 10-5600, Webster, TX). The inter-assay coefficient of variation (CV) for the ELISA was calculated using total IGF-I concentrations for SF (n= 58) and PL (n = 58) samples run in duplicate on two separate assays. The intra-assay CV was calculated using total IGF-I concentrations for separate SF (n= 11) and PL (n = 6) samples run in duplicate ≥ 2 times within the same assay. The total IGF-I ELISA demonstrated an inter and intra-assay CV for PL and SF of 13%, 5.6% and 22%, 5.5% respectively (Appendix I).

Morphometric Measurements

From birth through 16 mo of age, monthly measurements of total body weight, wither height and forearm length were recorded to assess growth (Figure 3). Body weights were measured using a calibrated electronic walk on scale (Tyrel platform, TC-105, Alweights Hamilton Scale Corp., Richmond, VA). Average daily body weight gain (**BWG**), wither height gain (**WHG**) and forearm length gain (**FLG**) were calculated for 90 day periods between 0-3 mo, 3-6 mo, 6-9 mo, 9-12 mo and 12-15 mo of age.

Statistical Analysis

Associations between measures of gain, PL and SF IGF-I concentrations were analyzed with Spearman correlations. A repeated measures analysis of variance was used to examine the effects of time, gender, and feed treatment on PL and SF IGF-I. All analyses were performed using statistical software (Intercooled Stata, StataCorp LP, College Station, Texas). Differences were considered significant at $P < 0.05$. Data are presented as means \pm SE.

RESULTS

Total IGF-I concentrations were not found to be different between fillies and colts ($P > 0.05$) (Table 3). No influence of diet on PL or SF IGF-I was detected ($P > 0.05$) (Table 4). Therefore, data for all horses were pooled for further analysis. With the exception of the samples from 9 mo of age, SF concentrations of IGF-I were consistently lower ($30.1 \pm 1.8\%$ of PL IGF-I) and positively correlated with PL IGF-I ($r = 0.48$, $P = 0.0003$) (Figure 4). Both PL and SF IGF-I followed a similar pattern of change throughout growth (Figure 5). There was an effect of age on both PL and SF IGF-I concentrations ($P < 0.05$). The highest values for PL and SF were detected at 3 (462.0 ± 13.7 , and 140.3 ± 8.9 ; respectively) and 12 mo of age (409.6 ± 15.7 , and 144.9 ± 18.1 ; respectively). Highest rates of gain occurred at 3 and 12 mo of age, corresponding with spring months (Figures 6-11). Both PL and SF IGF-I were lowest at 15 mo of age (281 ± 28 , 57 ± 5 ng/ml; respectively). There was a positive correlation between all measures of gain except FLG with PL and SF IGF-I ($r = 0.41$ to 0.55 , $P < 0.05$ Table 5).

DISCUSSION

This is the first longitudinal study to examine the correlation between SF and PL IGF-I concentrations as well as their relationship to skeletal development in Thoroughbreds. The results of this study suggest a moderately strong association between PL and SF IGF-I ($r = 0.48$, $P = 0.0003$). A similar association between SF and PL total IGF-I concentrations has previously been found in human subjects (Schneiderman et al., 1995). The difference in PL and SF total IGF-I concentrations may be partially explained by the limited permeability of the synovium (Schneiderman et al., 1995). Less than 1% of total IGF-I circulates in free form, with the remaining percentage bound to 1 of multiple structurally related IGF-I binding proteins (IGFBP).

The majority of IGF-I present in circulation (70-80%) is found in a 150 kDa ternary complex with IGFBP-3 and acid-labile subunit (Yakar et al., 2002). It is likely that the ternary complex is too large to pass through the synovial membrane. It has previously been shown that complexes of smaller size than the ternary complex such as IgG (67kDa) are partially excluded from the synovial space (Levick, 1991). The majority of bound IGF-I in SF is bound in small complexes (Schneiderman et al., 1995).

Concentrations of IGF-I in SF may also reflect local production of IGF-I by chondrocytes, which varies in response to ageing as well as nutrient availability (Martin et al., 1997, Richardson et al., 2003). Exposure to increasing concentrations of IGF-I *in vitro* promotes increased chondrocyte metabolism (Trippel et al., 1989). Resulting increases in nutrient transport as well as collagen and proteoglycan synthesis and deposition contribute to longitudinal bone growth (Jenniskens et al., 2006, Phillips et al.,

2005, Smith et al., 1989). This was reflected in this study by the increase in PL and SF IGF-I detected during periods of compensatory growth in the spring months.

In this study SF IGF-I concentrations were similarly or more closely correlated with measures of skeletal growth than PL IGF-I. Synovial fluid is likely to have a greater influence on chondrocyte metabolism than PL, particularly in articular cartilage due to its proximity to the articular cartilage itself. This highlights the importance of collecting SF as well as PL in order to provide an accurate assessment of how dietary treatments may affect cartilage metabolism.

As found in previous studies, IGF-I concentrations were highest in the younger more rapidly growing animals and followed a general decline with age (Cymbaluk and Laarveld, 1996, Staniar et al., 2007). Our results also characterize a secondary peak in both PL and SF IGF-I at 12 mo of age. It is notable that the timing of this peak corresponds with a compensatory growth period in the foals, as well as the improved nutrient availability associated with spring pasture (Staniar et al., 2004). During the spring months (April and May in northern Virginia) pasture DE, crude protein and non-structural carbohydrate content increases approximately 5-10% compared to the winter months (January and February) (Cubitt, 2004). Season-related changes in circulating IGF-I follow similar patterns to those found in previous research (Staniar et al., 2007). In addition to pasture nutrient availability, this may be partially attributed to changes in day length and temperature. Increased photoperiod and temperature have been positively correlated with circulating IGF-I in horses and other species (Dahl et al., 1997, Sarko et al., 1994, Staniar et al., 2001).

In contrast with earlier findings from our group, we did not detect an influence of dietary energy source on either PL or SF IGF-I concentrations (Staniar et al., 2007, Treiber et al., 2005). A previous study utilizing similar diets detected differences in PL IGF-I only in the months of April and May and corresponding with periods of rapid growth (Staniar et al., 2007). Another study found higher basal IGF-I concentrations in weanlings adapted to a high starch diet approximately 30d after weaning in the fall (Treiber et al., 2005). The infrequent sampling protocol utilized in the current study did not allow for full evaluation of these time periods. Our results are consistent with a study conducted in weanling Quarter horses between 5 and 8 months of age, where no differences were detected in serum IGF-I between those fed a soluble carbohydrate-based concentrate (DE 2.9 Mcal/kg, NSC = 33.9%) or those receiving a 10% fat supplemented, soluble carbohydrate-based concentrate (DE = 3.0 Mcal/kg, NSC = 24%) (Ropp et al., 2003). Again, this particular sampling protocol did not include time periods where differences in circulating IGF-I have previously been found. A frequent sampling protocol over a period of time may be necessary to detect adaptive changes to dietary energy source as demonstrated by changes in circulating IGF-I concentrations.

The results from this study may help to define how SF concentrations of IGF-I change in relation to age and skeletal growth. Future research should aim to further define these relationships, examining how the influence of dietary energy source on cartilage metabolism may change in relation to age, growth rate and time of year. The information obtained in this study highlights the need to examine SF concentration of IGF-I in addition to PL IGF-I when examining the influence of dietary treatments.

Table 1. Amount of supplement fed to mares, foals, weanlings and yearlings throughout the study period

Horses	Months fed	Kg/day ^a	DE, Mcal	DE requirement, %
Mares and foals, late lactation ^b	May-September	5.6 ^c	16.8	58
Weaned foals, moderate growth ^c	October-December	3.0	9.0	41
Yearlings, moderate growth ^d	January-April	4.0	12.0	63
Yearlings, moderate growth ^d	April - August	3.2	9.6	50

^aSplit into two meals/day.

NRC (1989) recommended daily DE: ^b29 Mcal/day, ^c15.5 Mcal/day, ^d19 Mcal/day.

^cMares and foals were fed together and shared 3.2 kg/day.

Table 2. Nutrient composition on a dry matter (DM) basis of the high starch (HS), and low starch (LS) supplements fed to Thoroughbred broodmares and their offspring through 15 mo of age.

	HS (n=6)		LS (n=6)	
	Mean	SE	Mean	SE
Crude Protein, %	15	0.20	15	0.10
Ether Extract, %	3.8	0.38	14	0.40
Acid Detergent Fiber, %	11	0.62	29	0.31
Neutral Detergent Fiber, %	19	0.88	43	0.34
Starch, %	39	2.0	4.8	0.70
Digestible Energy (MJ/kg)	13	0.08	3	0.02

Table 3. Total IGF-I concentrations in synovial fluid (SF) and plasma (PL) of growing Thoroughbred fillies and colts (mean \pm SE). No effect of gender on PL or SF IGF-I concentrations was detected at any time point ($P < 0.05$).

Month of Age	PL IGF-I (ng/mL)		SF IGF-I (ng/mL)	
	Colts	Fillies	Colts	Fillies
3	469.9 \pm 22.3	456.3 \pm 18.5	142.7 \pm 15.0	138.3 \pm 11.8
6	349.5 \pm 19.7	352.2 \pm 33	132.6 \pm 19.7	128.7 \pm 14.0
9	385.1 \pm 48.0	372.5 \pm 16.1	58.0 \pm 17.1	81.4 \pm 5.0
12	402.2 \pm 30.1	415.7 \pm 16.8	148.5 \pm 39.4	142.5 \pm 19.1
15	303.1 \pm 15.0	266.8 \pm 46.0	56.3 \pm 9.0	58.6 \pm 6.2

Table 4. Total IGF-I concentrations in synovial fluid (SF) and plasma (PL) of growing Thoroughbreds fed either high starch (HS) or low starch (LS) diets. No effect of diet on PL or SF IGF-I concentrations was detected at any time point ($P < 0.05$).

Month of Age	PL IGF-I (ng/mL)		SF IGF-I (ng/mL)	
	LS	HS	LS	HS
3	477.7 ± 15.1	446.3 ± 22.3	150.6 ± 14.6	127.9 ± 6.9
6	372.2 ± 30.0	330.0 ± 26.7	134.8 ± 16.7	125.9 ± 15.8
9	376.5 ± 28.8	378.9 ± 33.0	77.9 ± 15.2	65.4 ± 6.0
12	424.9 ± 16.0	391.2 ± 28.6	150.8 ± 23.6	138.9 ± 30.1
15	297.4 ± 22.1	257.3 ± 65.1	52.0 ± 8.9	62.1 ± 5.4

Table 5. Correlations between body weight gain (BWG), wither height gain (WHG), body weight gain (BWG), plasma (PL) and synovial fluid (SF) total IGF-I concentrations in Thoroughbred horses from birth through 15 mo of age.

Correlation Evaluated	r	P
PL and BWG	0.418	0.001
PL and WHG	0.489	0.000
PL and FLG	0.231	0.083
SF and BWG	0.554	0.000
SF and WHG	0.530	0.000
SF and FLG	0.455	0.001
SF and PL	0.482	0.000

Figure 3. Measurements of forearm length and wither height were used to calculate forearm length gain (FLG) and wither height gain (WHG) for 90 day periods between 0-3 mo, 3-6 mo, 6-9 mo, 9-12 mo and 12-15 mo of age . Forearm length was measured as the distance in cm between the point of the elbow and the accessory carpal bone. Total distance between the highest point of the withers and the ground was measured to determine wither height.

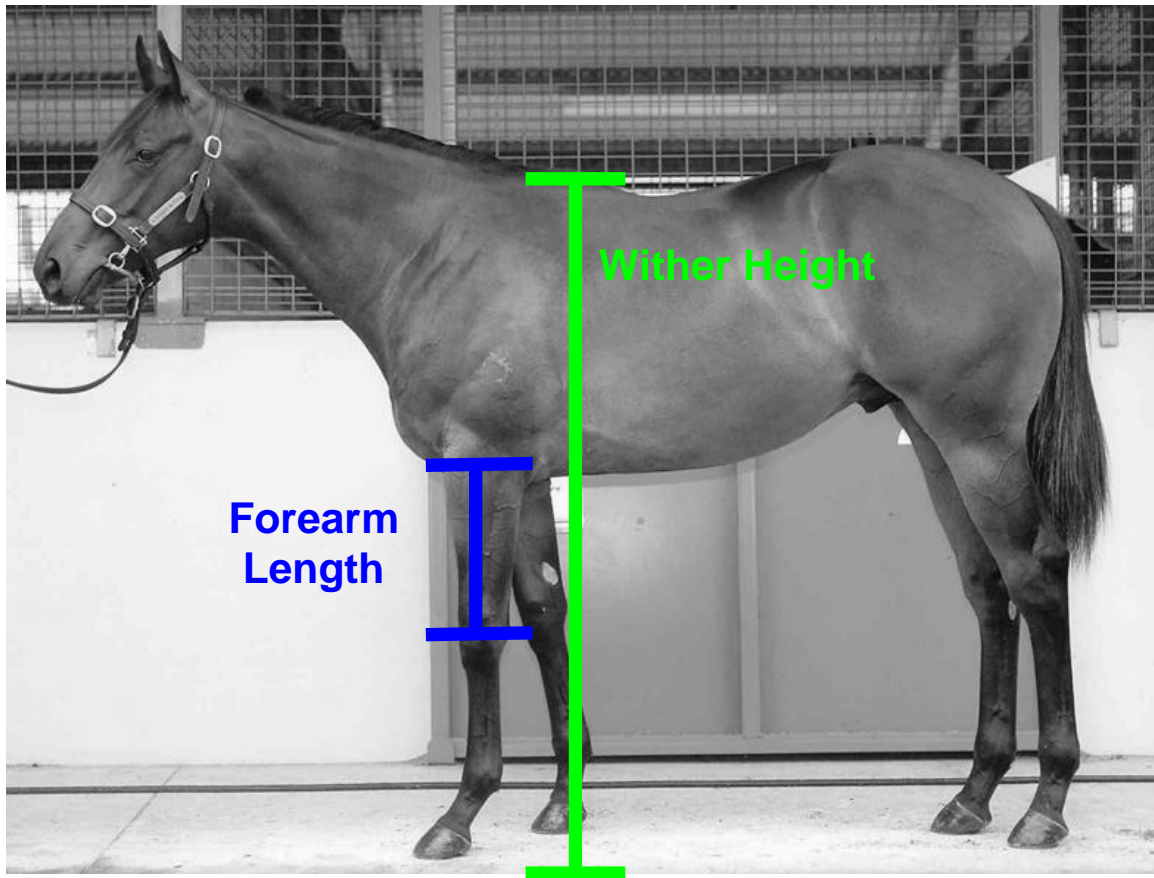


Figure 4. Correlation between plasma (PL) and synovial fluid (SF) total IGF-I concentrations in growing Thoroughbred horses as measured at 3, 6, 9, 12 and 15 mo of age ($r = 0.48$, $P = 0.0003$).

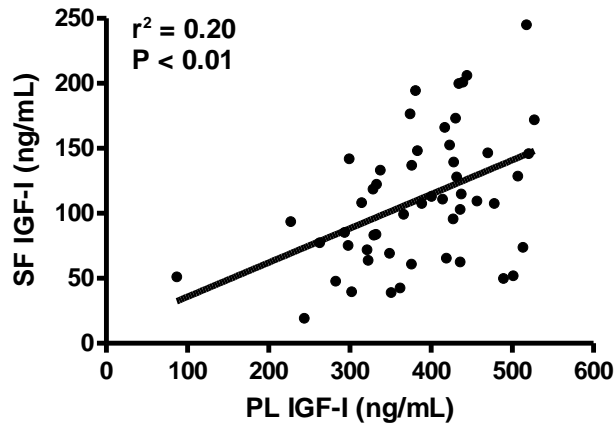
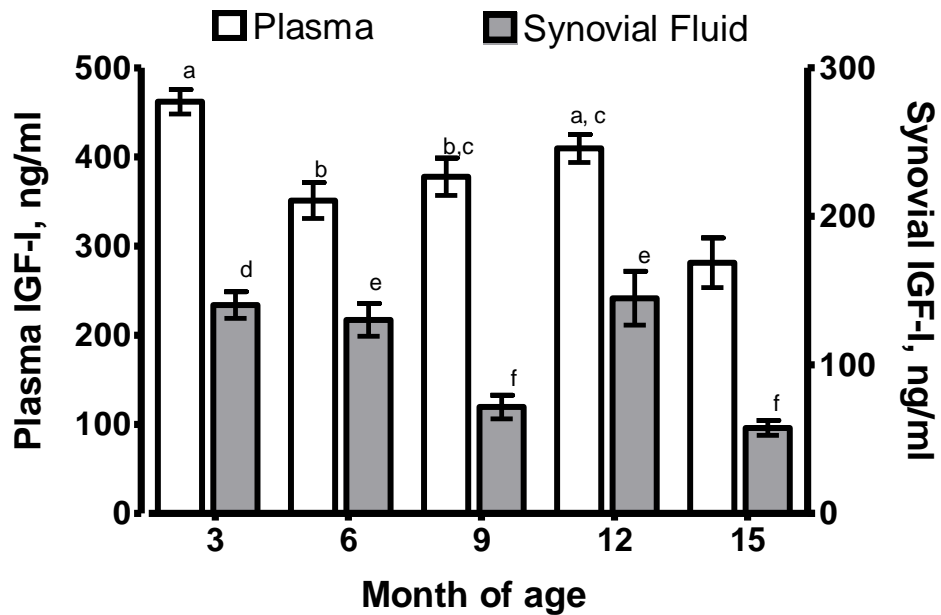


Figure 5. Synovial Fluid (SF) and plasma (PL) concentrations of total IGF-I (mean± SE) in Thoroughbred foals from 3 to 15 mo of age. There was a significant effect of month of age on both PL and SF IGF-I ($P < 0.05$).



a - c: PL IGF-I for groups without a common letter designation differ ($P < 0.05$)
d - f: SF IGF-I for groups without a common letter designation differ ($P < 0.05$)

Figure 6. Mean changes in wither height gain (WHG) and plasma (PL) IGF-I (mean± SE) throughout seasons in Thoroughbred foals from 3 to 15 mo of age.

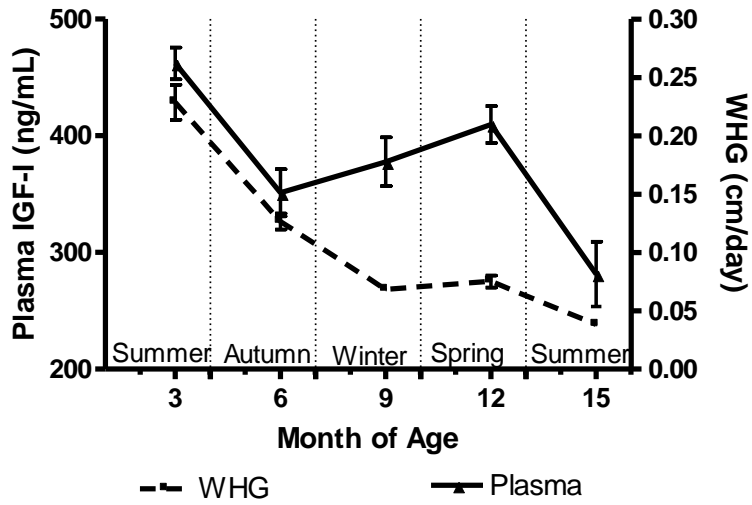


Figure 7. Mean changes in wither height gain (WHG) and synovial fluid (SF) IGF-I (mean± SE) throughout seasons in Thoroughbred foals from 3 to 15 mo of age.

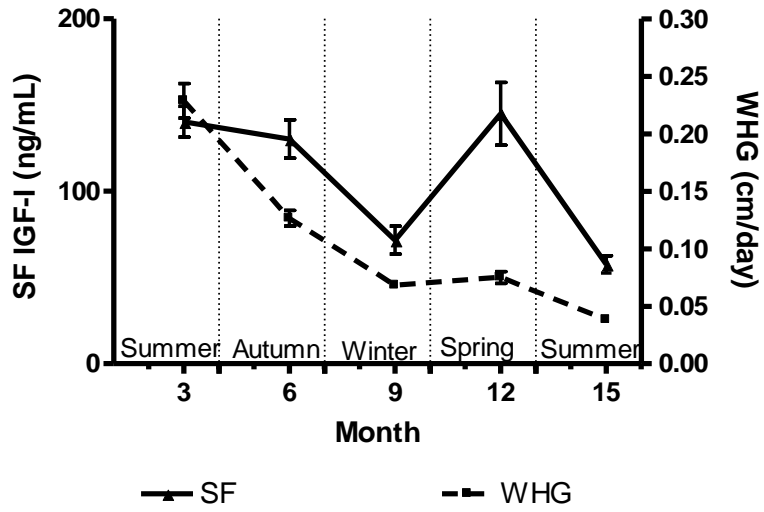


Figure 8. Mean changes in forearm length gain (FLG) and plasma (PL) IGF-I (mean± SE) throughout seasons in Thoroughbred foals from 3 to 15 mo of age.

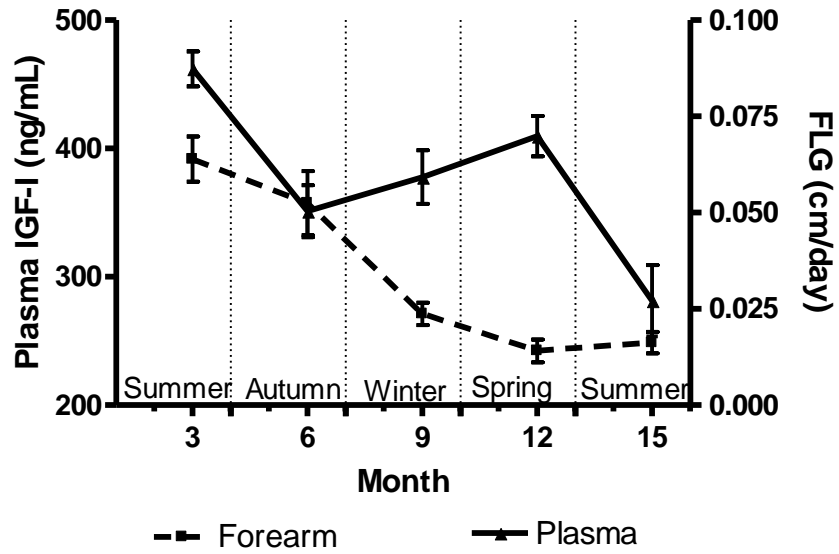


Figure 9. Mean changes in forearm length gain (FLG) and synovial fluid (SF) IGF-I (mean± SE) throughout seasons in Thoroughbred foals from 3 to 15 mo of age.

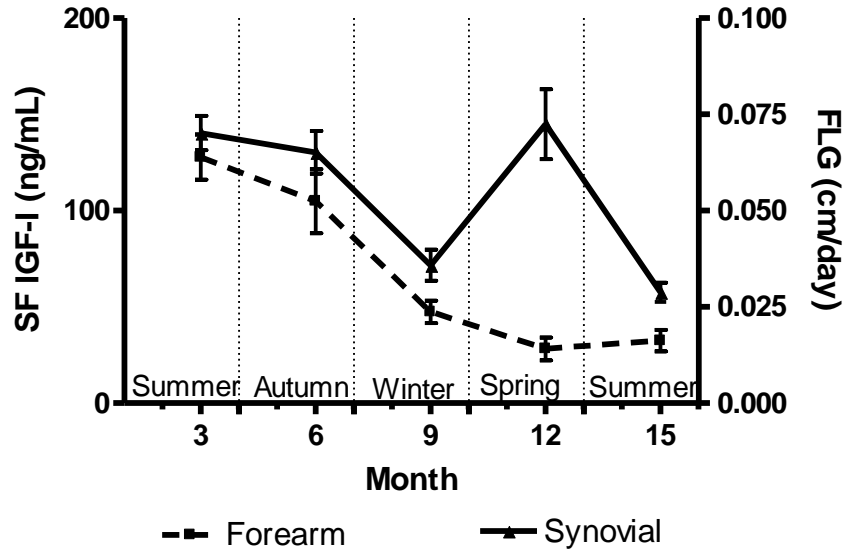


Figure 10. Mean changes in body weight gain (BWG) and plasma (PL) IGF-I (mean± SE) throughout seasons in Thoroughbred foals from 3 to 15 mo of age.

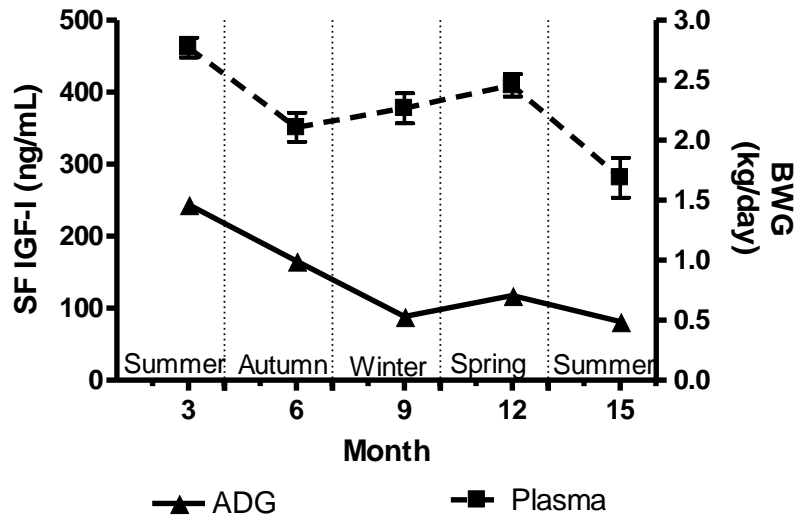
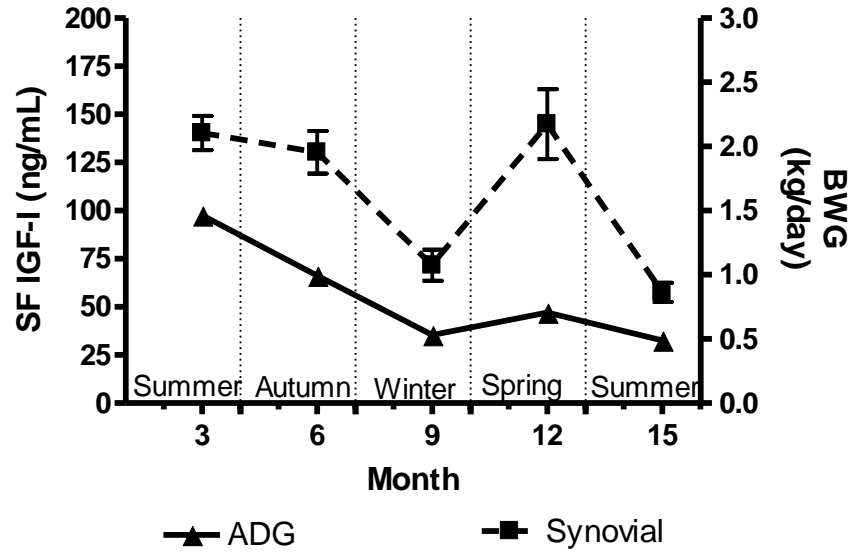


Figure 11. Mean changes in body weight gain (BWG) and synovial fluid (SF) IGF-I (mean± SE) throughout seasons in Thoroughbred foals from 3 to 15 mo of age.



CHAPTER III

Evaluation of methods for isolation of high-quality total RNA from small articular cartilage samples

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ABSTRACT

Obtaining chondrocyte RNA from articular cartilage that is of sufficient quality and quantity for gene expression analysis has proven challenging due to the acellular nature of tissue. We evaluated the use of a liquid-nitrogen cooled mortar and pestle, motorized freezer mill and rotor-stator homogenizer for homogenization of small (<50mg) articular cartilage samples. A rotor-stator homogenizer consistently produced quantifiable RNA yields, and was used to evaluate three different RNA isolation protocols. The first protocol included modifications of guanidinium isothiocyanate/acid-phenol extraction and cesium trifluoroacetate centrifugation procedures in combination with a commercial silica gel-based spin column protocol. The other protocols evaluated are commercially available and were modified to minimize proteoglycan contamination (Lipid RNeasy® Mini, Qiagen Inc U.S.A, Valencia, CA, Versagene® RNA Purification Kit, Gentra Systems, Minneapolis). All protocols yielded a sufficient quantity of quality RNA suitable for gene expression analysis. The combined average yield for all protocols

was 91.9 ng RNA/mg of cartilage. Here, modifications to RNA isolation procedures have been described that will permit and facilitate transcriptional profiling of chondrocytes

INTRODUCTION

Isolation of quality RNA from articular cartilage samples has proven challenging for researchers interested in studying gene expression. Articular cartilage is an acellular tissue with the limited number chondrocytes protected by the surrounding extracellular matrix. The extracellular matrix is composed of a high proportion of large, negatively charged proteoglycans which tend to co-purify with RNA (Carney and Muir, 1988). These obstacles create challenges when isolating RNA from small (< 100mg) sample sizes cartilage samples. Serial sampling of a single individual provides great insight into chondrocyte metabolism in response to experimental treatments. However, the limited size of cartilage biopsies that may be harvested without producing significant joint damage (<<250 mg) creates further challenges for researchers (Lee et al., 2000). A study utilizing a canine model found that harvest of samples as small as 250mg from the knee joint negatively affected cartilage strength and integrity at other sites within the joint (Lee et al., 2000). Yields from current RNA isolation protocols may be as low as 0.001-0.018 µg/mg of tissue (McKenna et al., 2000). Thus, more efficient RNA isolation protocols are needed in order to effectively evaluate chondrocyte gene expression.

The first and one of the most critical steps in isolating RNA from tissues is to ensure complete tissue disruption and homogenization. Use of a liquid nitrogen-cooled mortar and pestle is often successful but inappropriate for use with small samples (Fehr et al., 2000). A motorized freezer mill is also effective for homogenization of larger samples but inherit sample loss is problematic for smaller samples (McKenna et al.,

2000). The ability of rotor-stator homogenizers to handle small samples has led to their increased use with cartilage samples (Baelde et al., 2001, Smith et al., 2006). Our first objective was to evaluate the use of these three homogenization techniques with small (< 50mg) articular cartilage samples.

Techniques developed for isolation of RNA from articular cartilage are often designed for use with large samples (~ 2.5 g, McKenna et al., 2000). The multiple step protocols and homogenization methods result in excessive sample loss when used with small samples. The second objective was to evaluate the use of three separate protocols designed for use with small tissue samples. The first method has been used successfully with large (500mg) cartilage samples (MacLeod et al., 1996). This protocol includes modifications of guanidinium isothiocyanate/acid-phenol extraction and cesium trifluoroacetate centrifugation procedures (Adams et al., 1992) in combination with a commercial silica gel-based spin column protocol (RNeasy®, QIAGEN Inc., Chatsworth, CA). This protocol also includes precipitation of RNA using isopropanol and a high-salt solution after chloroform extraction.

The second method evaluated is a commercially available extraction protocol designed for use with lipid tissues (Lipid RNeasy Mini®, Qiagen Inc U.S.A, Valencia, CA). This method is unique in that it inherently incorporates phenol/chloroform extraction procedures with silica gel-based spin column procedure, eliminating the need to purchase separate reagents and supplies. Lastly, a commercial kit designed for use with fibrous tissue was evaluated (Versagene® RNA Purification Kit, Gentra Systems, Minneapolis). This particular protocol uses a unique Proteinase K solution for digestion of proteins in the extracellular matrix. A search of published data did not reveal

information regarding the use of these commercially available methods with articular cartilage samples. This study was aimed at developing a robust technique for isolating RNA from small articular cartilage samples. Our goal was to produce RNA of sufficient quantity and quality for microarray analysis and real time PCR.

MATERIALS AND METHODS

Tissue Homogenization

Three methods of tissue homogenization were evaluated in combination with a commercially available RNA isolation kit (RNeasy® Mini, Qiagen Inc U.S.A, Valencia, CA). Articular cartilage samples (39.1 ± 1.5 mg) were previously harvested immediately postmortem from a yearling. Samples were frozen in liquid nitrogen and stored at -80° C. First, a liquid nitrogen-cooled mortar and pestle was evaluated (Bel-Art Products, Pequannock, NJ). Further tissue disruption was performed using lysis buffer RLT (containing 10 ml/ml 2- β -mercaptoethanol (Qiagen Inc U.S.A.)) and needle and syringe homogenization. Sample lysate was then passed through a 20-gauge needle attached to an RNase free syringe a minimum of 5 times. Ethanol precipitation and silica membrane purification were completed according to manufacturer's protocol (RNeasy® Mini, Qiagen Inc U.S.A, Valencia, CA).

To evaluate the use of a motorized freezer mill, the vial and impactor portions of a commercially available freezer mill were pre-cooled in liquid nitrogen (6750 SPEX CertiPrep Freezer/Mill, Metuchen, NJ). The entire mill unit was also pre-cooled for 15 minutes prior to use. Samples were ground for 3 cycles of 2 min grinding and 2 min cool down with an impact frequency of 15 hits per s. Ethanol precipitation and silica

membrane purification were then completed according to manufacturer's protocol (RNeasy® Mini, Qiagen Inc U.S.A, Valencia, CA). Lastly, we evaluated the use of a rotor-stator homogenizer (Fisher Powergen 125, Thermo Fisher Scientific Inc. Waltham, MA). Frozen samples were placed in lysis buffer (Trizol® reagent, Invitrogen Carlsbad, California) and maintained on ice. Homogenization was completed at maximum speed for 30 sec followed by a 1 min cool down period. Homogenization intervals were continued until pieces of cartilage were no longer visible (≥ 3 cycles).

Total RNA was quantified using a RiboGreen® RNA Quantification kit (cat. # R-11490, Molecular Probes, Eugene, Oregon) according to manufacturers instructions. RiboGreen® dye Fluorescence was measured using a fluorescence plate reader set at 485nm excitation and 535nm emission wavelength (Tecan GENios™, TECAN, North Carolina).

Isolation of total RNA

Cartilage Samples. Articular cartilage samples were collected from a 3 d old foal within 30 min of euthanasia. All samples were harvested by sharp dissection from the lateral trochlear ridge of the femur. Samples were immediately frozen using liquid nitrogen and stored at -80°C until further analysis. For all experiments, 50mg of sample was weighed out and placed in lysis buffer for homogenization using a rotor-stator homogenizer (Fisher Powergen 125, Thermo Fisher Scientific Inc. Waltham, MA).

Protocol Evaluation. A protocol utilizing high salt precipitation of RNA in combination with silica gel-based spin columns was evaluated (MacLeod et al., 1996). Tissue homogenization was completed with a rotor stator homogenizer rather than a

freezer mill due to small sample size. All other methods were followed as described previously (Appendix II).

A commercially available kit utilizing monophasic phenol and guanidine isothiocyanate solution to facilitate tissue lysis was also evaluated (RNeasy® Lipid Tissue Mini, Qiagen Inc U.S.A, Valencia, CA). Several modifications were made to the manufacture's protocol, including steps to avoid proteoglycan contamination and maximize RNA yield. Samples were homogenized, subjected to chloroform extraction and centrifugation. These steps serve to separate the extraction solution into aqueous and organic phases with proteoglycans and other contaminants found in the organic and interphase, while RNA is present within the aqueous phase. Thus, care was taken to avoid disturbance of the interphase and organic phase. An additional wash step was included to further decrease proteoglycan contamination of the RNA. Prior to final elution, the silica-membrane spin column was washed with buffer RPE a total of 3 times. RNA was eluted from the column using two 20 µl aliquots of RNase free water heated to 65°. Centrifugation at 8000 x g for 1 min was performed after each elution. Total elution volume was 40 µl (Appendix II).

Lastly, we evaluated the use of an RNA purification kit designed for use with fibrous tissue (Versagene® RNA Purification Kit, Gentra Systems, Minneapolis MN). This kit incorporates the use of Proteinase K solution (Cat #V- 0550) for digestion of proteins in the extracellular matrix. RNA isolation was then performed according to manufacturer's instructions (Appendix II).

RNA quantification and quality analysis

Total RNA was quantified at 260 nm using a spectrophotometer (ND-1000 UV-Vis Spectrophotometer NanoDrop Technologies, Wilmington, DE). Absorbance at 230-280nm was also measured for calculation of A260:A280 and A260:A230 ratios.

Microfluidic capillary electrophoresis was completed with an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Available software was used for calculation of an RNA integrity number (RIN) as described previously (Mueller et al., 2006).

RESULTS AND DISCUSSION

Results obtained from each homogenization method are summarized in Table 6. The use of a liquid nitrogen-cooled mortar and pestle resulted in low, inconsistent yields as found previously (Fehr et al., 2000). Substantial sample loss was visible when a freezer mill was used to homogenize samples. The rotor-stator homogenizer produced consistent yields (7.72 ± 0.17 ng/mg) from articular cartilage samples. Another consideration for selection of homogenization tools is their initial purchase cost. The liquid nitrogen-cooled mortar and pestle (\$200) was the most economical, while the significantly higher cost of the rotor-stator homogenizer (\$1400) and the freezer mill (\$4500) should be noted. The quantity of RNA obtained by all homogenization methods was still lower than previously reported, highlighting the need to evaluate different protocols (McKenna et al., 2000). The rotor-stator homogenizer was used for evaluation of all RNA isolation protocols.

The average total RNA yield for all methods evaluated is greater than 10 fold of that previously reported (91.9 vs. 8.39 ng of total RNA/mg of cartilage, respectively)

(McKenna et al., 2000). RNA quality as determined by the RIN, A260:A280 and A260:A230 ratios indicate that RNA obtained by all methods was of sufficient quality for gene expression analysis (Imbeaud et al., 2005) (Table 7). The RIN is considered a reliable method for RNA quality assessment, and considers multiple factors such as 28S:18S ribosomal band ratio and RNA integrity (Schroeder et al., 2006b). An RIN \geq 7 is indicative of sufficient RNA quality for analysis of gene expression within articular cartilage (Clements et al., 2006). All methods evaluated in this experiment yielded total RNA which met this criterion (Table 7).

Proteoglycan contamination is a concern when isolating RNA from articular cartilage samples. Proteoglycan and organic compound contamination was considered through calculation of the A260:A230 ratio. High quality RNA from articular cartilage should have A260:A230 of >0.7 (Clemments et al, 2006). Our data show that all methods produced RNA without proteoglycan contamination. The high A260:A280 ratios are indicative of limited protein contamination, whereas high A260:A230 ratios are indicative of an absence of residual contamination by organic compounds such as phenol or alcohol.

Purification of RNA was facilitated through the use of silica-membrane spin columns. It is notable that additional wash steps added to the RNeasy® Lipid Kit protocol resulted in the highest A260/A230 ratio of all methods. Care to avoid disturbance of the organic and interphases in all methods also minimized proteoglycan content.

Phenol-chloroform extraction successfully precipitates RNA from tissue lysate. However, multiple phenol-chloroform extractions likely result in sample loss

(Chomczynski and Sacchi 1987). The high-salt precipitation protocol we evaluated incorporated multiple extractions, resulting in a lower average RNA yield than the RNeasy® Lipid Kit protocol. It should be noted that desirable yields were also obtained from the Versagene® protocol. This protocol does not incorporate a phenol-chloroform extraction. Exclusion of these hazardous materials may make this protocol desirable to some laboratories.

Another consideration for the use of these isolation protocols is their time requirement. Both the Versagene® and RNeasy® protocols may be completed in less than two hours, suggesting their suitability for high throughput sample handling.

In summary, we have evaluated the use of 3 protocols for the isolation of high-quality RNA from articular cartilage samples. All of these protocols produced a sufficient quantity and quality of RNA to be used with gene expression techniques. It should be noted that of all methods evaluated, the RNeasy® Lipid tissue kit produced the highest yields and quality as determined by all quality analysis measures.

Table 6. Comparison of homogenization methods used with articular cartilage samples in combination with the RNeasy®Mini kit (Qiagen Inc, U.S.A.).

Homogenization Method	Sample Size (mg)	Yield (ng/mg)
Mortar and Pestle	29.3	8.6
Mortar and Pestle	28.5	< 0
Mortar and Pestle	26.5	< 0
Freezer Mill	30.4	9.57
Freezer Mill	34.2	< 0
Rotor-stator Homogenizer	30	3.54
Rotor-stator Homogenizer	38.5	7.55
Rotor-stator Homogenizer	37.8	7.89

Table 7. Total RNA quantity and quality obtained from 50 mg articular cartilage samples as measured by the A260:A280 and A260:A230 optical density ratios and RNA integrity number (RIN) calculated by the Agilent Bioanalyzer.

Protocol	Yield (ng RNA/mg of cartilage)	A260:A280	A260:A230	RIN
High Salt Precipitation (n= 6)	56.6 ± 6.7	1.96 ± 0.16	1.11 ± 0.745	7.11 ± 0.66
Versagene® RNA Purification Kit, Gentra Systems, Minneapolis MN (n=4)	96.8 ± 19.4	2.18 ± 0.01	1.7 ± 0.145	8.1 ± 0.39
RNeasy® Lipid Tissue Mini Kit, Qiagen Inc U.S.A, Valencia, CA (n=4)	114.7 ± 14.4	2.17 ± 0.02	2.17 ± 0.05	8.5 ± 0.23

CHAPTER IV

CONCLUSIONS

The objectives of these studies were to determine the relationship between circulating and synovial fluid concentrations of total IGF-I, dietary energy source and measurements of skeletal growth. In addition, we aimed to develop a robust method for isolation of high quality total RNA from small (<50mg) equine cartilage samples for use in transcriptional profiling experiments.

The results from the first study suggest a significant but moderate correlation between circulating concentrations of IGF-I and concentrations of IGF-I in SF. The moderate strength of this relationship suggests investigation of circulating IGF-I provides little insight into IGF-I concentrations present at the level of articular cartilage. Thus, it is likely necessary to compliment PL IGF-I sampling with SF IGF-I sampling when investigating possible dietary treatment effects on cartilage metabolism.

The first study also helped to further characterize the link between skeletal development, circulating and SF IGF-I. With the exception of forearm length gain, all morphometric measurements obtained in this study were significantly correlated with both PL and SF IGF-I. Highest rates of growth and IGF-I concentrations were obtained at 3 mo of age. It is notable that a secondary peak in both rates of gain, PL and SF IGF-I occurred during the spring months of the horses' second year. This time point corresponds with a previously characterized period of compensatory growth in young Thoroughbreds, and provides additional support for the link between circulating and PL IGF-I concentrations and skeletal development.

In contrast with previous findings, this research did not detect an influence of dietary energy source on circulating concentrations of IGF-I. Future research designed to include a larger number of subjects and frequent sampling protocol would allow for a more powerful investigation of this p relationship.

The results from the second study will be useful for studying the molecular mechanisms directing cartilage development *in vivo*. Efficient RNA isolation is an important first step in studying chondrocyte gene expression analysis. All RNA isolation protocols examined in the second study yielded RNA of a sufficient quantity and quality for transcriptional profiling experiments. Modifications to the RNeasy Lipid Kit protocol helped to minimize proteoglycan contamination, as well as produce higher yields than the other protocols evaluated. These techniques will be useful in conducting further research investigating chondrocyte behavior in response to dietary treatments.

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APPENDIX I

Validation of a commercial human total IGF-I ELISA for use with equine plasma

INTRODUCTION

Several methods are available for evaluating concentrations of total IGF-I in biological samples. Early techniques utilized a radioimmunoassay (RIA) procedure, and more recently steps for the removal of IGFBP have been incorporated to improve the accuracy of RIA protocols (Clemmons, 2001, Furlanetto et al., 1977). Commercial immunoradiometric (IRMA) sandwich assays using antibodies specific to IGF-I are also available for use (Clemmons, 2001). These techniques are both sensitive and specific for measurement of total IGF-I, but may be time consuming and require special precautions as radioactive isotopes are used. Enzyme-linked immunosorbent assays (ELISA) have gained increasing popularity due to their ease of use. Several types of RIA and IRMA have been validated for use with equine samples (De Kock et al., 2001, Staniar, 2001), but the use of a commercial ELISA has not yet been evaluated. Although IGF-I is 100% homologous between human and equine, equine IGFBP share a lower degree of homology (75 to 95%) with human IGFBP (Dahlgren and Nixon, 2005). Differences in human and equine binding protein structure may alter the position of the binding proteins relative to IGF-I between these species and therefore interfere with antibody binding, necessitating validation of assays used for both species. The objective of this experiment was to evaluate the specificity, accuracy and precision of a commercially available human total IGF-I ELISA (DSL 10-5600, Webster, TX) for use with equine plasma.

MATERIALS AND METHODS

ELISA validation

Specificity. Biological specificity was determined qualitatively by measuring the biological change in IGF-I concentrations after Equine somatotropin (eST) administration to mature Thoroughbred mares. Intramuscular eST injections (EquiGen, BresaGen Limited, Australia) were administered daily to non-pregnant mares (25ug/kg BW) for a period of 5 days (n = 3). Sterile saline was injected into a group of control mares to serve as a control (n = 3). Venous blood samples were taken each day prior to the administration of eST, and plasma was separated via centrifugation and stored at -20°C until further analysis. Aliquots of each daily sample were used for ELISA and RIA analysis. Radioimmunoassay procedure for total IGF-I (Berry et al., 2001) was modified for use with equine plasma and results for RIA analysis were reported previously (Staniar, 2001). Dilutional parallelism was determined by comparison of serial dilutions of kit standards with serial dilutions of plasma samples (1:2, 1:3, 1:5, 1:9 and 1:17) as described previously (Plikaytis et al., 1994).

Accuracy. Accuracy is the extent to which the measurement of the hormone in a sample agrees with the exact amount that is present (Reimers et al., 1981). The accuracy of the IGF-I ELISA was evaluated by comparing results obtained with the ELISA to those obtained with a previously validated RIA (Staniar, 2001). This comparison was made by determining linear regression of concentrations for 5 basal plasma samples each measured by ELISA and RIA. Accuracy may also be evaluated by measuring the recovery after the addition of known amount of hormone. This was determined by

adding a high concentration plasma sample to a low concentration plasma sample and calculating total recovery.

Precision. Precision was determined by evaluating inter- and intra-assay variability. Inter-assay variability was evaluated by measuring 18 high (494.1 ± 15.0 ng/mL), 18 medium ($379.5.1 \pm 6.0$ ng/mL), and 18 low (290.8 ± 5.1 ng/mL) concentration plasma samples run in duplicate on two separate days and averaging inter-assay coefficients of variation (CV). Intra-assay variability was evaluated using 4 replicate measurements of low, medium, and high concentration samples run in within assay run.

Statistical analysis

Recoveries for dilutions and additions were calculated as (observed concentration \div expected concentration) * 100%. Average recovery values are presented as mean \pm SE. Comparison of RIA and ELISA was also made by robust linear regression as well as calculation of the coefficient of determination (r^2) and the P-value for the null hypothesis that the slope of the best fit line equals one (Intercooled Stata 9.0; StataCorp LP, College Station, TX). Non-linear regression lines for serial dilutions of kit standards and plasma samples were calculated using a polynomial straight line equation. An F Test was used to examine differences between the slope of these lines, with the null hypothesis that the slope was not different for each line. (Graphpad Prism 4.0; Graphpad Software Inc, San Diego, CA). Comparisons of ELISA and RIA measurements were also analyzed by Bland-Altman plots of the difference in ELISA and RIA values (ELISA – RIA) plotted against the mean value ($[\text{ELISA} + \text{RIA}] \div 2$) (Bland & Altman, 1999). Values $P < 0.05$ were considered significant.

RESULTS

Specificity. Daily administration of eST increased concentrations of IGF-1 in the plasma over time as detected by both RIA and ELISA (Figure 12). Plasma levels of IGF-1 increased from 140 ng/mL at Day 1 to 523 ng/mL as determined by ELISA. Plasma IGF-1 levels detected by RIA also increased from 103 to 355 ng/mL over the five day study. Values obtained by RIA tended to be lower than values obtained with ELISA, with an average difference between values of 112 ± 23 ng/ml. Results from both assays show a similar pattern to those from a study conducted in Thoroughbred geldings, where daily eST administration (25ug/kg BW) increased serum total IGF-I from 281 ng/ml to >530 ng/ml as measured by IRMA (De Kock et al., 2001). These results indicate that each of these methods demonstrate appropriate biological specificity, as they are able to detect increased systemic IGF-I production in response to GH stimulation.

When lines defining serial dilutions of high, medium and low concentration plasma samples and kit standards were analyzed, an F Test showed that the lines were not different ($F=0.92$, $P=0.44$, Figure 13). This indicates parallelism between serial dilutions of kit standards and plasma samples, suggesting that sufficient similarities exist between antibody binding to human and equine samples for appropriate estimation of IGF-I levels in diluted plasma samples (Plikaytis et al., 1994).

Accuracy. When linear regression was used to compare basal plasma samples measured by RIA and ELISA, results demonstrated that concentrations determined by each method were systematically different ($P = 0.001$), but correlated ($r^2 = 0.96$) (Figure 14). Bland-Altman plots of the data suggest a positive bias in the EIA measurements because the bias line, the mean difference in measurements (ELISA – RIA), is much greater than

zero (Figure 14). Both boundaries of the 95% limits of agreement, 2 SD above or below the bias line are also positive. This indicates that well over 95% of ELISA measurements would be greater than RIA measurements. Data points on the Bland-Altman plots reveal a positive slope to the data resulting from proportional error. There is a greater difference between the two methods at higher concentrations; indicating that accuracy as determined by RIA values is lower at higher concentrations.

While RIAs are often considered to be more sensitive than ELISAs, this is dependent on several factors including the location of antibody binding sites, antibody avidity, the enzymatic substrate of the ELISA and the ELISA amplification method used. This RIA utilizes mouse anti-human IGF-1 antibody and goat anti-mouse antiserum (Berry et al., 2001) while antibodies in the ELISA are proprietary mouse monoclonal antibodies (DSL Laboratories, personal communication). Differences in antibodies and binding properties likely contributed to differences in values obtained from the two assays. Although RIA may be considered a “gold standard” for measurements of hormone concentrations, the ability of this RIA to measure true total IGF-I concentrations is unknown. It is also important to note that this RIA was initially developed for use with cattle (Berry et al., 2001), whose IGFBP structure and therefore IGF-I binding properties may differ from equine.

When a low concentration of IGF-I was spiked with a higher concentration of IGF-I, recovery ranged from 94% to 104% (Table 8). These consistently high recovery values indicate that the ELISA has a high degree of accuracy for determining total IGF-I in equine plasma samples (De Kock et al., 2001).

Precision. For low, medium and high concentration samples run repeatedly in a single assay, the inter-assay CV was calculated as 2.1%, 1.04% and 4.11% respectively. For low, medium and high concentration samples run in duplicate using different assays, the intra-assay CV was 19.5%, 10.1%, 10.6% respectively (Table 9). The high intra-assay CV may be partially explained by the fact that samples were run a limited number of times between assays (n=2), decreasing the power of these CV calculations. Others that have used this assay (DSL 10-5600) have also found a high inter-assay CV (12.3-17.6%) for human serum (Berrigan et al., 2007). Variance was attributed to several factors, including plate, day, and week that the assay was run. For our experiment, the first assay used to calculate inter-assay CV was completed several months prior to the second assay.

Storage length and temperature have been shown to adversely affect IGF-I measurements in biological samples. Plasma samples used in this experiment were stored at -20°C immediately after collection and separation. While the IGF-I peptide is stable in plasma at -80°C, a study investigating IGF samples stored at -20°C found up to a 20% decrease in IGF-I concentrations over time due to proteolysis (Khosravi et al., 2005). Future sample collection procedure should be changed to include storage at -80°C to avoid possible proteolysis that may occur at - 20°C.

CONCLUSIONS

The commercial ELISA utilized for this experiment (ELISA, DSL 10-5600, Webster, TX) demonstrated acceptable specificity and accuracy for use with equine plasma samples. Low inter-assay precision should be noted, as well as differences in values previously obtained by the RIA and those obtained with this ELISA. The RIA used for this

experiment may be useful for comparison with the ELISA to determine biological specificity as evidenced by responses to eST.

Figure 12. Total IGF-I as measured by ELISA (■) and RIA (●) in response to daily eST administration (25ug/kg BW/day) or sterile saline (control) to mature Thoroughbred mares.

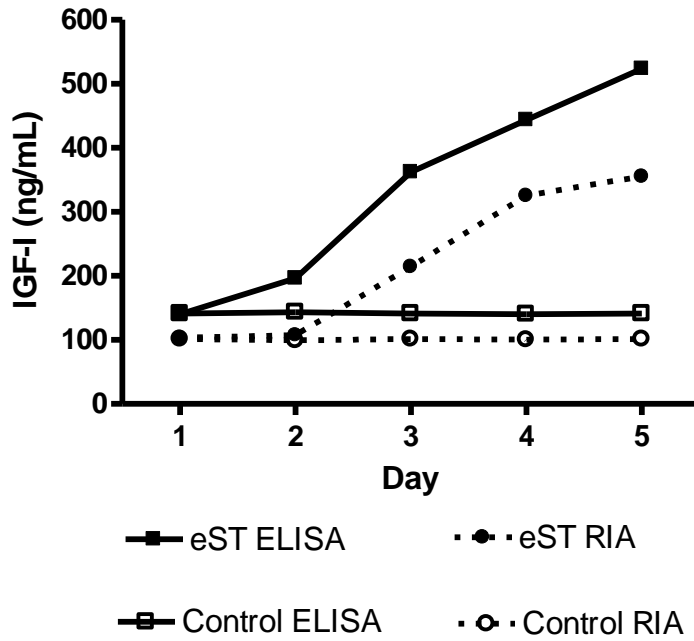


Figure 13. Parallelism between IGF-I concentration calculations created using a standard curve prepared from serial dilutions of IGF-I kit standard (slope = 0.016) and serially diluted high (slope = 0.012), medium (slope = 0.011) and low (slope = 0.010) concentration plasma samples. An F test performed on the slopes indicated that the slopes were not different (F=0.92, P=0.44).

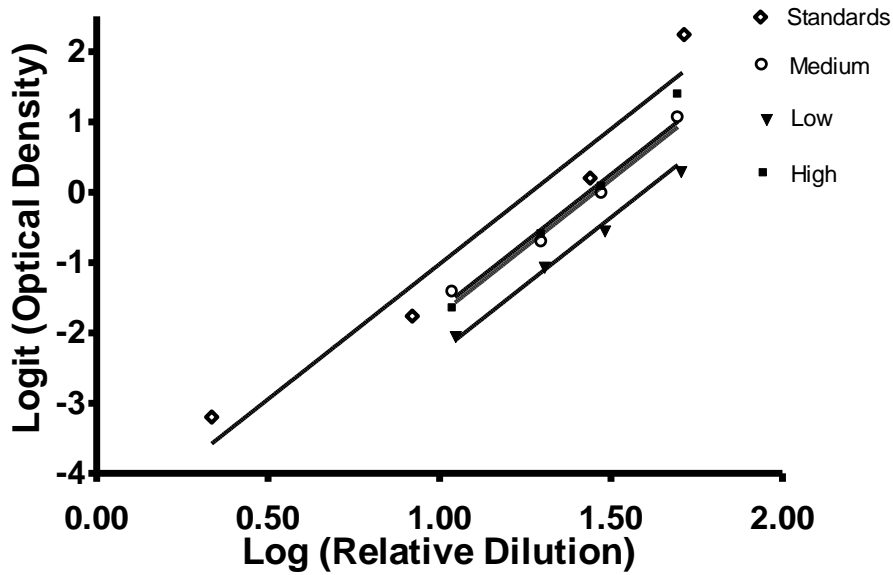


Figure 14. Comparison of total IGF-I values obtained from aliquots of basal plasma samples with RIA and ELISA. $R^2 = 0.96$.

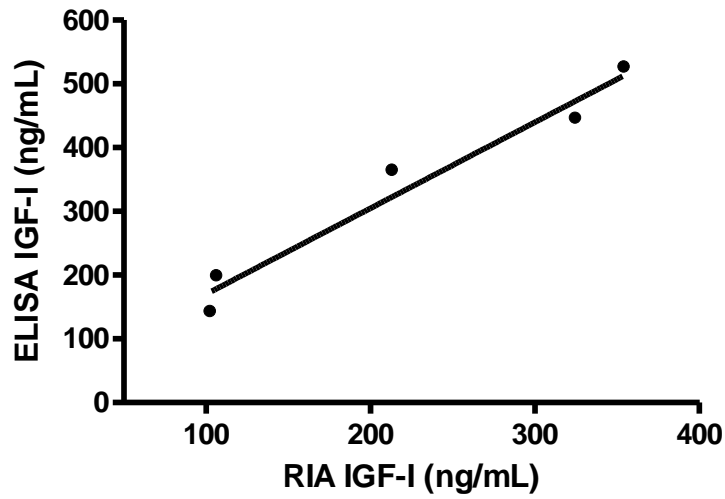


Figure 15. Bland-Altman plots of the difference in ELISA and RIA measurements against mean measurement for total IGF-I. The solid line represents the mean difference in the two methods (bias line), the broken lines represent two standard deviations above or below the mean difference (95% limits of agreement).

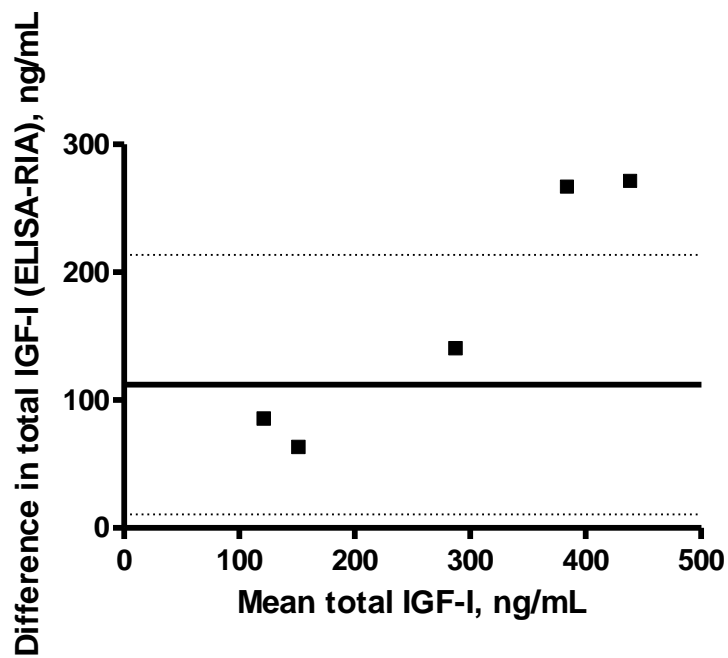


Table 8. Recoveries (%) for ELISA after addition of high concentration plasma samples to low concentration plasma samples

Dilution	Volume (ul) from high concentration sample (439 ng/mL)	Volume (ul) from low concentration sample (354 ng/mL)	Calculated Final Concentration (ng/mL)	Actual Final Concentration	% Recovery
1:2	30	30	397.02	418.39	94.89
1:5	20	80	371.49	369.94	100.42
1:9	10	80	363.93	346.79	104.94
1:33	10	320	357.05	372.86	95.76
1:65	10	640	355.79	347.91	102.26

Table 9. Coefficients of variation (CV, %) for total IGF-I measured by ELISA

	Concentration		
	Low	Medium	High
Intra-assay CV	2.1	1.04	4.11
Inter-assay CV	19.5	10.1	10.6

APPENDIX II

RNA ISOLATION PROTOCOLS

1.) High Salt Precipitation Protocol

I. References

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MacLeod, JN, Burton-Wurster N, Gu DN, Lust G. (1996) Fibronectin mRNA splice variant in articular cartilage lacks bases encoding the V, III-15, and I-10 proteins segments. *J Biol Chem*, 271:18954-18960.

Protocol RNA Cleanup, p79. RNeasy®Mini Handbook. QIAGEN. June 2001.

II. Chemical Reagents

A. Guanidinium Stock Solution

250 g Guanidinium thiocyanate (USB, 75818, FW 118.16), final concentration 4 M

293 ml de-ionized and sterile distilled water

17.6 ml 0.75 M sodium citrate, pH 7.0, final concentration 25 mM

26.4 ml 10% (w/v) Sarkosyl (FW 293.39), final concentration 0.5%

Note: If possible, mix components in the same container that the guanidinium thiocyanate is purchased in. **Do not use a stir bar.** It may require 24 hours for everything to go into solution. Heating to 65°C will accelerate everything going into solution. Store at room temperature for up to 3 months.

B. 0.75 M Sodium Citrate, pH 7

22.05 g Sodium Citrate (FW 294.1) dissolved in 100 ml de-ionized and sterile distilled water

Adjust pH to 7.0

Filter sterilize

C. Guanidinium Working Solution (**Solution D**)

Guanidinium stock solution (above)

0.1 M 2-mercaptoethanol (FW 78.13)

Note: Prepare fresh on the day of use. Add 0.36 ml 2-mercaptoethanol per 50 ml of guanidinium stock solution on the day of use. Add the 2-mercaptoethanol in a chemical fume hood.

D. 2 M Sodium Acetate, pH 4.0

136.1 g Sodium acetate, trihydrate (FW 136.08) dissolved in 350 ml de-ionized and sterile distilled water

Adjust pH to 4.0 with glacial acetic acid

Normalize volume to 500 ml with de-ionized and sterile distilled water

Filter sterilize

- E. Phenol (acidic), DEPC-water saturated Phenol or Tris-saturated lower pH
Phenol, (Fisher, BP1751I-400)

Saturated with DEPC-water (do not neutralize pH).

Store at 4° C.
- F. Chloroform/Isoamyl Alcohol (24/1, v/v), (Chloroform, Fisher, C574-4, Isoamyl Alcohol, Fisher, BP1150-500)

Store at 4° C.
- G. Chloroform/Isoamyl Alcohol (49/1, v/v)

Store at 4° C.
- H. High Salt Solution (0.8 M sodium citrate and 2 M NaCl in double-autoclaved water)
- I. DEPC-treated water

Note: add 1 ml Diethyl Pyrocarbonate (DEPC) to 1000 ml distilled water and shake well, incubate at room temperature overnight in a fume hood, and autoclave twice.

Note: sterile/distilled water without DEPC treatment is also fine, provided you take all precautions to avoid Rnase contamination.
- J. Isopropyl Alcohol (Fisher, A416-500)
- K. 100% Ethanol (AAPER, 200 Proof)

III. Protocol

A. Powder cartilage tissue with a Spex Mill or other freeze fracturing instrument, keeping the tissue frozen by using liquid nitrogen.

B. Starting with 0.5 g to 1 g of powdered tissue, add 10 ml Solution D.

Homogenize within a 50 ml disposable polypropylene tube at room temperature for 30 seconds three times.

Note: To minimize the potential for RNA degradation, do not allow the tissue to thaw. Homogenize the cartilage powder immediately in the Solution D.

Homogenize on ice.

C. After homogenizing the tissue, spin the homogenate using a Sorvall SH-3000 rotor or equivalent at 2700 rpm (1506 g) for 2 minutes at 4 °C to reduce the foam in the tub produced by the homogenization process.

D. Transfer homogenate to 15ml disposable polypropylene tube. Spin the homogenate again using a Sorvall SH-3000 rotor or equivalent at 2700 rpm (1506 g), this time for 10 minutes at 4° C, transfer the supernatant to a 28 ml Oak Ridge tube and store on ice.

E. Add sequentially:

1 ml (0.1 volumes) 2M Na Acetate (pH 4)

10 ml (1.0 volumes) Phenol (acidic)

2 ml (0.2 volumes) Chloroform: isoamyl-alcohol (**49/1, v/v**)

Mix by shaking and vortexing vigorously for 10 seconds after *each* addition.

- E. Cool in ice bath for 15 minutes and centrifuge at 14,994 g (11,200 rpm) in an SS-34 rotor for 20 minutes at 4° C.
- F. Remove (upper) aqueous phase being careful to avoid contamination with the white interface material (denatured proteins) and the phenol/chloroform (this should be approximately 5-6 ml). Transfer the aqueous phase to clean, double-autoclaved microcentrifuge tubes in 1 mL aliquots. (Note – additional aqueous sample can be recovered from the region close to the white interface by collecting this material into a microcentrifuge tube and re-spinning, spin at max speed for 10 minutes.)
- G. To each microcentrifuge tube, add 250 uL (0.25 volumes) of High Salt Solution. Mix well by vortexing briefly. The solution should become white with the addition of High Salt Solution.
- H. To each microcentrifuge tube, add 250 uL (0.25 volumes) of Isopropanol. Mix well by inverting and vortexing until the solution has cleared. Allow the tubes to sit at room temperature for 10-15minutes.
- I. Centrifuge the microcentrifuge tubes at 4° C for 10 minutes at 12,000 g.
- J. Remove the supernatant and wash the RNA pellet with 75% Ethanol. Add 1 mL per microcentrifuge tube and mix by pipetting carefully a few times or by brief vortexing. Afterward, centrifuge the tubes for 10 minutes at 4° C at 14,000 rpm.
- K. Repeat the 75% Ethanol wash as in Step J.
- L. Fully redissolve the pellets in 20-30 µl DNase/RNase-free water at 4° C; this may take several hours and is dependent on size of the pellet. The tubes may be

heated at 55-65° C for 10 minutes to help pellets dissolve into solution. If done, please briefly spin the tubes to collect all the solution at the bottom of the tube.

- M. Combine those centrifuge tubes as necessary. Then quantitate the RNA by NanoDrop and normalize the RNA concentration to 1 µg/µl (or 0.5 µg/µl) by adding additional water as necessary. Also prepare an aliquot for Bioanalyzer analysis. Remember to fully label final tubes, using a printed cryolabel that includes sample identification, RNA concentration, date of preparation, and your name. Store the sample in the -70°C freezer.
- N. It is recommended that an RNeasy column purification step be considered as well. RNeasy maximum capacity is up to 100 µg of the RNA per single column.
- O. Add 350 µl of Lysis Buffer RLT (QIAGEN, RNeasy kit) to the each 100 µl of the RNA sample and mix thoroughly. **Note: Ensure that 2-ME is added to Buffer RLT before use (10 µl 2-ME per 1 ml RLT).**
- P. Add 250 µl of **96-100%** Ethanol and thoroughly mix by pipetting.
- Q. Apply the mixture to an RNeasy Spin Column (in aliquots of 700 µl for each spin) and centrifuge 25 seconds at 8,000 g (corresponds to ~ 8,700 rpm for eppendorf centrifuge). Save the flowthrough from this and later steps until you confirm that the RNA isolation procedure has worked. Re-use the collection tube.
- R. Optional: Add 700 µl Wash Buffer RW1 onto the spin column, centrifuge at 8,000 g for 25 seconds.

- S. Place the RNeasy spin column into a new collection tube. Add 500 μ l Wash Buffer RPE (make sure an ethanol is added into Buffer RPE) onto the spin column and centrifuge at 8,000 g for 25 seconds. Re-use the collection tube.
- T. Add 500 μ l of Wash Buffer RPE onto the spin column and centrifuge for 2 minutes at 8,000 g.
- U. Optional: Place the RNeasy column in a clean autocleaved microcentrifuge tube, and centrifuge in microcentrifuge for 1 minute at full speed (14,000 rpm in the microcentrifuge).

Note: It is important to ensure that the RNeasy spin column matrix is dry, because residual ethanol may interfere with subsequent experimental applications using the RNA. This 1 minute spin assures that no residual ethanol will be carried over during elution.

- V. Transfer the spin column to a new 1.5 ml collection tube and elute the RNA with 50 μ l of DEPC-treated water. Centrifuge for 1 minute at 8000 g. If the expected total yield of RNA is above 30 μ g, repeat the elution step with a second 50 μ l of DEPC-treated water. Pool the 2 elution fractions for spectrophotometric analysis.

Note: Warming up the elution buffer to 65° C and set the tube on the bench for 5 minutes after adding the elution buffer may help increasing the yield.

- W. Optional: Prior to spectrophotometric quantitation, remove any insoluble particulate material from the sample by microcentrifugation. Transfer the aqueous supernatant to a new 1.5 ml tube.

X. Quantitate the RNA by NanoDrop and normalize the RNA concentration to 1 $\mu\text{g}/\mu\text{l}$ (or 0.5 $\mu\text{g}/\mu\text{l}$) by adding additional water as necessary, if needed. Also prepare an aliquot for Bioanalyzer analysis. Remember to fully label final tubes, using a printed cryolabel that includes sample identification, RNA concentration, date of preparation, and your name. Store the sample in the -70°C freezer.

IV. Notes

- A. If a clear distinct pellet is not evident at the RNA precipitation step, something is wrong. Save the supernatant and ask someone for help.

- B. Always follow the necessary precautions to avoid RNase contamination (see Maniatis). Final analysis of RNA structural integrity should be determined by electrophoresis on a formaldehyde agarose gel or on Bioanalyzer.

2. Modified RNeasy Lipid Tissue Mini Kit

(Qiagen Inc U.S.A, Valencia, CA)

Note: Perform Steps 1-9 under the hood

- 1.) Place cartilage in Qiazol, weigh out starting amount of cartilage, not to exceed 100mg.
- 2.) Use #10 scalpel blade to manually slice cartilage into pieces of suitable size for homogenizer (Fisher Powergen 125). Do not remove samples from Qiazol solution.
- 3.) Transfer cartilage and Qiazol to round bottom 5mL polypropylene tube.
Homogenize immediately using a rotor-stator homogenizer until pieces of cartilage are no longer visible. To avoid overheating, keep tube containing homogenate on ice and homogenize in 30s intervals with a 1 minute cool-down period between each homogenization interval.
- 4.) Place the tube containing the homogenate on the benchtop for 5 min after return to room temperature (15-25°C).
- 5.) Transfer homogenate to a 1.5 ml collection tube and add 200 µl chloroform. Cap the tube containing the homogenate securely, and vortex for 15 s.
- 6.) Place the tube containing the homogenate on the benchtop at room temperature for 2 - 3 min.
- 7.) Centrifuge at 12,000 x g for 20 min.
- 8.) Transfer the upper aqueous phase to a new collection tube, taking care not to disturb the interphase and organic phase. Measure total volume of aqueous phase removed.

- 9.) Add 1 volume (usually around 600 μ l) of 70% ethanol, and mix thoroughly by vortexing. **Continue without delay** with step 10.
- 10.) Pipet up to 700 μ l of the sample, including any precipitate that may have formed, onto an RNeasy Mini Spin Column in a 2 ml collection tube (supplied). Close the tube gently, and centrifuge at $\geq 8000 \times g$ ($\geq 10,000$ rpm) for 15 s at room temperature (15-25°C). Discard the flow-through.*Reuse collection tube in step 11.
- 11.) Pipet 350 μ l Buffer RW1 into the RNeasy Mini Spin Column, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash. Discard the flow-through.
- 12.) Optional: DNAase Digestion
- Mix DNase solution from Qiagen RNase-Free DNase Set.
 - Add 10 μ l DNase I stock solution (see above) to 70 μ l Buffer RDD. Mix by gently inverting the tube. Do not vortex.
 - Pipet the DNase I incubation mix (80 μ l) directly onto the RNeasy silica-gel membrane, and place on the benchtop at room temperature for 15 min.
 - Pipet 350 μ l Buffer RW1 into the RNeasy Mini Spin Column, and centrifuge for 15 s at $\geq 8000 \times g$. Discard the flow-through
- 13.) Transfer the RNeasy Spin Column into a new 2 ml collection tube and pipet 500 μ l Buffer RPE onto the RNeasy Spin Column. Close the tube gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the column. Discard the flow-through.
- 14.) Repeat step 13 to ensure thorough washing of spin column.

- 15.) Add another 500 μ l Buffer RPE to the RNeasy Spin Column. Close the tube gently, and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to dry the RNeasy silica-gel membrane.
- 16.) Place the RNeasy Spin Column into a new 2 ml collection tube (not supplied), and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1 min.
- 17.) To elute, transfer the RNeasy Mini Spin Column to a new 1.5 ml collection tube (supplied). Pipet 20 μ l RNase-free water (65°C) directly onto the RNeasy silica-gel membrane. Close the tube gently, and place on benchtop at room temperature for 1 minute. Centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute.
- 18.) Pipet an additional 20 μ l RNase-free water (65°C) directly onto the RNeasy silica-gel membrane. Close the tube gently, and place on benchtop at room temperature for 1 minute. Centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute. Final elution volume is 40ul.
- 19.) Store sample at -80°C for further analysis.

3.) Genra Versagene™ RNA Purification Kit

Introduction

Certain types of tissues contain high levels of contractile proteins and connective tissue, making disruption and homogenization of the tissue difficult. Incomplete disruption and homogenization can result in degradation of the RNA and lower than expected yields. To obtain the best yields of high quality RNA, we recommend treating fibrous tissue samples with Genra Proteinase K Solution (Cat.# V- 0550, 30 mg/ml in buffer) during the Versagene RNA Isolation Process as outline in the procedure below.

Before Starting:

1. Add TCEP to the Lysis Solution (8ul TCEP per every 800 ul Lysis Solution) for all samples that will be processed. TCEP is stable in the Lysis Solution for 30 days at room temperature.
2. Mix Lysis Solution by shaking prior to each use.

Tissue Homogenization and Cell Lysis

Weight of tissue	Volume of Lysis Solution	Volume of TCEP (0.05 M)	Number of Preclar Columns	Number of purification columns
40-80 mg	800 ul	8 ul	2	1

1. Add 8 µl TCEP to 800µl Lysis Solution
2. Add 40-80 mg of tissue to the Lysis Solution containing TCEP and homogenize thoroughly.

3. If excessive foam is present in the lysate after homogenization, centrifuge at 400 x g for 1 minute to eliminate the foam. Gently rock solution to resuspend any pellet formed during centrifugation.
4. Keep lysate on ice until continuing with the purification protocol.
5. Add 20µl Gentra Proteinase K Solution (20 mg/ml) per 800 µl of lysate and vortex sample for 10 seconds to mix.
6. Keep samples on ice for a 10 minute incubation.

Preclear Column

1. Pipette 400 µl of lysate onto each of 2 Preclear Columns in green tubes. This is a total of 800 µl of lysate/80 mg tissue sample.
2. Centrifuge at 400 x g for 1 minute to obtain a clear lysate free of tissue pieces
3. Discard the baskets containing the Preclear columns.
4. Gently pipette the lysates up and down to resuspend any tissue particulates

RNA Binding Wash I

1. Pipet the lysate (~400µl) from one Preclear green tube onto a purification column in a clear tube.
2. Change centrifuge setting to maximum speed. Centrifuge at 13,000-16,000 x g for 1 minute. The white membrane of the column may become discolored, but this will have no effect on the purified RNA.
3. Discard waste after second centrifugation and reuse collection tube for the final centrifugation.
4. Pipet the second ~400 µl lysate from the second Preclear column onto the Purification column

5. Centrifuge at 13,000-16,000 x g for 1 minute.
6. Rotate the tubes 180 degrees and centrifuge at 13,000-16,000 x g for 1 minute.
7. Transfer basket containing Purification column to a new tube.

Wash 2

1. Add 200 µl Wash 2 solution to the Purification column.
2. Centrifuge at 13,000-16,000 x g for 1 minute.
3. Add an additional 200 µl Wash 2 solution to the Purification column
4. Centrifuge at 13,000-16,000 x g for 2 minutes.

RNA Elution

1. Carefully transfer basket containing Purification column to a new tube. Do not allow the Purification column to come into contact with the waste tube.
2. Add 50-100 µl Elution Solution to the Purification column to elute the RNA. Be sure that the entire surface of the Purification column is saturated with Elution solution.
3. Centrifuge at 13,000-16,000 x g for 1 minute.
4. Discard basket containing Purification Column.
5. Place the tube containing RNA on ice. Store RNA at -70° to -80°C.

VITA

Rebekah Stacey Cosden graduated from Westminster High School in 2001 in Westminster, MD. She then attended the University of Maryland at College Park where she graduated with a B.S. in Animal Science: Equine Studies in 2005. Rebekah has always liked working with horses and enjoys foxhunting as a hobby. After completing her M.S. in Animal and Poultry Sciences at Virginia Tech, she plans to earn her PhD in Veterinary Science at the University of Kentucky beginning in the fall of 2007.