mRNA expression profile differences before and after enzymatic digestion of equine articular cartilage

by

Rishi Sharma

A Thesis
presented to
The University of Guelph

In partial fulfillment of the requirements
for the degree of
Master of Science
in
Biomedical Sciences

Guelph, Ontario, Canada

ABSTRACT

mRNA EXPRESSION PROFILE DIFFERENCES BEFORE AND AFTER ENZYMATIC DIGESTION OF EQUINE ARTICULAR CARTILAGE

Rishi Sharma
University of Guelph, 2017

Advisor:
Thomas G. Koch

Focal cartilage defects of developmental or traumatic origin affect horses and humans in similar manners. Current treatments have poor long-term outcomes. *Ex vivo* tissue-engineered cartilage is a potential treatment option. Tissue engineering benchmarks and process checkpoints rely on accurate knowledge of the normal biological properties of cartilage. Assessment of cartilage gene expression is commonly compared to chondrocytes isolated from enzymatically digesting cartilage. However, studies have demonstrated that gene expression of cultured chondrocytes change over time making them poor proxies for gene expression of chondrocytes within normal native cartilage tissue. More accurate knowledge about normal cartilage biology is needed to inform future cartilage-engineering approaches. The overall hypothesis of work presented is that enzymatic digestion of articular cartilage causes rapid changes in the levels of specific chondrocyte RNAs. First an RNA isolation protocol was optimized to enhance yield and quality for mRNA analysis, next generation sequencing, and miRNA studies. Subsequently, the expression of SOX9, COL1A2, COL2A1, ACAN, and COLX was compared between native and enzymatically-digested cartilage from weight bearing and non-weight bearing regions 24 hours after initiation of digestion and without an *in vitro* chondrocyte culture period. Homogenization method significantly impacted RNA yield, with large ball homogenization producing greater yields of RNA. Digested cartilage showed a significant decrease in the expression of COL1A2, COL2A1, and ACAN compared to native cartilage and an increase in COLX expression in non-weight bearing digested cartilage. Thus, enzymatic digestion of cartilage rapidly and significantly impacts gene expression profiles. Determination of normal cartilage biology should be conducted on native untreated cartilage.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Thomas Koch, who believed in my abilities and offered me an opportunity to be a part of his lab. You always provided me with support throughout this process, but also gave me the independence to enrich my learning. You not only encouraged me to pursue research, but also supported my extracurricular ventures. Thank you for always being a great advisor and guiding my both academically and personally.

I would also like to thank my advisory committee member, Dr. Jonathan Lamarre. Thank you for your incredibly valuable feedback and guidance. You were always available to bounce ideas off of and helped to elevate my research experience tremendously.

I would like to thank the members of the Koch lab, without whom I cannot imagine this experience. Sarah, words cannot describe your impact on me these past two years. Thank you for taking me under your wing and teaching me everything I know in the lab. Not only were you a great support system in the lab, but you were also a great friend to have outside of the lab. Our daily life chats were extremely meaningful and I could not have asked for a better friend! Hamed, thank you for always being a supportive friend and colleague in the lab, my experience would not have been the same without you. Ritesh, thank you for being like an older brother and guiding me through this challenging experience. I want to thank you for also keeping it real with me and motivating me when I most needed it. Keith, thank you for the life experience you imparted and for your continuing support. Josh, thanks for being a lab bud and introducing me to a lot of new EDM music. David, thank you for transitioning me into the

lab and for your support in the first half of this project. Cris, thanks for your encouragement. Naomi, thanks for playing Pokémon Go and the frapps. Megan, thank you for being someone I could talk to and bounce ideas off of. Alex, thanks for being a great friend and ultimate teammate! Olivia, thanks for your presence in the lab. Rames and Dustin, thank you both for sharing your veterinary experience with me. Amy, thank you for being such a kind lab mate. Lisa and Evan, although you both were new to the lab, you made the final stretch enjoyable.

A special thank you to Dr. Monica Antenos, Allison MacKay, and Ed Reyes for all your support in making sure that the lab ran smoothly. Us students in the RHB lab are extremely lucky to have your assistance and we would not be nearly as efficient without your guidance. I would also like to thank Dr. Jing Zhang for her expertise and assistance in using the bioanalyzer, you truly are an expert and helped me immensely, so thank you! Thank you Megan MacAlpine for coordinating equine cartilage sample retrieval. Thank you to Gabrielle Monteith for helping me through the statistical analysis, I am extremely grateful for your assistance and for taking the time to help explain the concepts to me. Thank you to each and every member of the RHB Lab that has helped shape my experience over the past two years and for creating such a positive learning environment.

I want to give a big thank you to Dr. Leanne Stalker and Allison Tscherner, without whom I cannot imagine this project being successful. I refer to you both as my “google searches” because you both are extremely knowledgeable and were always willing to answer any questions I had, even if they were stupid. Thank you for sharing your expertise, I am extremely appreciative of your support!

Thank you to my lab friends who made my experience in Guelph a positive one. Thank you to the OG MBS crew: Rachel, Alana, Jess, Lucas, Karson, Allie, Melissa, Paige and Mitchell. Coming to Guelph was daunting, but you all made the transition very easy. You were all very supportive and friendly and I am extremely grateful to have met such amazing people! Karson, thanks for always being there to grab coffee and vent about the struggles we were having in the lab. I could not have gotten through this without you, so thank you for your support. I am so happy that we are finishing this journey together! I also want to thank Alyssa Hooper, for being such an awesome friend this year. Thank you for your support during all night marking sessions, qPCR struggles, the door decorating contest, and the challenges of life. I am thankful for our friendship and I wish you nothing but the best! Thank you Vashine for being so friendly and helping out with door decorating. I also want to thank the Alumni House Call Centre and the friends I made there. Thank you Cass, Dan, Jill, Michelle, Maggie, Temi, Bree, Eva, and Caroline.

I want to thank my friends and family. Emma, Liz, David, Cynthia, Komal, and Ani – thank you for being the best friends I could have ever asked for. You accept me for who I am and have always been supportive, through the ups and downs (and we know there have been many downs). I am blessed to have such amazing friends, I wouldn't be where I am today without you! Mom, Dad, and Shiva, thank you for your unconditional love and support all my life. You believe in me at times when I have given up on myself. Thank you for driving back and forth to Guelph to pick me up, drop off food, or help me run errands. I am privileged to have you in my life and to be blessed with such a loving family.

Lastly, I want to thank my dog and best friend Bruno. You were the spark of hope and positivity that my life needed. Thank you for being the greatest companion and making my life infinitely better. My work is in honour of you, as a dog diagnosed with joint disease, I hope this research can make advances to help treat/prevent your condition one day!

DECLARATION OF WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in this thesis was performed by me:

Dr. Jing Zhang performed analysis of RNA quality using the Agilent 2100 Bioanalyzer.

Gabrielle Monteith performed the statistical analysis for the assessment of the various parameters evaluated with respect to RNA isolation for Objective 1.

David Dukoff assisted in tissue homogenization and extraction of RNA as part of the optimization study for Objective 1.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

18S - 18S ribosomal ribonucleic acid

ABAM - anti-biotic anti-mycotic

ACAN - aggrecan

ACI - autologous chondrocyte implantation

ACTB - beta actin

ANOVA - analysis of variance

B2M - beta-2-microglobulin

cDNA - complementary deoxyribonucleic acid

COL1A2 - collagen type I (alpha 2 chain)

COL2A1 - collagen type II (alpha 1 chain)

COLX - collagen type X

DMEM - Dulbecco Modified Eagle's Medium

DMEM/F12- Dulbecco Modified Eagle's Medium : Nutrient Mixture F-12

ECM - extracellular matrix

FBS - fetal bovine serum

GAG - glycosaminoglycan

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GUSB - beta-glucuronidase

mRNA - messenger ribonucleic acid

miRNA - micro ribonucleic acid

MSC - mesenchymal stromal cell

NGS - next-generation sequencing

NRT - no reverse transcriptase control

NTC - non-template control

OA - osteoarthritis

qPCR - quantitative polymerase chain reaction

RIN - RNA integrity number

RNA - ribonucleic acid

RPLP0 - ribosomal protein lateral stalk subunit P0

RT - reverse transcriptase

SD – standard deviation

SOX9 - SRY-box 9 transcription factor

INTRODUCTION

Equine joint disease and implications for the equine industry

Joint injuries are one of the leading causes of lameness in horses, which has severely impacted the equine industry. Horse owners and trainers are burdened by significant financial and emotional stress due to veterinary costs, decreased use of the horse, and loss of market value. The equine industry comprises a very large market, supporting 154,000 jobs and contributing more than $19 billion to the Canadian economy (Evans, 2010). Therefore, successful and efficient treatment for joint disease is crucial to limit the financial burden on the owner and to provide the horse an opportunity to return to a healthy state. However, it is difficult to treat cartilage injuries due to the lack of endogenous healing capabilities of the tissue. Cartilage is avascular, aneural, and chondrocytes, the principal cell within cartilage, are embedded in an extracellular matrix. When damaged, cartilage is repaired and replaced with fibrocartilage scar tissue, which is unable to mimic the functions of healthy articular cartilage. In horses post-traumatic osteoarthritis (OA) is the leading cause of cartilage damage, which is manifested due to the accumulation of acquired injuries.

Currently, many treatment options for focal cartilage defects exist, however none are truly sufficient to heal the damage inflicted on the tissue. Most treatments options, both intrinsic and extrinsic, promote the formation of fibrocartilage tissue, a type of cartilage that is unable to mimic the force and shock absorbing properties of articular cartilage. As a result, the joint is unable to withstand force long-term and the patient succumbs to pain and restricted movement. Alternatively, stem cell-based treatment options have been investigated in order to resolve the aforementioned issues. To

validate the structure and integrity of engineered cartilage, comparisons have been made to the expression profile of chondrocytes from enzymatically-digested cartilage. Unfortunately, this comparison may be inaccurate and a false benchmark as digestion of cartilage may result in an expression profile that is not representative of chondrocytes *in vivo*. The dispute is whether this enzymatically-altered tissue represents the same expression profile of native articular cartilage. Additionally, creation of tissue-engineered cartilage requires that conditions mimic the environment present *in vivo*. Thus, the correct biomechanical stimulation, growth factor environment, and developmental conditions are required. In an attempt to better understand those conditions, it is imperative to look at the expression profile of key mRNA and microRNA (miRNA) transcripts.

LITERATURE REVIEW

Structure & function of articular cartilage

Articular cartilage is a specialized connective tissue that lines the surface of bones in diarthroidal joints. It serves as a smooth surface that reduces friction during articulation and is a compressible tissue, which facilitates the distribution of forces on the joint (Hu & Anathasiou, 2003). Normal articular cartilage consists of chondrocytes embedded in an extracellular matrix, which is composed of collagen, proteoglycans, and water (Buckwalter et al, 1988). In humans, articular cartilage is a very cell poor tissue, with chondrocytes comprising roughly 1-5% of the total tissue. Additionally, collagen makes up 10-30% of the tissue, proteoglycans roughly 3-10%, and the majority of the tissue is water and dissolved electrolytes that make up 60-85% of the tissue (Cohen, Foster, & Mow, 1998). Approximately 30% of the water exists in the collagen fibres, in the intrafibrillar space. The remaining water resides in the pores of the extracellular matrix (ECM), where it exists as a gel-like substance providing force distributing properties (Maroudas et al., 1991).

Chondrocytes are the main cell type found in articular cartilage, however they comprise roughly 1-5% of the tissue. Their main role is to secrete ECM materials, namely collagen and aggrecan, while synthesizing and maintaining the ECM. Each chondrocyte is also responsible for producing a microenvironment, in which they are trapped ultimately restricting their migration potential (Bhosale & Richardson, 2008). There are no cell-to-cell contacts between chondrocytes, preventing cell

communication. However, chondrocytes are stimulated by the microenvironment by means of pressure, growth factors, and mechanical loads. The survival of chondrocytes is largely dependent on the optimal chemical and mechanical stimuli imposed on them (Buckwalter et al., 1988). There is a variation in chondrocyte morphology across the different zones of articular cartilage, from flatter chondrocytes near the surface to more spherical chondrocytes in subsequent zones.

The majority of collagen found in articular cartilage is type II collagen (COL2), providing the tensile strength in the tissue (Basser et al., 1998). While other types of collagen are present, they are few and rare and function to stabilize the type II collagen network. Collagen consists of a triple helix of 3 α chains that form a fibril, which is further stabilized by intermolecular crosslinks (Eyre, 1987; Maroudas, 1979).

The final component of articular cartilage are proteoglycans, which are glycosylated proteins consisting of a core protein with 1 or more glycosaminoglycan (GAG) chains attached via a covalent bond (Yanagishita, 1993; Sophia Fox, Bedi, & Rodeo, 2009). Proteoglycans play a role in resisting compressive forces and are abundant throughout the matrix to allow for force dispersal (Korhonen et al., 2003: Mow, Holmes, & Lai, 1984). Aggrecan (ACAN) is the predominant proteoglycan found in articular cartilage, which is covalently bound to chondroitin sulphate and keratin sulphate GAG chains (Kiani et al., 2002). Within articular cartilage, aggrecan plays a role in maintaining the fluid and electrolyte balance in the ECM. Due to aggrecan's negatively charged sulphate and carboxyl groups, it attracts positively charged molecules and repels negatively charged molecules. This increases the concentration of inorganic ions in the ECM and contributes to the osmotic properties of cartilage by

which a lot of water is absorbed, ultimately aiding in its shock absorbing properties (Buckwalter & Mankin, 1997; Hardingham & Fosang, 1992).

Zonal stratification

Articular cartilage is organized into 4 distinct zones, the superficial (tangential) zone, the middle zone, the deep zone, and the calcified zone. The superficial zone is a thin layer that protects the subsequent zones from shear stress. The two main collagen fibres, type II and IX are tightly packed and align parallel to the articular surface. There is an abundance of flattened chondrocytes in the superficial layer. The superficial zone is in direct contact with the synovial fluid and provides protection against sheering forces (Sophia Fox et al., 2009).

Deep to the superficial zone is the middle zone, which makes up the majority of the tissue. The middle zone contains proteoglycans and thicker collagen fibres, which are arranged in a randomized oblique fashion (Clark, 1985). The chondrocytes in this layer are sparse and spherical. The middle zone acts to provide resistance to compressive forces placed on the tissue (Sophia Fox et al., 2009).

Finally, the deep zone is responsible for a majority of the resistance to compressive forces on articular cartilage. This is due to the widest collagen fibrils being arranged perpendicular to the articular surface. The deep zone also has the highest content of proteoglycans and the lowest water content; further increasing its resistive properties to force. The chondrocytes in this zone are also arranged perpendicular to the articular surface, are spherical, and more abundant than the middle zone (Sophia Fox et al., 2009).

The final zone, the calcified zone, is separated from the deep zone by the tidemark. The function of the calcified zone is to anchor the collagen fibres and securing the cartilage tissue to the underlying bone. There are very few chondrocytes in this layer, those present are hypertrophic (Mankin, Mow, Buckwalter, & Iannotti, 1994).

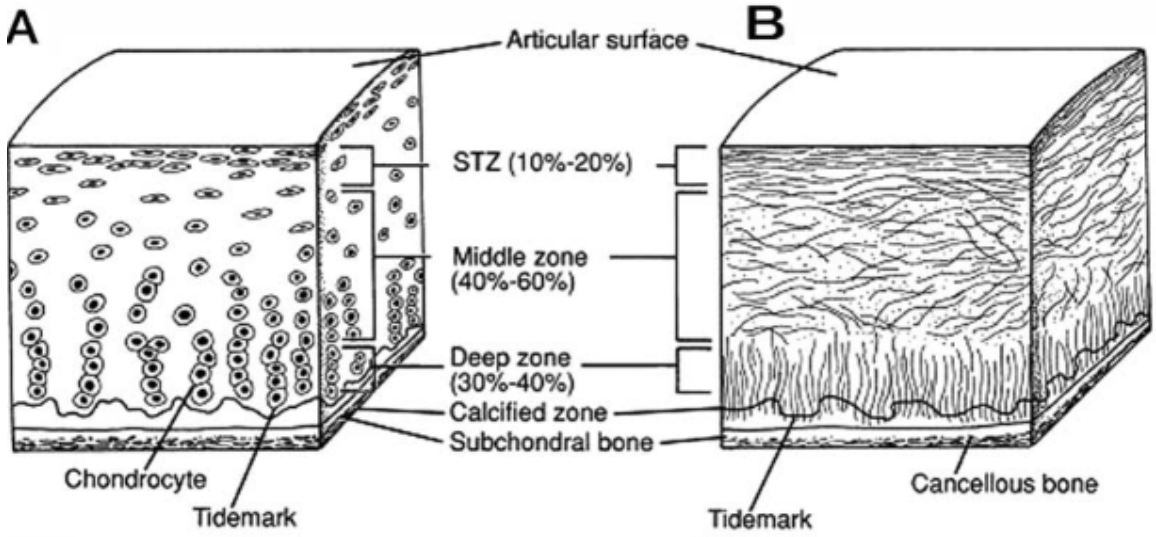

Figure 1: Zonal stratification and composition of articular cartilage. A) Chondrocyte organization and morphology across different zones. B) Arrangement of collagen fibres and orientation in different zones. Adapted and obtained with permission from *Buckwalter et al., 1994.*

Extracellular matrix organization

In addition to zonal stratification, the matrix is characterized by 3 distinct regions. The pericellular region, the territorial region, and the interterritorial region are distinguished by their proximity to chondrocytes, composition, and distribution of collagen and proteoglycan molecules (Sophia Fox et al., 2009). The pericellular region is the layer of matrix adjacent to the cell membrane of chondrocytes. This layer is composed mostly of proteoglycans and other minor glycoproteins. It is suggested that this layer plays a role in initiating signal transduction (Eggli, Herrmann, Hunziker, &

Schenk, 1985). The territorial matrix instead is composed mostly of thin collagen fibres and forms a protective network around the chondrocytes to protect from mechanical stress imposed on cartilage (Muir, 1995; Poole, 1993; Szirmai, 1969). Lastly, thick collagen fibres and proteoglycans are both abundant in the interterritorial matrix. It is the largest of the 3 matrix regions and provides articular cartilage with its characteristic biomechanical properties (Mow & Guo, 2002).

The distribution and ratio of collagen and proteoglycans within the matrix is what dictates the load-bearing abilities of cartilage tissue. In the superficial zone, where collagen fibres are parallel to the articular surface, the tissue is better suited for tensile strength. As the collagen fibres become more perpendicular in the subsequent layers, the compressive strength increases (Sophia Fox et al., 2009). As mentioned previously, the ability of proteoglycans to create an osmotic gradient facilitate absorption of water allowing the matrix to swell, whereas collagen limits the swelling ability of cartilage (Martel-Pelletier, Boileau, Pelletier, & Roughley, 2008). Therefore, the ratio of collagen to proteoglycans in the matrix regulates the swelling ability of cartilage, allowing for force-dependent fluid movement in and out of the cartilage that facilitates force distribution and lubrication (Broom & Poole, 1983; Kempson, 1980). Thus, there is a greater content of proteoglycan in the middle and deep zones compared to the superficial zone, while collagen is predominant in the superficial zones than the deep zone (Sophia Fox et al., 2009).

Equine cartilage as a model for human cartilage disease and treatment

Animal models are commonly used to explore disease pathologies and treatment options prior to initiating clinical trials in humans due to the ethical and logistical complications of obtaining human tissue. Mice and rats are usually the preferred animal model due to the ability to generate genetically modified models, whether that be knocking out a gene or creating transgenic mice to model disease (Vandamme, 2014). However, mice and rats are not reliable surgical models for cartilage disease as they have smaller joints and thin articular cartilage, which hampers surgical manipulation and outcome assessment. Although larger animal models such as pigs, rabbits, cows, and dogs are used in induced surgical models of cartilage repair, horses remain an attractive model for human medicine for several reasons (Chu, Szczodry, & Bruno, 2010). Firstly, horses are the most similar model with respect to the manifestation of focal cartilage defects and/or post-traumatic OA from athletic injuries like humans (McIlwraith, Frisbie, & Kawcak, 2012; Lacourt et al., 2012). Secondly, the thickness of articular cartilage is similar in both horses and humans. Horse articular cartilage on the stifle joint, which is analogous to the human knee joint, is 1.5-2.0mm whereas the human knee joint cartilage is 2.2-2.5mm thick (Frisbie, Cross, & McIlwraith, 2006). Other animal species have largely different thicknesses, for example rabbits have a thickness of 0.3mm, dogs 0.6-1.3mm, and goats 0.7-1.5mm. Furthermore, due to the ability to generate large focal defect sizes in horses, there are substantially more repair outcome parameters that can be assessed as compared to other animal models. Some of these parameters include clinical examination of lameness, pre and post-treatment

radiographs, ability to measure flexion, sequential arthroscopies, gross post-mortem examinations, and many more (McIlwraith, Fortier, Frisbie, & Nixon, 2011).

Cartilage injuries

Focal cartilage defects are produced due to trauma or inflammation and if left untreated will continue to deteriorate the tissue and ultimately lead to OA (Wang et al., 2006). Unfortunately, cartilage lacks endogenous healing capabilities due to the lack of vasculature, which deprives the tissue with any nutrient supply. Furthermore, the extensive ECM prevents cells from migrating to the defect site within the tissue. The inability to repair these defects ultimately leads to the onset of post-traumatic OA, which predominantly affects the fetlock, knee and hock joints (Kidd, Fuller, & Barr, 2010). Currently, if lesion size is less than 0.5cm^2 non-surgical treatment options are used to address defects. Nonsteroidal anti-inflammatory medications, intra-articular corticosteroid injections, and hyaluronic acid viscosupplementation have been utilized to relieve inflammation and pain. Furthermore, limiting the movement of the joint to reduce further damage as well as weight loss have been recommended (Strauss et al., 2011). For lesions greater than 0.5cm^2, surgical options such as microfracture, autologous chondrocyte implantation, and mosaic arthroplasty are currently used for treatment (Williams et al., 2010).

Current treatments for cartilage defects

For smaller lesions less than 2cm^2 in size that have not impacted the subchondral bone, debridement and microfracture are utilized (Knutsen, 2007). First the defect site is debrided to remove any damaged tissue and to expose the underlying

subchondral bone. Then small holes are drilled into the subchondral bone to promote healing by creating a blood clot as well as releasing chondroprogenitor cells from the underlying bone marrow to promote the formation of repair tissue (Steadman, Rodkey, & Rodrigo, 2001; Steadman, Rodkey, & Briggs, 2002). However, the repair tissue generated does not serve as a functional repair and patients have reported functional deterioration and pain in as little as 18 to 36 months post-microfracture (Mithoefer et al., 2009). The repair tissue formed consists of fibrocartilage, a biomechanically inferior tissue compared to hyaline cartilage. Additionally, there is incomplete filling of the defect site and the newly generated repair tissue integrates poorly with the surrounding tissue (Williams & Harnly, 2007). In horses, Frisbie and colleagues (1999) showed similar results where the repair tissue generated contains more collagen type II, but less GAG content than native tissue that compromises the compressive properties of the tissue.

Autologous chondrocyte implantation (ACI), a cell-based therapy, has long been used for treatment of cartilage defects. It is a two-step procedure that involves first harvesting native cartilage from a non-weight bearing region of cartilage from the patient. This cartilage is then enzymatically digested to isolate the chondrocytes, which are then expanded in culture and reimplanted into the defect site (Brittberg et al., 1994; Knutsen et al., 2004). There are several issues with this procedure, one being donor site morbidity as harvesting cartilage from those sites deteriorates that tissue, causing further damage (Lee et al., 2000). Furthermore, there is a limited amount of chondrocytes that can be extracted, which is further complicated by the fact that chondrocytes exhibit dedifferentiation in culture, losing the characteristics of articular cartilage (von der Mark et al., 1977). After multiple passages, chondrocytes in

monolayer expansion begin to upregulate collagen type I while downregulating collagen type II and aggrecan (Cheng et al., 2011; Saris et al., 2008). However, ACI has shown more promise than other procedures. Specifically in humans, Brittberg and colleagues (2003) reported that 81% of their patients had good-to-excellent results and 83% at 5-11 years. Pascual-Garrido and colleagues (2009) also reported that patients treated with ACI had significantly better results than other established treatment methods based on several functional parameters. In horses, ACI has also been proven successful as the repair tissue maintains hyaline-like characteristics compared to defects left untreated (Frisbie et al., 2008). Nixon and colleagues (2011) also reported that ACI produced better matrix organization with significantly higher proteoglycan content. Although ACI has been proven as a better treatment option compared to microfracture, it still is inferior to mosaic arthroplasty (Horas et al., 2003; Dozin et al., 2005).

Mosaic arthroplasty, also known as osteochondral autograft transplantation, is predominantly used for full thickness defects where cartilage and subchondral bone have been impacted. It involves a one-step procedure where a osteochondral “plug” is removed from a non-weight bearing region from the donor, in this case the patient, and press-fitted into the defect site (Hangody et al., 2008). This procedure is beneficial in that it replaces the defect site with hyaline cartilage from the patient, limiting immunologic reactions. However, as with ACI, the limitations include donor site morbidity, limited availability of tissue, as well as gaps at the lesion/autograft interface. Additionally, since these plugs are taken from a non-weight bearing area, the composition of the matrix in these plugs varies from that of a weight-bearing region, further complicating the functional capabilities of this procedure (Esquisatto, Pimentel, &

Gomes, 1997). Hangody & Fules (2003) reported that 92% of patients treated for femoral condyle lesions and 79% of patients treated for patellar lesions reported good-to-excellent results in a 10-year follow up study. In horses, Bodo and colleagues (2004) conducted mosaic arthroplasty to treat subchondral cysts. Of the 11 horses treated, 10 had successful outcomes of which 7 went on to perform at high activity levels. Once again, the issues of donor site morbidity and cartilage composition variance exist. In order to combat this allografts have been investigated, however complications such as fresh allograft sources and risk of infection or immune rejection exist (Strauss & Galos, 2013).

Alternatively, to combat the issue of donor site morbidity and limited cell quantity, scientists have explored the use of tissue engineering approaches to treat cartilage defects. Tissue engineering involves creating an osteochondral construct, which is seeded with chondrocytes to mimic the osteochondral “plug”, used in mosaic arthroplasty. Seeded chondrocytes are generated either by isolating chondrocytes and expansion in monolayer culture or differentiation of mesenchymal stromal cells (MSC) into chondrocytes (Tuli, Li, & Tuan, 2003). Chondrocytes are isolated in a similar fashion to the first step of ACI, native cartilage tissue is harvested and enzymatically digested to isolate the chondrocytes, which are then cultured in monolayer culture until a sufficient number of cells are achieved for tissue-engineering purposes. On the other hand, MSCs are pluripotent cells that have self-renewal properties, which can be cultured in chondrogenic media to induce chondrogenesis for the generation of chondrocytes (Dragoo et al., 2003).

Complications with comparison to primary chondrocytes

One of the major complications with tissue-engineering, which was prevalent in ACI techniques as well, was the potential for dedifferentiation of chondrocytes which would then take on a fibroblast-like morphology and result in the formation of fibrocartilage repair tissue, unlike native cartilage (Cancedda et al., 2003). The issue with MSC-derived chondrocytes becomes that the cells are not being programmed under the right biomechanical conditions to mimic the development of chondrocytes in vivo. It has been shown that compressive mechanical forces aid in chondrogenesis by upregulating the expression of key chondrogenic genes such as SOX9, collagen type II, and aggrecan (Takahashi et al., 1998). As a result, MSC-derived chondrocytes may exhibit a different phenotype and may not be able to create cartilage with the same characteristics as native cartilage. Assessment of the gene expression of chondrocytes seeded onto osteochondral constructs have therefore been compared to native chondrocytes as a parameter of assessment. Due to the nature of articular cartilage, chondrocyte retrieval from native tissue is particularly challenging. As a result, native cartilage is enzymatically digested to break down the extensive ECM, allowing for increased chondrocyte retrieval for comparison. However, it has been proposed that enzymatic digestion of cartilage results in altered gene expression of key chondrogenic genes such as collagen type II, aggrecan, and GAPDH in chondrocytes (Hayman, Blumberg, Scott, & Athanasiou, 2006). Similar findings were also reported in knee meniscus tissue, where there was abnormal gene expression compared to native tissue. The use of collagenase type II produced the least changes to the expression profile of chondrocytes as compared to native cartilage, nonetheless chondrocytes

displayed a phenotypical change towards a fibrocartilage phenotype (Sanchez-Adams & Athanasiou, 2012). Thus, the use of chondrocytes from enzymatically-digested cartilage for gene expression comparisons may not be accurate as they may not be representative of chondrocytes *in vivo*.

Role of ECM in chondrocyte gene expression

The extensive and intricate structure of the ECM not only contributes to the organization of cartilage and the various functions of the tissue, but it also contributes to signal transduction that affects the expression profile of chondrocytes. ECM molecules have been shown to interact with chondrocytes through adhesion receptors found on the surface of chondrocytes. Specifically, integrin receptors present on the surface of chondrocytes have been shown to interact with ECM molecules and have altered gene expression of chondrocytes as a result of these interactions (Damsky & Werb, 1992). Integrins are transmembrane heterodimeric receptors that can bind to ligands of the ECM (Horwitz et al., 1985). In a study done by Werb and colleagues (1989), they demonstrated that the integrin family of adhesion receptors bind to ECM molecules such as collagen and fibronectin. Disruption of the ECM ligand and chondrocyte integrin interaction resulted in a change in ECM gene expression, matrix deposition, as well as regulation of the ECM environment. The depletion of ECM components in bovine articular cartilage explants resulted in 9-10 fold increases in aggrecan, which were likely caused by the interaction between ECM ligands and chondrocyte integrins (Lee, Bentley, & Archer, 1994). Binding of ECM ligands to the integrin receptor causes a change in the cytoskeleton of cells, contributing to the increase or decrease in gene expression (Ruoslahti, 1991). In a study conducted by Hering and colleagues (1994)

they also reported an increase in collagen type II, aggrecan, and chondrocyte link protein upon enzymatic depletion of ECM components, which was suggested to be due to cytoskeleton reorganization.

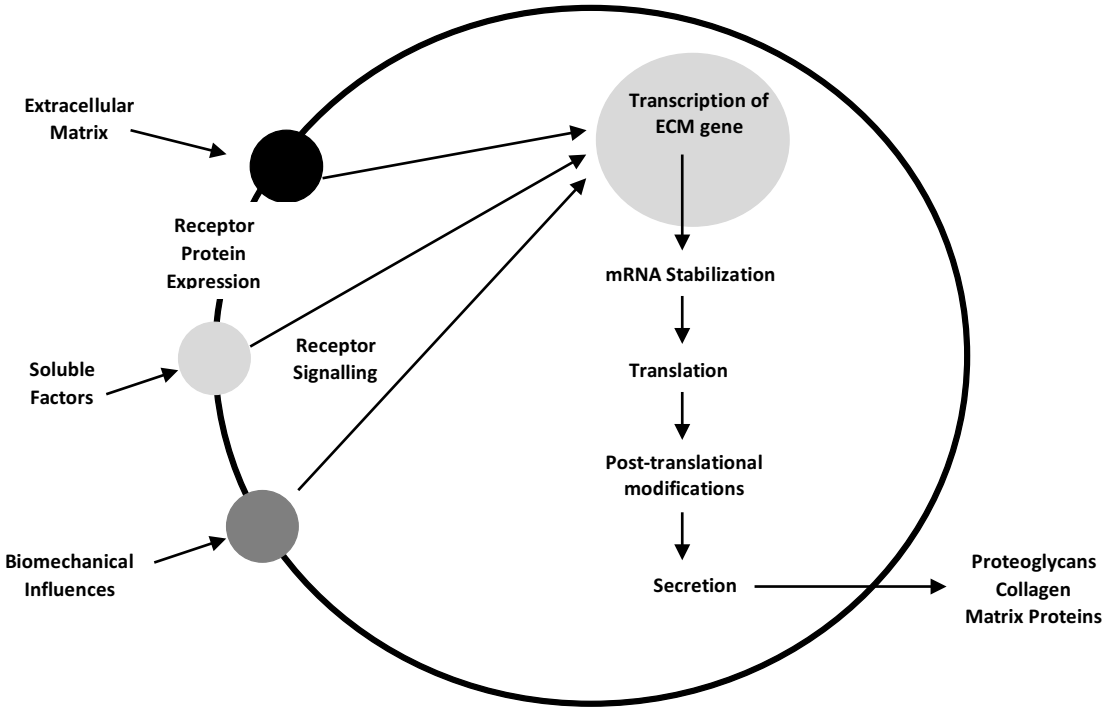

Figure 2: Extracellular matrix effects on signal transduction and gene expression in chondrocytes. Binding of ECM ligands to cell receptors influences the production of matrix proteins. Adapted with permission from *Hering, 1999.*

Chondrocyte shape has been known to have an effect on gene expression. Particularly, cells that retain the typical spherical shape of a chondrocyte maintain a normal phenotype (Benya & Shaffer, 1982). However, as the cells take on a fibroblast-like morphology and flatten, the result is a change in phenotype that leads to dedifferentiation (von der Mark, 1986; Zanetti & Solursh, 1989). Takigawa and colleagues (1984) explored the effects of altering cytoskeleton structure on gene expression in rabbit chondrocytes. When cells were changed from a polygonal to

spherical morphology, there was an upregulation of GAG synthesis. In contrast, when cells were changed from a polygonal to flattened morphology by microtubule inhibition, there was an inhibition of GAG synthesis. However, complete disruption is not required to cause a change in phenotype, modifications of the microfilaments in a cell have also been reported to produce changes in gene expression in chondrocytes (Benya, Brown, & Padilla, 1988). Ultimately, changes to the ECM composition, whether that be through enzymatic digestion or matrix reorganization, results in a change in the intracellular mechanisms of chondrocytes causing a change in gene expression.

Chondrocyte phenotypes and characteristic gene expression

There are 3 distinct chondrocyte phenotypes that can be identified based on expression profiles of ECM components. Firstly, there are chondrocyte precursor cells, which help form cartilage during development. These chondrocytes namely synthesize collagen type I, a form of cartilage that does not lend the same biomechanical strength. However, cells that are cultured in monolayer can dedifferentiate and take on a fibrocartilage-like morphology and produce similar products as precursor chondrocyte cells. Upon dedifferentiation, these cells replace the production of type II collagen with type I collagen, a biomechanically weaker collagen molecule. Secondly, upon maturation chondrocytes that have differentiated can be found in developed hyaline cartilage in adults. These chondrocytes predominantly express collagen type II, which contributes to the biomechanical properties of developed articular cartilage. These chondrocytes also express collagen type IX and XI, however collagen type II is abundant and predominant in comparison. Lastly, hypertrophic chondrocytes have terminally differentiated and are commonly associated with OA (Aigner et al., 1992).

These chondrocytes express collagen type X, which is normally not expressed in healthy human articular cartilage but is detectable in osteoarthritic cartilage. Additionally, hypertrophic chondrocytes are characterized by the expression of matrix metalloproteinase 13 (MMP13) and Runx2, which are associated with calcification of articular cartilage (van der Kraan & van den Berg, 2012).

The production of these different forms of collagen and the temporally differential expression pattern is regulated, similar to many other tissues, by transcription factors. Sox9, the major chondrogenic transcription factor, is responsible for the activation of gene expression for several genes such as collagen type I, type II, type IX, and aggrecan (de Crombrugghe et al., 2000). In coordination with two other transcription factors, Sox5 and Sox6, Sox9 binds to the promoter regions of several important chondrogenic genes (Lefebvre, Li, & de Crombrugghe, 1998). It has been shown that when Sox9 is knocked out, mice fail to produce chondrocytes due to a block in the mesenchymal condensation phase. Thus, Sox9 plays an important role in not only in promoting the expression of ECM molecules, but also in controlling the expression of cell surface markers that are involved in condensation during chondrogenesis (Bi et al., 1999).

RNA isolation, importance and challenges with articular cartilage

One way in which gene expression can be assessed is through the determination of messenger RNA (mRNA) levels of target genes in a sample of interest. Gene expression begins by the transcription of DNA sequences into RNA sequences, which are then modified to produce mRNA and subsequently translated into a protein

(Bogenhagen & Brown, 1981). This protein is the product of gene expression and ultimately has effects within the cell or is transported to exert its effects on the extracellular environment. Analysis of gene expression provides the ability to determine the phenotype of cells and tissues, but is also very important in identifying disease states as mRNA levels can be used as biomarkers of disease (Mendrick, 2011). Thus, analysis of mRNA expression is crucial and provides scientists with valuable information.

Unfortunately, isolation of RNA from articular cartilage has long been a problem for scientists investigating the molecular biology of cartilage. There are several factors that make RNA isolation from cartilage difficult, namely the low cell content (1-5% of tissue mass) found in human cartilage. Additionally, the highly cross-linked ECM molecules prove challenging for tissue homogenization. There is also a high proteoglycan content, which may impede isolation protocols using several commercial kits. Lastly, the collection of cartilage samples from adult specimen introduces the age-associated degradation of cartilage that affects yield (Ruettger et al., 2010). Several studies have been conducted to enhance the RNA isolation procedure from cartilage, but no “gold standard” is available for consistent RNA isolation.

The first step in RNA isolation of articular cartilage is to homogenize the tissue in order to break down the dense ECM. This allows the isolation protocol chemicals to take effect and allows greater contact with chondrocytes. Geyer and colleagues (2008) reported that the use of a mill-based homogenization technique as superior to the conventional methods of using a mortar and pestle or stator-rotor homogenizer. Subsequently, an RNA isolation kit is required to extract high-quality RNA in large

quantities, a necessity for downstream analysis such as qPCR and/or microarray. There are several kits available currently for RNA isolation, the mirVana and miRNeasy isolation kits utilize a phenol/chloroform extraction process whereas other kits such as the RNeasy isolation kit is a phenol/chloroform-free extraction process (Ruettger et al., 2010).

Assessment of RNA quality

Ultimately, effective RNA isolation strategies require a high RNA content yield as well as high purity, a measure of stable RNA material. RNA quality can be assessed using absorbance ratios or the use of RNA integrity numbers. Absorbance ratios are determined using a spectrophotometer, where the absorbance of the molecules in a sample is determined and ratios are then calculated to assess quality. Absorbance ratios 260/280 and 260/230 are the standards most commonly used to assess RNA quality. For RNA samples, 260/280 ratios should be between 1.8-2.2 whereas 260/230 ratios should be greater than 1.7. Nucleic acids are known to absorb at 260nm, while protein compounds are known to absorb around 280nm and absorbance at 230nm is indicative of other contaminants that may be present in the isolation kits such as guanidine thiocyanate (Wilfinger, Mackey, & Chomczynski, 1997). One of the major disadvantages of the use of absorbance ratios is the lack of specificity of contaminants. Furthermore, the accuracy of the instrument is compromised with low quantity samples (Wieczorek, Delauriere, & Schagat, 2012). Using articular cartilage, the concentration of RNA yield is fairly low and thus the use of absorbance ratios is not an ideal parameter for RNA quality, instead the use of gel electrophoresis to assess the degradation of RNA has emerged as a superior technique.

RNA Integrity Number

Historically, gel electrophoresis has been used as an assessment tool for RNA quality. The process works by analyzing RNA in agarose gel electrophoresis stained with ethidium bromide. The resulting bands of RNA are then analyzed; specifically the ratio of 28S rRNA to 18S rRNA is measured to determine degradation in the sample (Sambrook, Fritsch, & Maniatis, 1989). One of the major drawbacks to this method is the subjectivity and room for human error. The emergence of the Agilent 2100 bioanalyzer helped address this issue, in which the RNA molecules are separated based on molecular weight in a microchip. The software then analyzes the chip and detects the separated bands of RNA content using laser-induced fluorescence (Mueller et al., 2000). Once again, assessment of 28S to 18S ratios was used to determine the extent of degradation in the sample being analyzed. However, it has been reported that the use of the ribosomal ratio of 28S/18S is weakly correlated with the integrity of RNA molecules (Imbeaud et al., 2005). As a result, the RNA integrity number (RIN) was developed as a superior assessment measure. The RIN value is calculated using a software algorithm programmed into the Agilent 2100 bioanalyzer, which looks at the entirety of the curve produced on the electropherogram. After assessing several features and regions of the electropherogram, in addition to the 28S/18S ratio, the software produces a RIN value ranging from 1-10 (Schroeder et al., 2006). This value is substantially more valuable and is based on several important markers of degradation. Thus, modern research has utilized the RIN value as a staple assessment measure for determining the quality of RNA in samples.

Downstream applications using longer stranded RNA, such as messenger RNA, require high RIN values to ensure that the RNA sample has not degraded. RNA degradation would ultimately affect downstream analyses and may cause erroneous results. qPCR, microarray, and next generation sequencing (NGS) methods are recommended to be done using RNA with a high RIN value as a measure of integrity of the sample. Although no consensus on the threshold RIN value to perform downstream analyses has been stipulated, researchers have shown that expression studies significantly vary with progressively lower RIN values (Romero et al., 2014). Furthermore, RIN values are not a suitable predictor of miRNA stability as the basic premise of a RIN value is to evaluate the stability of longer stranded RNA molecules. With miRNA samples, the RIN value is not a robust method of assessment of integrity. Instead, qualitative observation of the bioanalyzer generated electropherograms and gels are a preferred method of assessment. Additionally, miRNAs have been found to be very stable as compared to mRNA transcripts. Specifically a study that observed degradation in mRNA samples with altered expression results saw no impact on miRNA samples (Jung et al., 2010).

miRNA role in gene expression

miRNAs are small non-protein coding single-stranded RNA molecules that range from 22-25 nucleotides in length. miRNAs are transcribed by RNA polymerase II from either independent genes or introns of coding genes as a primary miRNA or pri-miRNA, which consist of a hairpin structure (Berezikov, 2011). pri-miRNA molecules are then processed and the hairpin loop is cleaved at the stem by Drosha, creating a 60-70 nt hairpin structure known as the pre-miRNA (Ha & Kim, 2014). pre-miRNA are then

transported from the nucleus to the cytoplasm where they are further processed to form mature miRNAs. In the cytoplasm, Dicer cleaves the miRNA molecule to roughly 22 nt, generating a RNA duplex molecule (Ketting et al., 2001). Upon formation of an RNA duplex molecule, argonaute proteins (AGO) associate with the duplex to form the RNA-induced silencing complex (RISC) (Ha & Kim, 2014).

MiRNA molecules act post-transcriptionally to regulate gene expression (Wahid et al., 2010). They do so either through translational repression of target mRNA transcripts or by degradation of target mRNA transcripts. The degree of complementarity between miRNA and their target mRNA transcripts determines whether translational repression or transcript degradation occurs (Bartel, 2009; Huntzinger, 2011). High complementarity between miRNA and target mRNA transcripts results in degradation, whereas incomplete complementarity simply causes translational repression (Wahid et al., 2010).

As mentioned above, miRNA quality cannot be assessed using conventional methods for total RNA such as the bioanalyzer. Instead, assessment of miRNAs is usually done through qPCR, microarray or NGS.

Next generation sequencing

NGS, also commonly defined as high-throughput sequencing or deep sequencing, is a process in which small fragments of DNA are sequenced in parallel to determine gene expression but also to sequence transcripts. Traditionally, Sanger sequencing was used, however this technique is a lot more expensive, requires more sample and is less accurate (Behjati & Tarpey, 2013). There are many different

sequencing platforms such as Roche 454, Illumina, and SOLiD sequencing, but for the purposes of our study Illumina was chosen as our preferred platform. Illumina sequencing begins by fragmentation of the DNA sample to create many small fragments. These fragments are then ligated to adapters, which are then seeded onto a flow cell that has attached short oligos complementary to the adapter sequences to facilitate capturing. The fragments are then amplified and clusters of the same fragment are created, known as cluster generation. The fragments are then sequenced by the addition of DNA polymerase and nucleotides. The nucleotides are fluorescently labelled with a distinct colour for each type (adenine, guanine, cytosine, and thymine) with a chemically inactivated 3'-OH to facilitate termination and prevent additional bases from being attached. Bases are thus incorporated one by one, and after each cycle of one base being added, an imaging step follows. After imaging, the fluorescent signal is removed and the 3' end is unblocked to allow for the addition of one more base. This cycle continues until the fragment is sequenced. Finally, the sequences are mapped by looking at the fluorescent signal for successive images of cycles to create a sequence (Mardis, 2007). The generated sequences are then aligned to a reference genome to observe for any mutations, insertions or deletions, as well as to generate read counts for RNA. NGS is a lot cheaper than previous sequencing methods due to the chemical reaction step and detection step being combined, whereas in Sanger sequencing these steps were separate. Additionally due to the parallel sequencing technology, less initial sample is required and fragments are amplified that generates multiple reads for the same fragment of DNA (Metzker, 2010).

Use of NGS technologies requires samples of high quality and quantity to ensure accurate results. Specifically, Exiqon guidelines require that 300 ng of RNA sample be submitted for NGS analysis. Although there are definitive requirements for quantity, quality is not as robustly stated. RNA quality is assessed using the Agilent 2100 Bioanalyzer for total RNA samples, with samples with RIN values ranging between 7-10 being considered stable. RIN values ranging from 5-7 indicate partial degradation, whereas RIN values below 5 have high degradation. For total RNA samples, RIN values above 7 are considered acceptable for NGS experiments. However, for small RNA enriched samples, namely miRNA samples, the RIN value is not a reliable method of assessment due to its assessment parameters requiring longer stranded RNA (Exiqon, 2015). As a result, Exiqon will analyze small RNA enriched samples that have low RIN values cannot be accurate. It has been shown that miRNA transcripts have shown no change in expression upon successive degradation, whereas degradation has altered expression of mRNA transcripts (Jung et al., 2010). Thus, miRNA samples may be more stable and less prone to degradation. Ultimately, small RNA enriched samples can be assessed qualitatively for the presence of small fragment RNA by observing the gel electrophoresis images and electropherograms produced from the Agilent 2100 Bioanalyzer.

RATIONALE

Articular cartilage is a complex tissue wherein structure and function are modulated through precise gene expression in order to achieve the optimal structural and function properties to be able to withstand high loads. Currently, no ideal treatments are available to improve the repair of focal cartilage defects. However, the use of tissue-engineered constructs is being explored in an attempt to combat the issues with other treatment options. In order to assess the expression profile of engineered cartilage, studies are comparing gene expression in tissue-engineered constructs to chondrocytes isolated from enzymatically digesting articular cartilage. However, many studies have shown that chondrocytes isolated in said manner are prone to dedifferentiation and have a change in phenotype resulting in a change in expression profiles. Thus, the use of chondrocytes from cartilage that has been enzymatically digested may not be an accurate benchmark and may provide inaccurate gene expression findings.

This study was conducted using the equine model, as it is the closest model in terms of similarity in articular cartilage as well as the manner in which cartilage injuries are sustained to humans. Discoveries in the equine model have translational potential for human medicine, which makes this a powerful model to investigate further. First, an optimal protocol for RNA isolation from native equine cartilage was determined, isolating both total RNA and small RNA enriched samples. Secondly, this study investigated the effects of enzymatic digestion on articular cartilage to explore the effects of digestion on gene expression profiles in order to determine whether chondrocytes isolated in such fashion are truly an accurate representation of chondrocytes in native (undigested) cartilage *in vivo.*

HYPOTHESIS

Enzymatic digestion of articular cartilage causes rapid changes in the levels of specific chondrocyte RNAs.

OBJECTIVES

Objective 1: To optimize a total RNA isolation procedure for extracting RNA from native equine articular cartilage with high yield and integrity suitable for NGS, mRNA and miRNA expression studies.

Objective 2: To determine mRNA expression profile of important chondrocyte associated genes in enzymatically digested and undigested (native) equine articular cartilage.

MATERIALS & METHODS

Equine Articular Cartilage Harvesting

Equine articular cartilage samples were collected from the fetlock and stifle joints of cadaver limbs from post-mortem specimen that were euthanized. Limbs were stored for a maximum of 24 hours at 4°C prior to harvesting of cartilage and efforts were made to harvest cartilage immediately after euthanizing. The limbs were aseptically prepared prior to opening the joint space by scrubbing with antibacterial detergent and swabbing the area with 70% ethanol alcohol. The joint space was exposed using a number 20 scalpel blade using care not to make contact with or contaminate the articular cartilage surface. 1 mm shavings were taken tangential from the surface of the joint, ensuring to not include tissue from the underlying bone. Shavings were taken from both weight bearing (distal medial/lateral trochlear surfaces and sagittal ridge) and non-weight bearing regions (dorsal and palmar medial/lateral trochlear surfaces and sagittal ridge) of the fetlock joint for and the weight bearing (condyle) and non-weight bearing regions (lateral condyle) of the stifle joint. Cartilage shavings that were utilized for native cartilage samples were snap frozen in liquid nitrogen and stored at -80°C for RNA isolation, shavings that were enzymatically digested were placed in PBS and subsequently digested.

For objective number 1, samples were collected from six fetlocks and two stifle joints of 8 independent horses; one joint was used per horse. For objective number 2, equine articular cartilage shavings were collected from one fetlock joint of 5 independent donors.

Isolation of Chondrocytes

For enzymatic digestion of cartilage, shavings were transferred from the PBS solution to a petri dish with 15mL of Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM-F12) (Sigma Aldrich) containing 1% type I collagenase (Sigma Aldrich). The shavings were cut into 1x1x1 mm pieces using a number 10 scalpel and digested overnight at 37°C. The following morning, the digested samples were pipetted several times to facilitate digestion. After 4-6 hours, chondrocytes were retrieved by straining the digested sample through a 70μM filter followed by 3 washes of 10mL of DMEM-F12 with centrifugation at 900X*g* for 6 minutes between washes. The supernatant was removed and chondrocytes were resuspended in DMEM-F12 + 5% fetal bovine serum (FBS) (Invitrogen; Burlington, ON, Canada) and counted using a haemocytometer. Chondrocyte morphology was also assessed to characterize cell viability. Chondrocytes were then homogenized through pipette aspiration in mirVana total RNA isolation kit (ThermoFisher) lysis buffer prior to proceeding to extraction.

Tissue Homogenization

Snap frozen shavings were removed from -80°C storage and placed in either a pre-cooled stainless-steel canister with a 25mm stainless steel grinding ball (large ball) or in an Eppendorf Biopur RNase/DNase free tube with a 5mm stainless steel grinding ball (small ball). For large ball homogenization, the canister was closed and immersed in liquid nitrogen for 2 minutes. The tissue was then disrupted using an oscillating milling machine (Retsch Mixer Mill MM 400; RETSCH; Haan, Germany) for 2-3 minutes at 30Hz, in 1-minute intervals stopping to assess the degree of homogenization of the tissue. If the tissue was not homogenized to a powdered consistency, the canister was

immersed in liquid nitrogen and milled for an additional minute. Once the tissue was pulverized to a powder consistency, the tissue was moved to an Eppendorf Biopur RNase/DNase free tube (Sigma Aldrich) for RNA isolation. For small ball homogenization, individual Eppendorf tubes were put into a milling block, which was able to hold a maximum of 8 tubes. Tubes were loaded onto the block and then immersed into liquid nitrogen for 2 minutes. The tissue was then disrupted, following the same procedure as above, stopping after each minute to assess the degree of homogenization.

RNA Isolation – mirVana and miRNeasy Kits

For objective 1, total RNA was isolated from disrupted native (undigested) cartilage tissue using the mirVana total RNA isolation kit (Life Technologies) or miRNeasy isolation kit (Qiagen), following the protocols as outlined by each manufacturer for total RNA isolation. Homogenized cartilage tissue was subjected to either an incubation period with lysis buffer or no incubation prior to extracting RNA using either kit. Isolated RNA was purified using the RNA Clean & Concentrator Kit (Zymo Research; Irvine, CA, USA) and stored at -80°C.

For objective 2, total RNA was isolated from both native and digested cartilage tissue. Native cartilage was homogenized using large ball homogenization. RNA was extracted from both native and digested cartilage samples using the mirVana total RNA isolation kit (Life Technologies), following the protocols as outlined by each manufacturer for total RNA isolation. Isolated RNA was purified using the RNA Clean & Concentrator Kit (Zymo Research; Irvine, CA, USA) and stored at -80°C. Samples were analyzed using the Nanodrop 2000 Spectrophotometer and Agilent 2100 Bioanalyzer.

Generation of cDNA using qScript

Isolated RNA was first DNase treated using the PerfeCTa DNase I Kit (Quanta Biosciences; Beverly, MA, USA) following the protocol as outlined by the manufacturer in the kit. Samples were then reverse transcribed using the qScript cDNA Synthesis Kit (Quanta BioSciences; Beverly, MA, USA) following the protocols as outlined by the manufacturer.

Validation of Reference Genes

Determination of the most stable reference genes was undertaken using three separate software programs. 8 candidate genes (ACTB, GAPDH, 18S, B2M, GUSB, RPLP0, COL1A2, SOX9) were tested using a randomized set of 10 independent samples of RNA from cartilage tissue and chondrocytes. A quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in 10µL reaction volumes containing 5µL of SsoFast EvaGreen Supermix (BioRad), 1µL of forward and reverse primers for the targeted gene (Sigma Aldrich), 2µL of UltraPure DNase/RNase-free distilled water (Invitrogen), and 2µL of cDNA. The qPCR program consisted of 95°C for 30 seconds, and 40 cycles of either 95°C for 3 seconds and 60°C for 3 seconds (18S, B2M, GUSB, RPLP0, SOX9, COL1A2) or 95°C for 3 seconds and 65°C for 3 seconds (ACTB, GAPDH), followed by a melt curve gradient. All plates included a non-template control (NTC) and no reverse transcriptase control (NRT).

The three software platforms used to analyze qRT-PCR data for reference gene stability were: BestKeeper, NormFinder, and geNorm, which all utilize an algorithm to determine the stability of a set of candidate reference genes.

geNorm assigns reference genes a M score, which indicates the average expression stability of the genes. Reference genes with M scores below 1.5 are considered stable, but geNorm sets an arbitrary threshold at 0.8. The algorithm also utilizes a V score, which indicates the pairwise variation of genes and the software sets a threshold of 0.15.

NormFinder, an excel-based software, evaluates candidate reference genes across various samples and assigns genes with a stability value. Lower stability values represent more stable reference genes. One of the advantages of using NormFinder is the ability to differentiate between samples and assign samples under different treatment groups to allow the algorithm to take into account treatment group variance.

Lastly, BestKeeper is another excel-based software that evaluates stability of reference genes by comparing standard deviation against the BestKeeper index, which is a reference of expression of a variety of genes within the BestKeeper database. The algorithm assigns candidate reference genes a standard deviation value based on the average Ct value for that gene in the BestKeeper index. Reference genes with higher standard deviation values are less stable than those with smaller standard deviance.

Cartilage associated mRNA expression

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on the CFX96 Touch™ Real-Time PCR detection system (BioRad; Hercules, CA, USA). The genes of interest included SOX9, COL1A2, COL2A1, COLX, and ACAN. A quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed in triplicate in 10μL reaction volumes containing 5μL of SsoFast EvaGreen

Supermix (BioRad), 1μL of forward and reverse primers for the targeted gene (Sigma Aldrich), 2μL of UltraPure DNase/RNase-free distilled water (Invitrogen), and 2μL of cDNA. The qPCR program consisted of 95°C for 30 seconds, and 40 cycles of 95°C for 3 seconds and 60°C for 3 seconds, followed by a melt curve gradient. All plates included a NTC and NRT control. Expression levels of genes of interest were calculated relative to the reference genes previously established as being stable for the selected samples (RPLP0 & B2M) in BioRad CFX Manager 3.1. Expression levels were normalized to native cartilage samples. Gene expression analysis was conducted using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

For objective 1, data was analyzed using a 4-way ANOVA with a 95% confidence interval. Data showing inhomogeneity was log transformed prior to statistical analysis. A significance level of $p<0.05$ was used for all statistical analysis. The Tukey-Kramer method was used for post-hoc analysis.

For objective 2, data was normalized to native cartilage expression levels and standard deviations were calculated prior to statistical data analysis conducted on GraphPad Prism 6. A significance level of $p<0.05$ was used for all statistical analysis. Data was analyzed using a 2-way ANOVA, with a 95% confidence interval. Comparisons were made between native and digested cartilage samples in both weight bearing and non-weight bearing regions, but both native and digested samples were also compared between weight bearing and non-weight bearing regions, respectively. The Tukey-Kramer method was used for post-hoc analysis.

RESULTS

Effect of homogenization method on RNA yield and quality

Homogenization of native equine articular cartilage with a large ball (25mm) resulted in increased yield of RNA content as compared to small ball (5mm) homogenization ($P<0.001$) (Figure 5). Tissue homogenized with a large ball yielded on average an adjusted concentration of RNA of 51.07 ng/g of tissue used whereas small ball homogenization resulted in a concentration of 11.37 ng/g. Qualitatively, there was no difference between both homogenization methods. Large ball homogenized tissue resulted in a RNA Integrity Number (RIN) of 2.21, whereas small ball homogenized tissue had a RIN value of 1.93 ($P<0.16$).

Effect of isolation kit on RNA yield and quality

miRNeasy and mirVana isolation kits were used to isolate RNA from native articular cartilage. No difference in RNA yield was noted between the miRNeasy and mirVana isolation kits (Figure 6). RNA isolated using the miRNeasy kit had an adjusted concentration of 23.25 ng/g of tissue used whereas the mirVana kit resulted in an RNA concentration of 24.99 ng/g ($P<0.07$). From a qualitative standpoint, the kits performed similarly. RNA isolated using the miRNeasy kit had a RIN value of 2.16, while RNA isolated using the mirVana kit had a RIN value of 1.98 ($P<0.35$).

Effect of buffer incubation on RNA yield and quality

Native articular cartilage was homogenized and subjected to either a 24-hour incubation in lysis buffer or RNA was immediately extracted. Quantitatively, there was no difference in RNA yield between samples incubated in lysis buffer and those not

(Figure 7). Samples that were incubated in lysis buffer resulted in an adjusted RNA concentration of 21.20 ng/g of tissue used versus samples that were not incubated in lysis buffer resulted in a concentration of 27.41 ng/g ($P<0.23$). There was no difference in terms of the quality of the RNA isolated with or without a buffer incubation either (Figure 6). RNA isolated after the sample had been incubated in lysis buffer produced an average RIN value of 2.18 whereas the average RIN value of samples that were not incubated was 1.96 ($P<0.29$).

Effect of Clean & Concentrator kit on RNA yield and quality

All samples were subjected to an *RNA Clean & Concentrator Kit*™ to investigate the efficacy of the kit on RNA yield and quality. There was no difference observed in RNA yield between samples that were or were not subjected to the cleaning protocol (Figure 8). The adjusted concentration of RNA prior to cleaning was 40.00 ng/g of tissue used whereas after cleaning the concentration of RNA was 33.59 ng/g ($P<0.40$). Similarly, there was no difference in RNA quality between both conditions. The average RIN value for pre-cleaned samples was 2.04, whereas post-clean samples had an average RIN value of 2.09 ($P<0.78$).

miRNA enriched fragments

miRNA enriched samples were isolated utilizing the same methods as detailed for total RNA isolation above, except for using the small RNA enrichment modification to the protocols. Although miRNAs are not reliably assessed for integrity using the bioanalyzer as the algorithm is not designed for small RNA fragments, the stability and detection of their presence can be qualitatively observed on gel electrophoresis images

and electropherograms generated by the bioanalyzer. Samples isolated that were enriched for small RNA fragments displayed strong expression bands in the low nucleotide length range (15-35 nt) on gel electrophoresis images, indicating that there is presence of RNA fragments within that range. Furthermore, electropherograms showed defined peaks of RNA within those ranges, specifically at 25 nt which is within the typical range of miRNAs, thus indicating the presence of miRNAs in our samples (Figure 9).

Reference gene stability

Assessment of reference genes using geNorm with combined samples (both native and digested cartilage) resulted in only RPLP0 meeting the M score threshold of 0.8. Using an M score threshold of 1.5, RPLP0, GAPDH, SOX9, 18S, GUSB, and B2M (in that order) were found to be stably expressed across all samples (Figure 11). ACTB & COL1A2 however had M scores greater than 1.5 and were deemed unstable. However, none of the V scores for combinations of reference genes fell below the geNorm threshold of 0.15 (Figure 11). When assessing native cartilage samples only, geNorm ranked the following reference genes as stable (M score<0.8) in this particular order: GUSB, B2M, GAPDH, RPLP0, SOX9, 18S (Figure 11). Once again, ACTB & COL1A2 were deemed unstable. The V score for the native cartilage samples resulted in several combinations of reference genes falling below the minimum V score of 0.15 (Figure 11). Lastly, looking at digested cartilage only, geNorm ranked the following as most stable with an M score below 0.8: 18S, RPLP0, GAPDH, B2M, and SOX9 (Figure 11). With respect to the V score for digested cartilage samples only, only a combination

of the two most stable reference genes, which in this case were 18S and RPLP0, resulted in a V score below 0.15 (Figure 11).

Using NormFinder, an excel-based algorithm, there was an option to separate treatment groups and thus native and digested cartilage groups were separated to allow for the software to assess that variation. NormFinder assigned stability values to each candidate reference gene. The most stable reference genes were 18S (stability value = 0.321), B2M (0.497), RPLP0 (0.600), SOX9 (0.655), GAPDH (0.854), and GUSB (0.863) (Figure 12). The software also produced a combination of reference genes that was most stable across both treatment groups, the combination of B2M + RPLP0 resulted in a stability value of 0.220 (Figure 12). The unstable genes were COL1A2 and ACTB, with stability values of 2.991 and 4.125, respectively.

Lastly, the results from BestKeeper were incongruent with the results from the two other software programs. BestKeeper assessed the reference genes on their variation of expression amongst the randomized samples using a standard deviation value. According to this assessment, the genes with the least to most variation with respect to standard deviation were as follows: GAPDH (1.20), COL1A2/SOX9/ACTB (1.22), RPLP0/18S (1.23), and GUSB/B2M (1.24) (Figure 13).

In conclusion, after analyzing the results for reference gene stability using all three platforms, B2M and RPLP0 appeared to be the most reliable reference genes. This combination was supported by NormFinder with the best stability, both genes were deemed to be very stable using the geNorm algorithm. Specifically, RPLP0 was the only gene across both treatment groups to meet the geNorm stability cutoff of 0.8. Lastly, both genes were utilized and published in the literature by Mienaltowski and colleagues

(2008; 2010) in regards to work with equine articular cartilage, both native and cultured cells.

RNA isolation yield from native cartilage vs. enzymatically-digested cartilage

For objective 2, RNA from native cartilage that was homogenized using a large ball, isolated using the mirVana kit without a lysis buffer incubation was significantly lower ($P<0.05$) in concentration compared to RNA isolated following the same procedure from cartilage digested using collagenase type II (Figure 10). The cumulative concentration of total RNA from native cartilage was 113.9 ng while the cumulative concentration of total RNA from digested cartilage was 3864.9 ng.

mRNA expression in chondrocytes from native and enzymatically-digested cartilage

mRNA expression was assessed in weight bearing and non-weight bearing cartilage where RNA had been isolated either from native cartilage or from digested cartilage. The chondrogenic genes Sox9, collagen type I, collagen type II, aggrecan, and collagen type X were evaluated. Expression levels of digested cartilage were compared to the expression levels from native cartilage.

Sox9 expression was not different between native and digested cartilage samples in both weight bearing and non-weight bearing regions of the joint ($P<0.690$, $P<0.806$) (Figure 14). In contrast, collagen type 1 expression was lower in digested cartilage samples compared to native cartilage samples in both weight and non-weight bearing regions ($P<0.017$,$P<0.022$) (Figure 15). There was a 0.25-fold decrease in Col1A2 expression in non-weight bearing digested cartilage than native cartilage,

whereas there was a 0.16-fold decrease in digested non-weight bearing cartilage compared to native cartilage. Collagen type II expression was also decreased in expression in weight bearing and non-weight bearing digested cartilage ($P<0.017$, $P<0.023$) compared to native cartilage (Figure 16). In digested weight-bearing cartilage, there was a 0.02-fold decrease in expression of COL2A1 and in non-weight bearing digested cartilage there was a 0.03-fold decrease. ACAN was also decreased in expression in both weight and non-weight bearing digested cartilage samples ($P<0.032$, $P<0.027$) (Figure 17). There was a 0.26-fold decrease in the non-weight bearing digested cartilage samples whereas the weight bearing samples showed a 0.29-fold decrease in expression. Lastly, COLX expression was increased in non-weight bearing digested cartilage compared to native cartilage ($P<0.0001$), but there was no difference between native and digested weight bearing cartilage ($P<0.949$) (Figure 18).

Furthermore, there were no differences in mRNA expression between any weight bearing and non-weight bearing samples for SOX9, COL1, COL2, or ACAN ($P<0.924$, $P<0.985$, $P<0.159$, $P<0.948$). However, there was increased expression ($P<0.001$) of COLX in non-weight bearing cartilage as compared to weight bearing (Figure 18).

Tables

Gene	Forward (5'-3')	Reverse (3'-5')	Reference
18S rRNA (18S)	GTAACCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	Chen et al., 2012
Beta-actin (ACTB)	ATGGTGGCAATGGGTCAGAAGGAC	CTCTTTGATGTCACGCACGATTTC	Nagy et al., 2011
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	GGGTGGAGCCAAAAGGGTCATTCAT	AGCTTTCTCCAGGCGGCAGGTCAG	Iqbal et al., 2004
Beta-glucuronidase (GUSB)	GCTCATCTGGAACTTTGCTGATTTT	CTGACGAGTGAAGATCCCCTTTT	Mienaltowski et al., 2010
Beta-2-microglobulin (B2M)	CGGGCTACTCTCCCTGACT	GTGACGTGAGTAAACCTGAACCTT	Mienaltowski et al., 2008; Mienaltwoski et al., 2010
Ribosomal protein lateral stalk subunit P0 (RPLP0)	CTGATTACACCTTCCCACTTGCT	AGCCACAAATGCAGATGGATCA	Mienaltowski et al., 2008
Sox9 (SOX9)	ATCTGAAGAAGGAGAGCGAG	TCAGAAGTCTCCAGAGCTTG	Co et al., 2014
Collagen IA2 (COL1A2)	GAAAACATCCCAGCCAAGAA	TGATGTTTTGAGAGGCATGG	Co et al., 2014
Collagen IIA1 (COL2A1)	GACAACCTGGCTCCCAAA	ACAGTCTTGCCCCACTTAC	Co et al., 2014
Aggrecan (ACAN)	GCGTGGCTGCTGTCCCCTTA	CCCAGGGGCCTTCTGTGCTC	This work
Collagen XA1 (COLX)	CTTGGTTCATGGCGAGTTTT	GTCCAGGGCTTCCATAACCT	Co et al., 2014

Table 1. Primer pairs used for qPCR

Sample ID	Type of Joint	Total Weight of Cartilage Used (mg)
EQ 1	Fetlock	337.1
EQ 2	Stifle	416.7
EQ 3	Fetlock	364.3
EQ 4	Fetlock	509.2
EQ 5	Fetlock	480.6
EQ 6	Stifle	372.9
EQ 7	Fetlock	382.8
EQ 8	Fetlock	243.3

Table 2. Equine sample identification, joint type, and total weight of cartilage used for objective one.

Sample ID	Age	Sex	Breed	Cause of Death	Weight of undigested cartilage (mg)	Weight of cartilage used for digestion (mg)
REQ1	16	Male - castrate	Warmblood	Suspect small intestinal problem	157.8	162.9
REQ2	3	Male - castrate	American Paint Horse	Acetabular fracture	124.7	118.3
REQ3	6	Female	Thoroughbred	Equine multinodular pulmonary fibrosis	202.1	182.6
REQ4	18	Female	Thoroughbred	Chronic right front pastern subluxation & associated flexural deformity	130.4	152.6
REQ5	20	Female	Unknown	Equine asthma	163.5	193.5

Table 3. Equine sample information (age, sex, breed, cause of death) for objective two mRNA expression study.

Figures

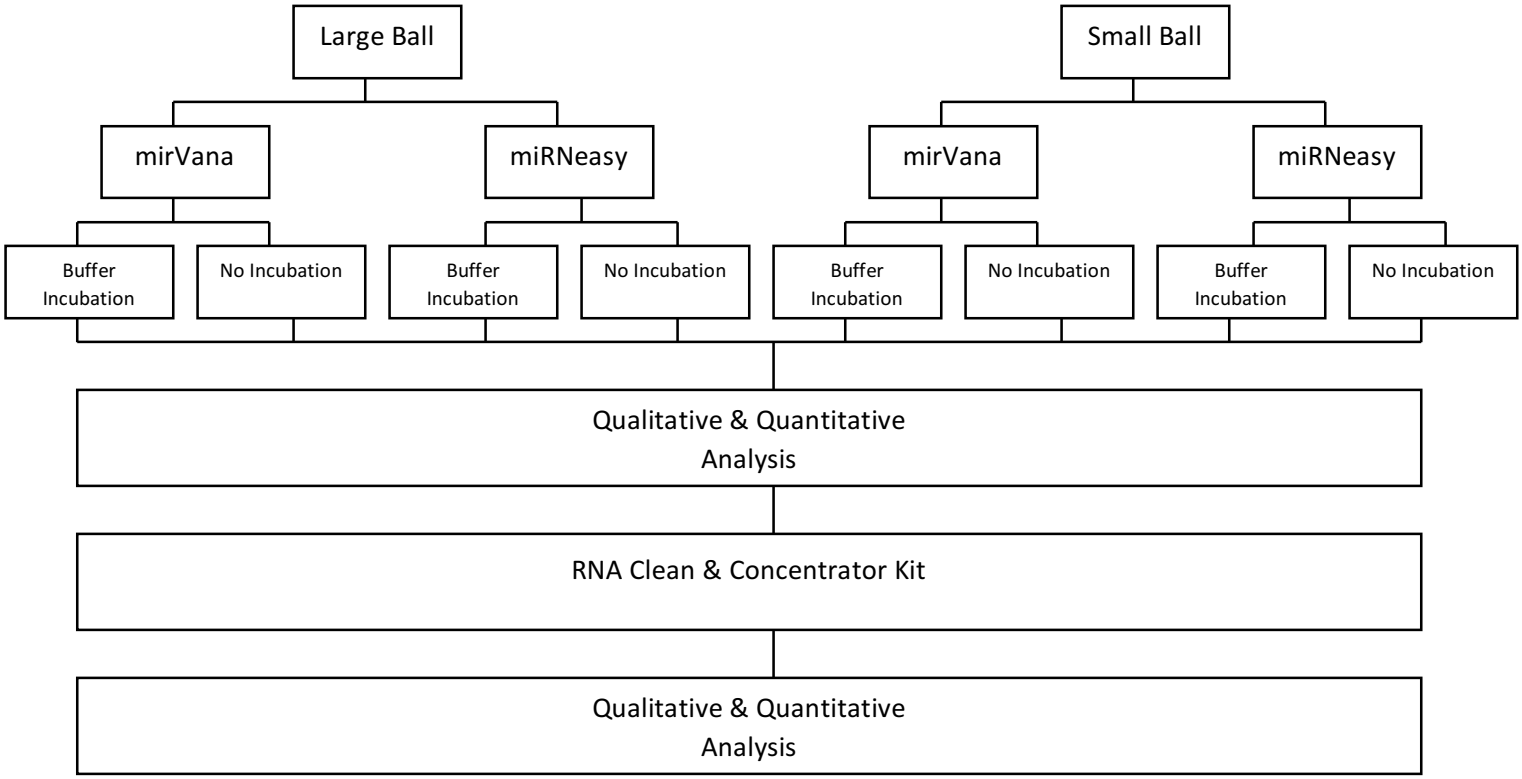

Figure 3: Study design for objective 1. Native equine articular cartilage samples were subjected to different homogenization methods, isolation kits, and presence or lack of buffer incubation. All samples were then assessed for quantity and purity, following which they were cleaned and assessed again.

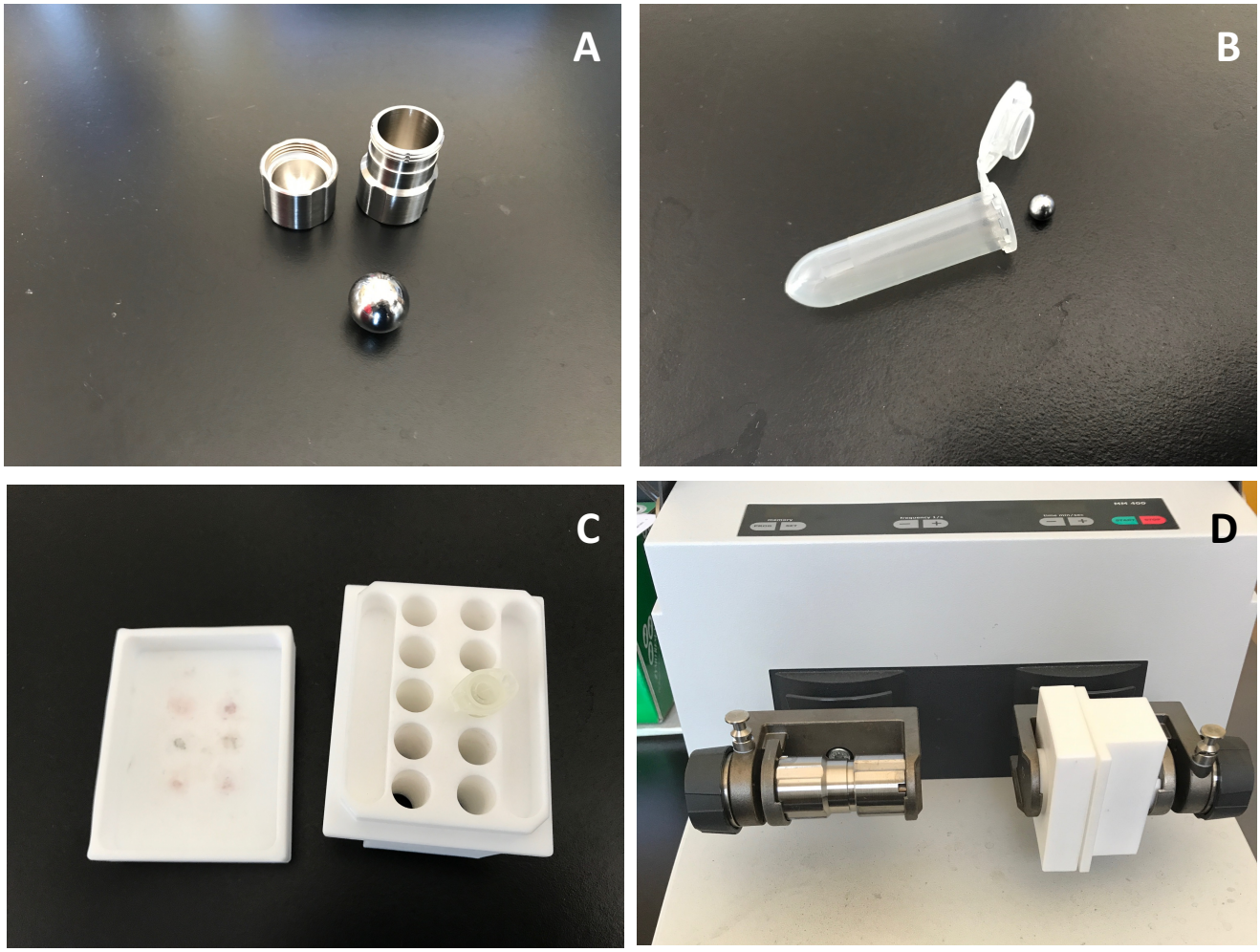

Figure 4: Retsch Mixer Mill 400 MM Apparatus (large and small ball homogenization). (A) Large ball (25mm) and canister assembly. (B) Small ball and Eppendorf RNase-DNase free tube assembly. (C) Small ball homogenizing vessel, can homogenize a maximum of 10 samples at a time. (D) Large ball canister and small ball block loaded onto Retsch Mixer Mill 400 MM prior to homogenization.

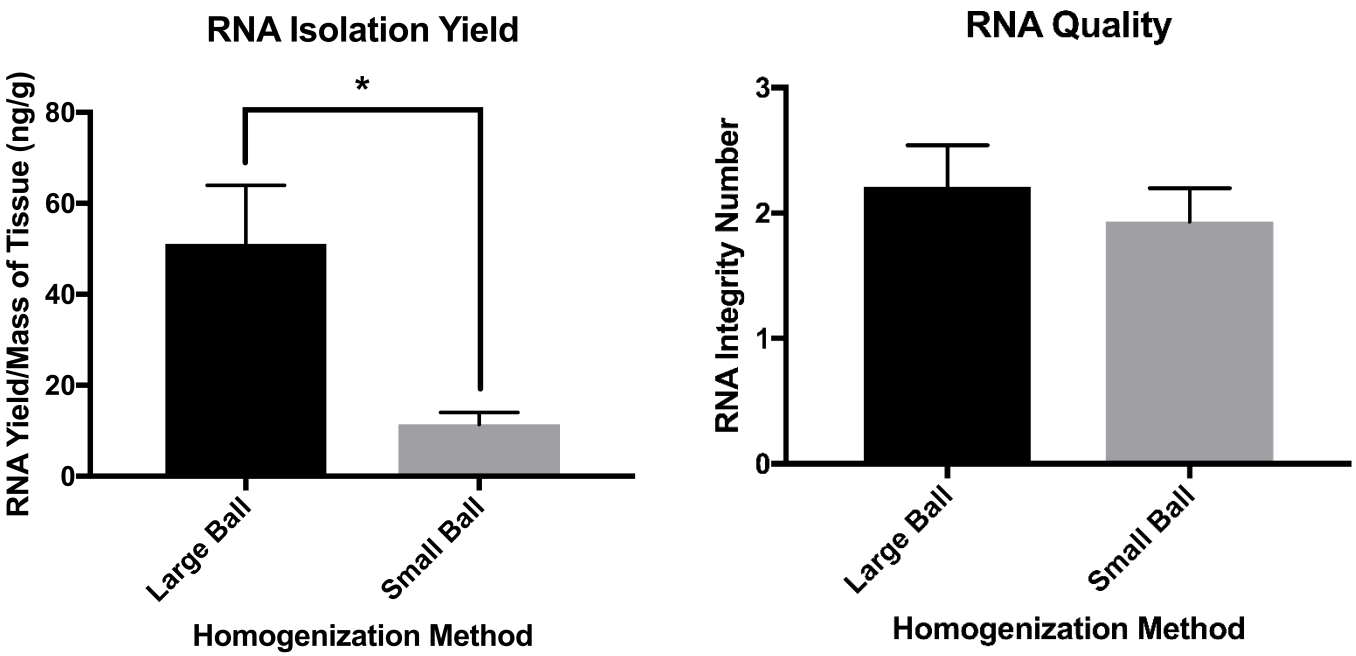

Figure 5: Effect of homogenization method on RNA isolation yield and quality. Data represented as mean values of all samples where errors bars represent SD. 4-way ANOVA statistical analysis was conducted, *p<0.05. Articular cartilage was harvested from 8 horses, N=8.

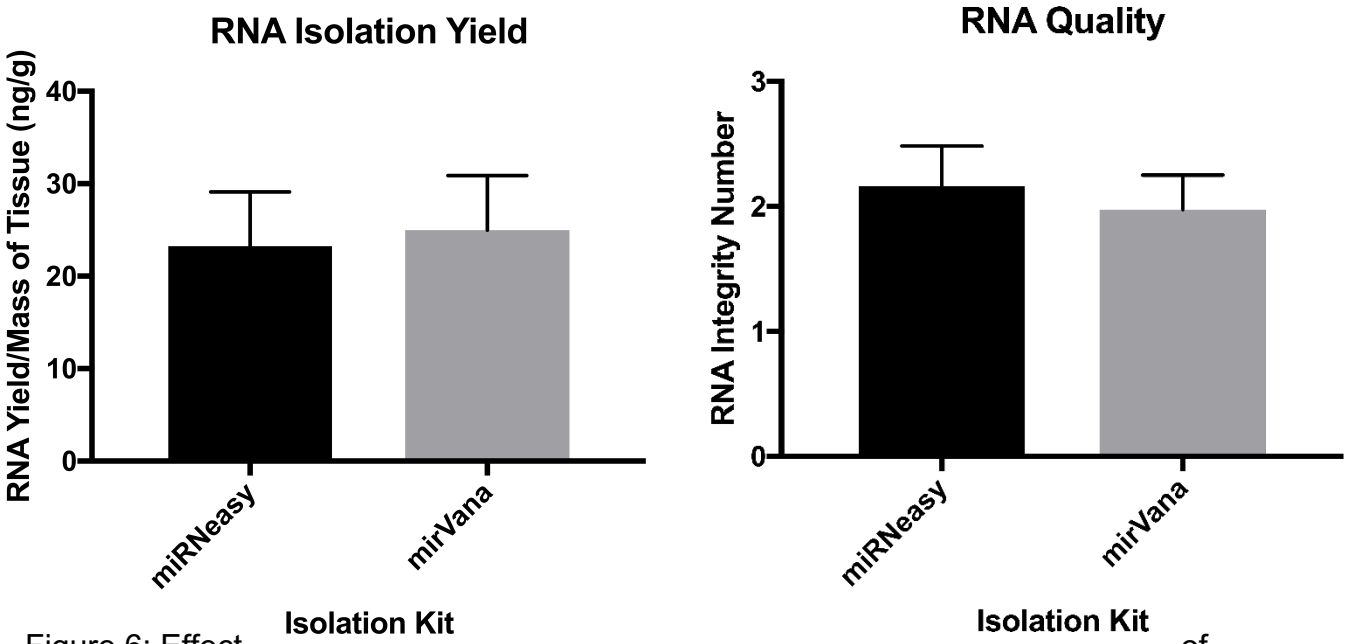

Figure 6: Effect of isolation kit (mirVana or miRNeasy) on RNA isolation yield and quality. Data represented as mean values of all samples where errors bars represent SD. 4-way ANOVA statistical analysis was conducted. Articular cartilage was harvested from 8 horses, N=8.

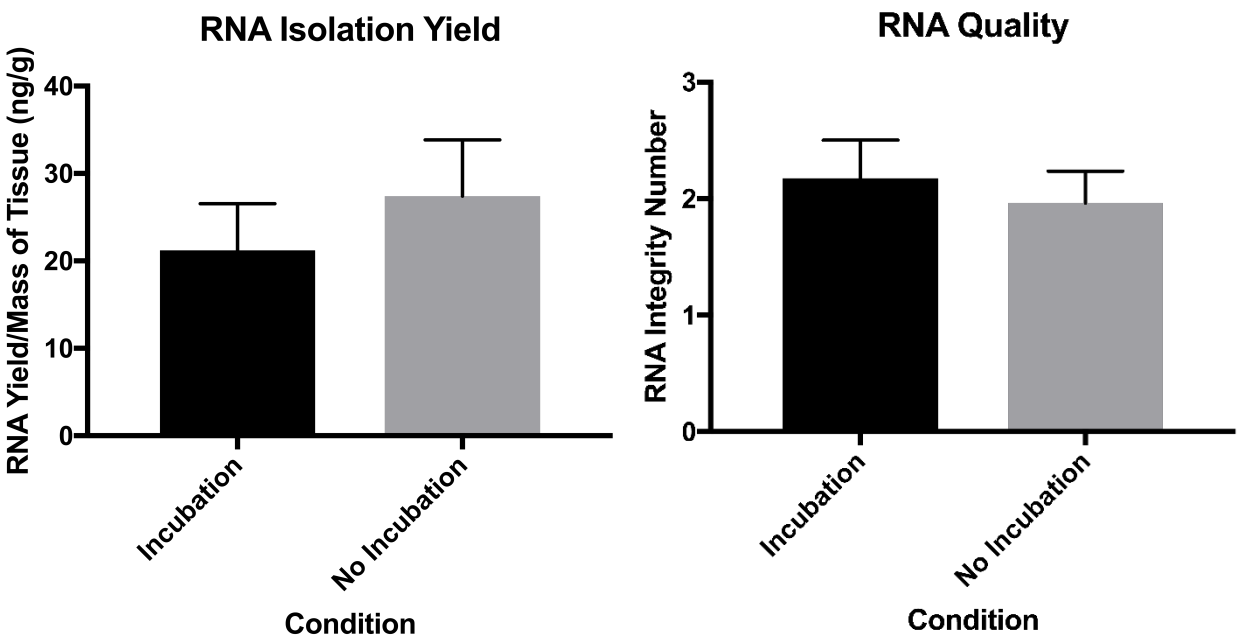

Figure 7: Effect of lysis buffer incubation on RNA isolation yield and quality. Data represented as mean values of all samples where errors bars represent SD. 4-way ANOVA statistical analysis was conducted. Articular cartilage was harvested from 8 horses, N=8.

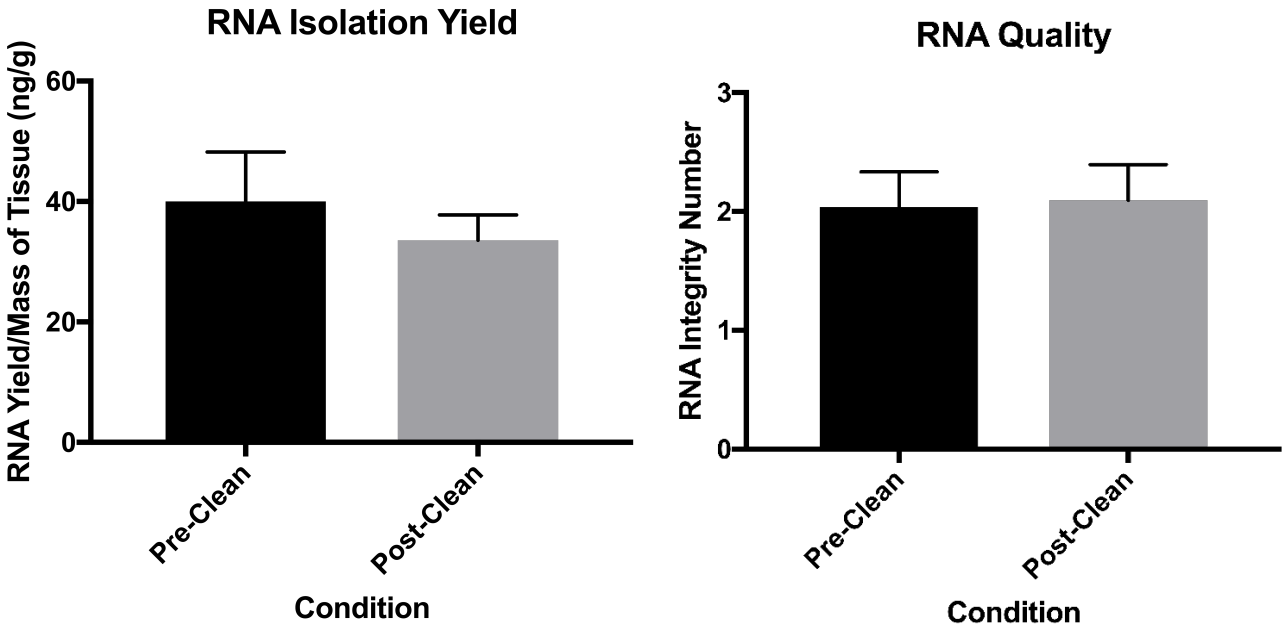

Figure 8: Effect of RNA Clean & Concentrator kit (Zymo) on RNA isolation yield and quality. Data represented as mean values of all samples where errors bars represent SD. 4-way ANOVA statistical analysis was conducted. Articular cartilage was harvested from 8 horses, N=8.

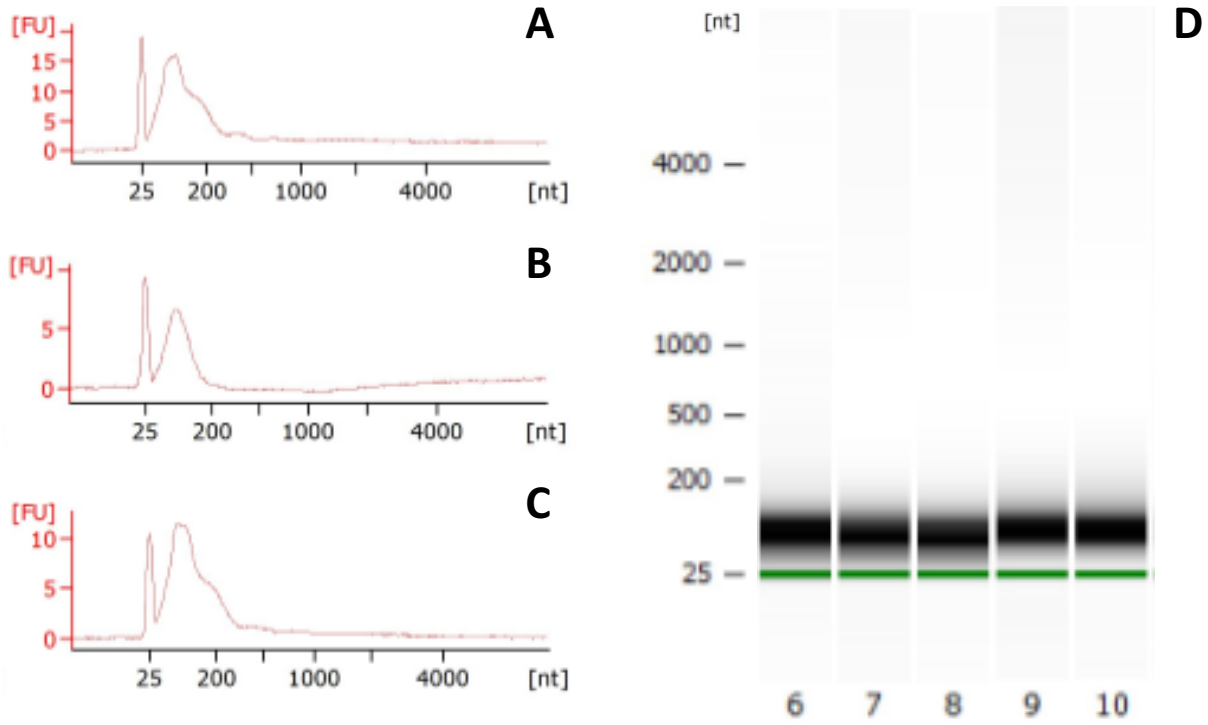

Figure 9: Agilent 2100 Bioanalyzer representative electropherograms and gel electrophoresis images of miRNA enriched samples suitable for NGS. (A-C) Gel electropherograms of 3 individual small RNA enriched samples. (D) Gel electrophoresis image showing 5 samples (lanes 6-10) with distinct expression of RNA at a lower nucleotide fragment lengths.

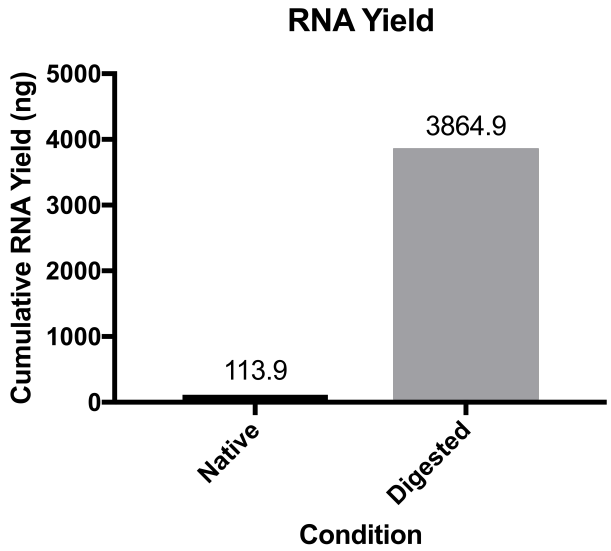

Figure 10: Cumulative RNA yield from tissue isolated from native and enzymatically-digested cartilage. Data represented as sum of RNA per gram of tissue used. Articular cartilage was harvested from the fetlock joint of 5 independent horses, N=5.

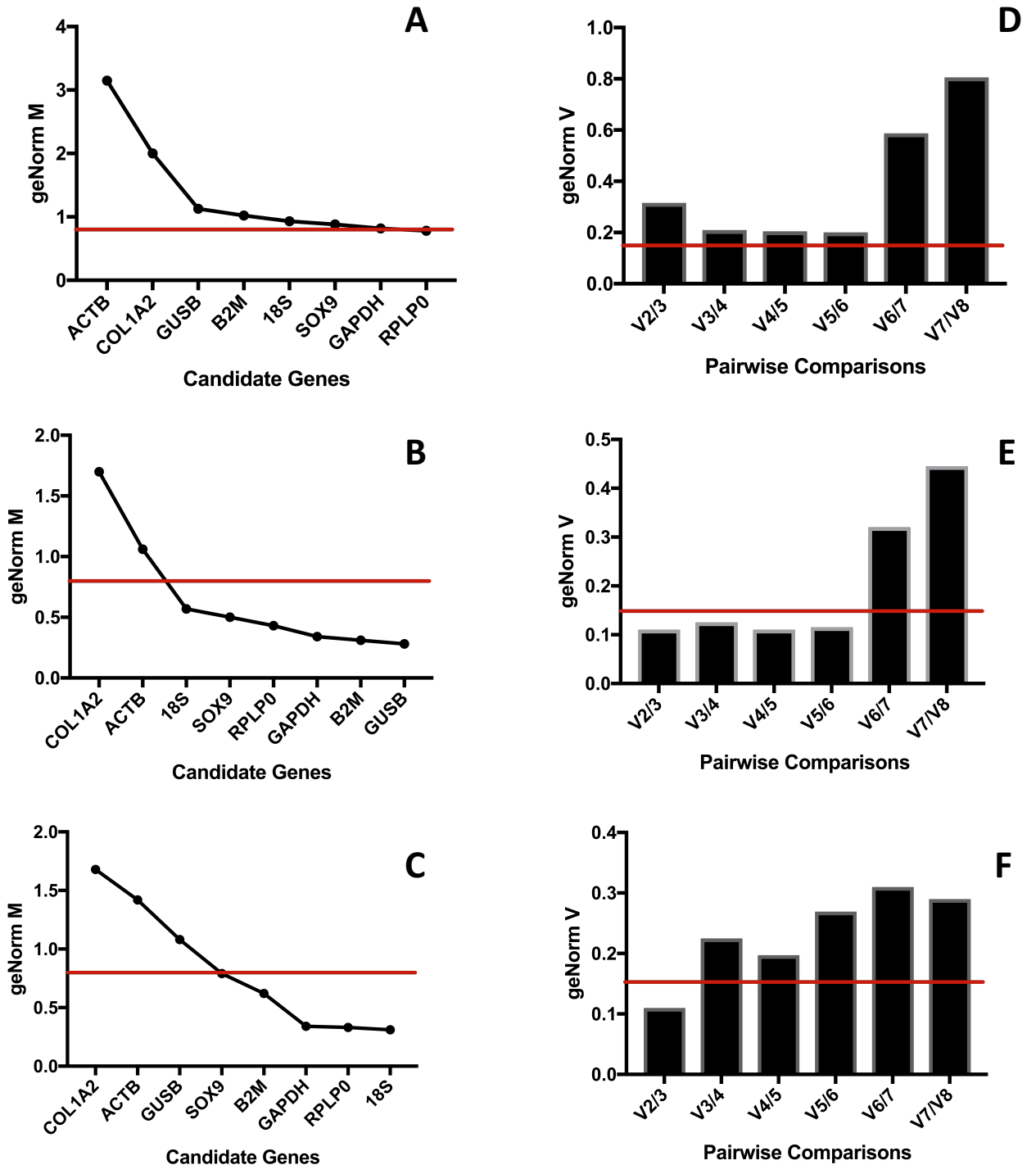

Figure 11: geNorm analysis of candidate reference genes. Reference gene stability, geNorm M score, of combined samples (A), native samples (B), and digested samples (C) are displayed. Pairwise variance results, geNorm V score, of combined samples (D), native samples (E), and digested samples (F) are displayed. Data represented as mean values, n=10. Minimum acceptable (by geNorm) M score is <0.8 and V score is <0.15, as indicated by the red lines.

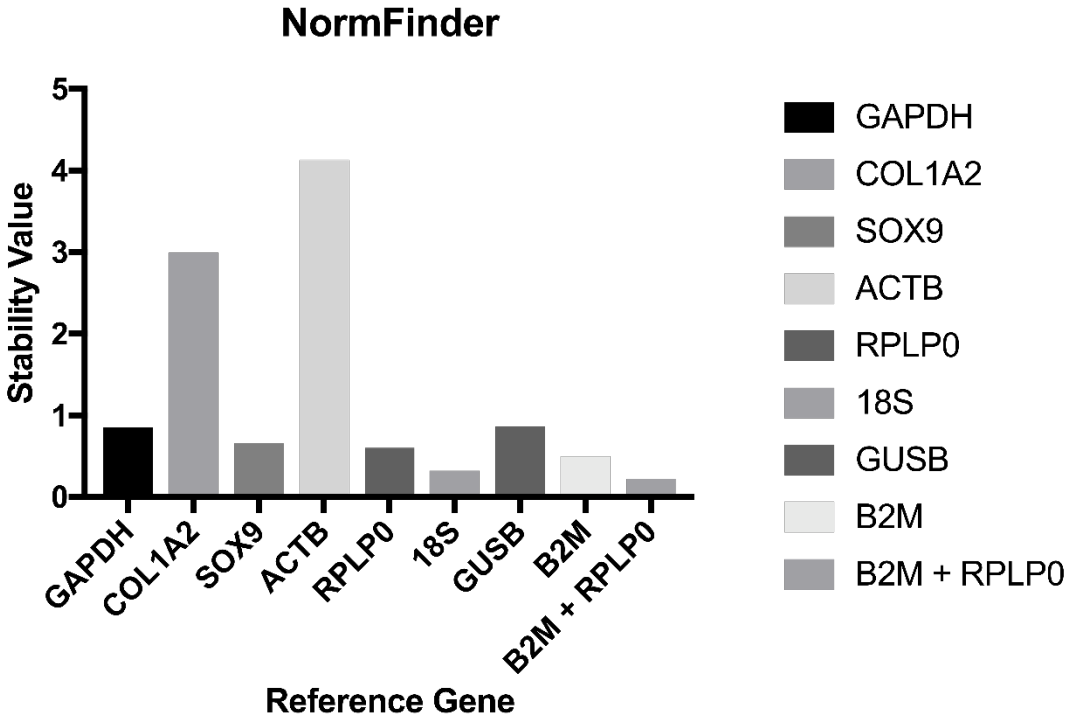

Figure 12: NormFinder analysis of candidate reference genes. Reference gene stability values are indicated for candidate reference genes. Lower stability values are indicative of a more stable reference gene. Data represented as mean values, n=10.

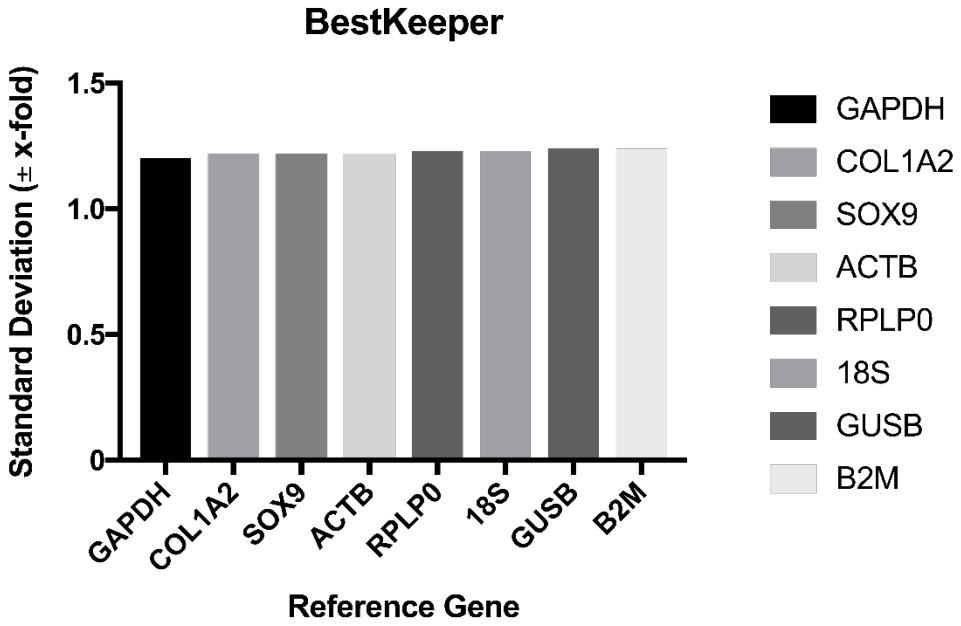

Figure 13: BestKeeper analysis of candidate reference genes. Reference gene stability is indicated as variance of expression. Greater variance, as represented by standard deviation, is indicative of weaker reference genes. Data represented as mean values, n=10.

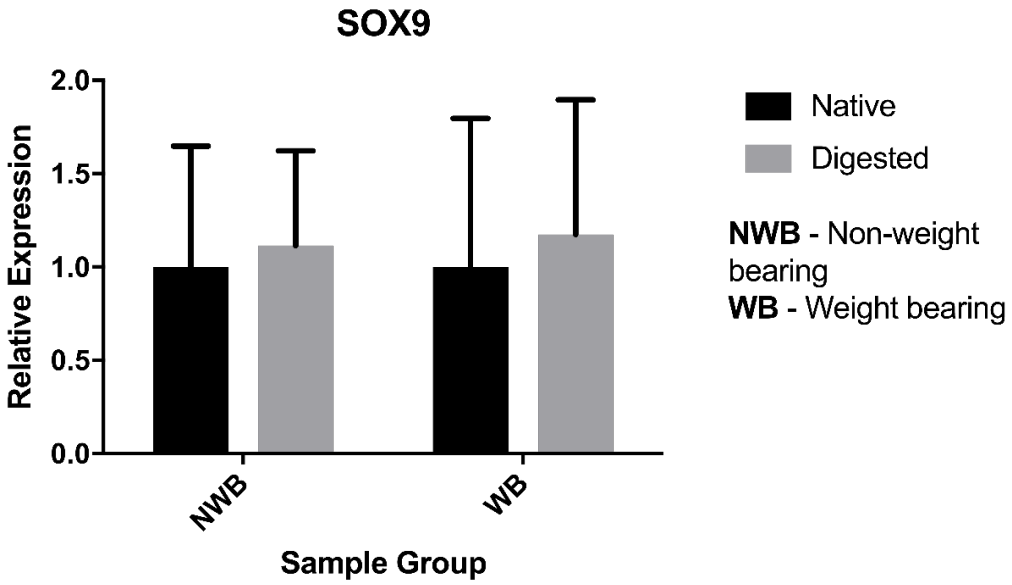

Figure 14: Gene expression of SOX9, a “master chondrogenic transcription factor”, in native and digested cartilage from weight bearing and non-weight bearing regions of articular cartilage from equine fetlock joints. mRNA levels were assessed using qPCR with RPLP0 and B2M as reference genes. Expression levels were normalized to native cartilage expression. Error bars represent SD between replicates, N=5. 2-way ANOVA statistical analysis was conducted, *p<0.05.

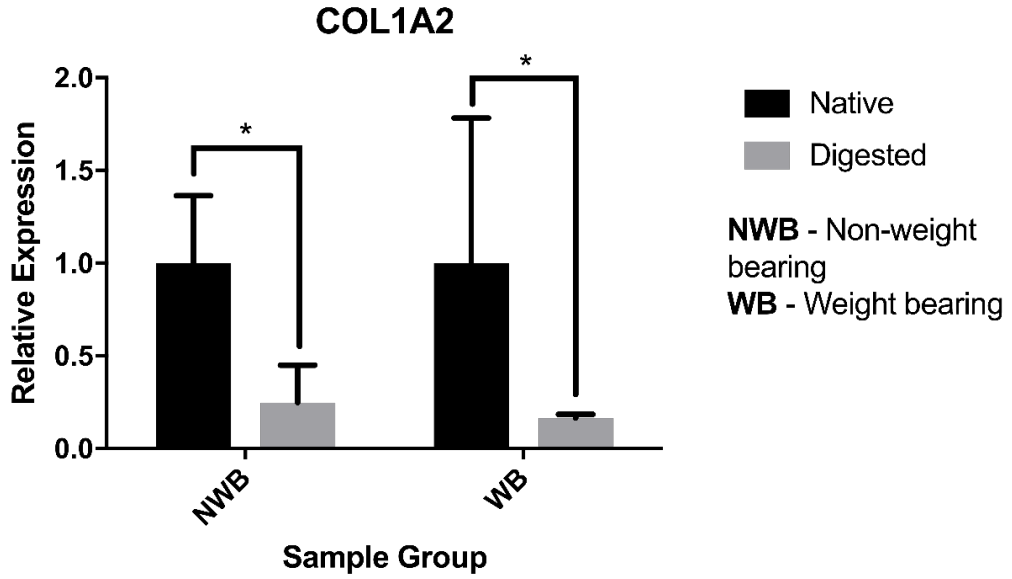

Figure 15: Gene expression of COL1A2 in native and digested cartilage from weight bearing and non-weight bearing regions of articular cartilage from equine fetlock joints. mRNA levels were assessed using qPCR with RPLP0 and B2M as reference genes. Expression levels were normalized to native cartilage expression. Error bars represent SD between replicates, N=5. 2-way ANOVA statistical analysis was conducted, *p<0.05.

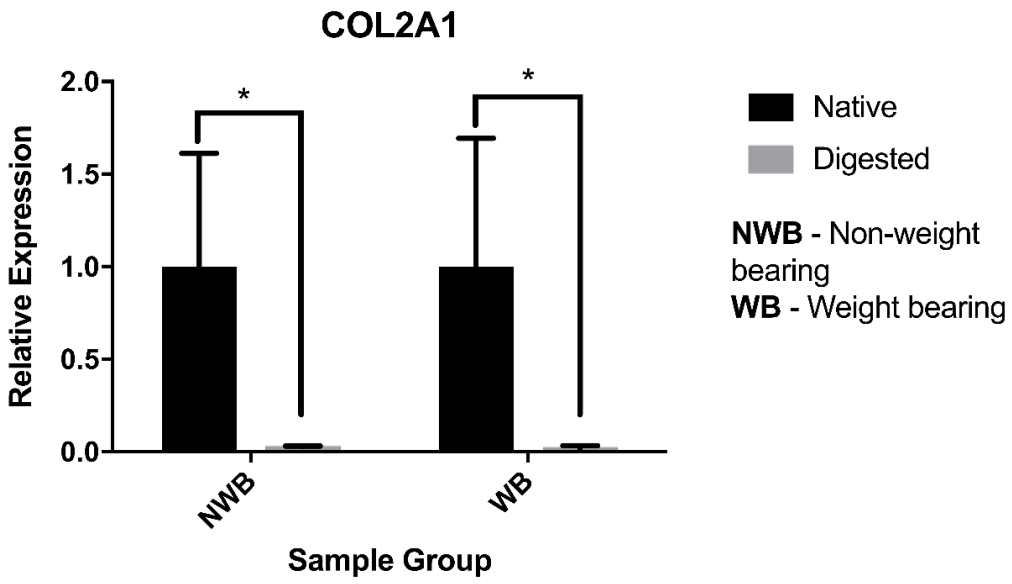

Figure 16: Gene expression of COL2A1 in native and digested cartilage from weight bearing and non-weight bearing regions of articular cartilage from equine fetlock joints. mRNA levels were assessed using qPCR with RPLP0 and B2M as reference genes. Expression levels were normalized to native cartilage expression. Error bars represent SD between replicates, N=5. 2-way ANOVA statistical analysis was conducted, *p<0.05.

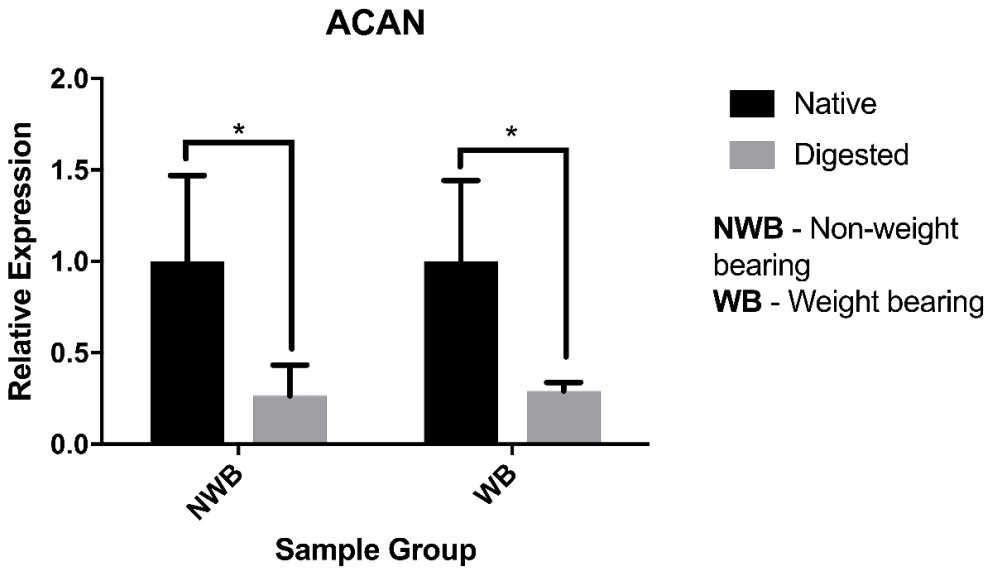

Figure 17: Gene expression of ACAN in native and digested cartilage from weight bearing and non-weight bearing regions of articular cartilage from equine fetlock joints. mRNA levels were assessed using qPCR with RPLP0 and B2M as reference genes. Expression levels were normalized to native cartilage expression. Error bars represent SD between replicates, N=5. 2-way ANOVA statistical analysis was conducted, *p<0.05.

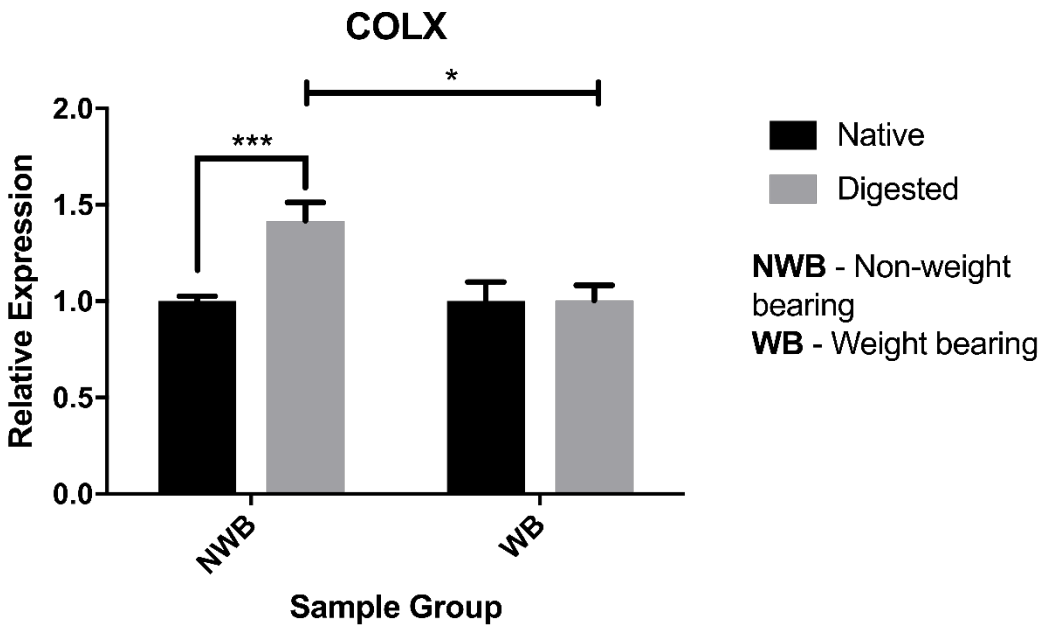

Figure 18: Gene expression of COLX in native and digested cartilage from weight bearing and non-weight bearing regions of articular cartilage from equine fetlock joints. mRNA levels were assessed using qPCR with RPLP0 and B2M as reference genes. Expression levels were normalized to native cartilage expression. Error bars represent SD between replicates, N=5. 2-way ANOVA statistical analysis was conducted, *$p<0.05$ and ***$p<0.001$.

DISCUSSION

The aims of this study were to evaluate the effects of enzymatic digestion of equine articular cartilage on gene expression and to optimize an RNA isolation protocol that would yield high quality RNA in high quantity for downstream analyses such as qPCR and NGS. Additionally, a protocol for miRNA-enriched fragments was explored. At present, RNA isolation from cartilage tissue is extremely difficult due to the low cellularity of the tissue, the thickness of the tissue, as well as the presence of many ECM molecules that interfere with the isolation process.

Several parameters were assessed to determine their efficacy in RNA isolation, including homogenization method, the isolation kit used, the use of lysis buffer incubation, and the effects of a clean and concentrator kit. Quantity and quality assessment of RNA isolation methods were determined using the Agilent 2100 Bioanalyzer. This study found that the use of large ball homogenization produced significantly higher yields of RNA than the small ball homogenization, however there were no significant differences in quality. There were no significant differences in quantity or quality of isolated RNA from using either the mirVana or miRNeasy kit. Lysis buffer incubation also had no significant impact on RNA yield as well as quality of the isolated product. Lastly, the use of a clean and concentrator kit had no significant influence on quantity or quality either.

In order to investigate the impact of enzymatic digestion of articular cartilage on gene expression, RNA isolation was first conducted using the aforementioned methods. This study found there to be a significantly higher yield of RNA from cartilage that had

been digested prior to extraction in comparison to native cartilage that had not been digested. Qualitatively, the RNA extracted from digested cartilage was superior to native cartilage. Subsequently, the differences in expression profiles between native cartilage and digested cartilage was analyzed. Five important chondrogenic genes, collagen type I, collagen type II, aggrecan, Sox9, and collagen type X, were evaluated. Candidate reference genes were analyzed using the geNorm, NormFinder, and BestKeeper softwares. This study concluded that RPLP0 and B2M were the most stable reference genes. SOX9 expression was not significantly different between native and digested cartilage from both weight bearing and non-weight bearing regions of the fetlock joint. However, there was a significant decrease in expression of COL1A2, COL2A1, and ACAN in digested cartilage as compared to native cartilage in both weight bearing and non-weight bearing regions. In contrast, COLX expression was significantly higher in digested cartilage in the non-weight bearing region, but not significantly higher in digested cartilage from the weight-bearing region. Furthermore, there were no significant differences in expression of SOX9, COL1A2, ACAN, or COL2A1 between weight bearing and non-weight bearing regions of the joint. However, there was a significant difference in COLX expression between the weight bearing and non-weight bearing regions of the joint.

Geyer and colleagues (2008) assessed the use of a mortar and pestle, a stator-rotor homogenizer, as well as a swing mill microdismembrator on human cartilage. They concluded that the swing mill was significantly better at producing greater RNA yield with high integrity compared to the other methods. The use of a closed system where the sample can be immersed in liquid nitrogen periodically helps to prevent deterioration

of the tissue through degradation as well as limits the potential for it to come in contact with any RNases. Additionally, another study observed no differences between the MagnaLyser and a freezer mill (ball homogenization) when it came to RNA yield or quality in goat cartilage. They ultimately chose the MagnaLyser due to its ability to homogenize more samples at a time (Peeters et al., 2016). Another study that isolated RNA from human articular cartilage reported success with the use of a SPEX Freezer/Mill, where they were able to achieve high yields as high as 8.39 ug/g from healthy cartilage tissue (McKenna et al., 2000). However, they only reported qualitative assessment using Nanodrop absorbances, which are not entirely an accurate assessment parameter. The increased yield in this study using the large ball homogenization method could be explained due to the strength of the ball when making contact with the tissue and producing greater disruption than the small ball. Additionally, the small ball is used in a plastic Eppendorf tube, whereas the large ball is used in a steel canister. The steel canister provides a stronger surface for the ball to make contact with and is able to retain the cold temperatures for a longer time. The small ball homogenization was also flawed in that the plastic tubes were prone to shattering during disruption. The vendor of the milling unit was consulted and it was determined that shattering of plastic tubes is a recognized problem. One solution is to decrease the frequency of milling. However, a decrease in frequency decreases the capability of disrupting the tissue. The similarity of the quality of RNA extracted from both homogenization methods can be explained by the efforts made to ensure that the tissue did not come into contact with any RNases or allowing the tissue to warm up and allowing degradation of the RNA molecules. Both homogenization methods were carried

out in intervals, ensuring to keep the tissue frozen. However, the small ball homogenization method is superior in limiting the exposure of the tissue as the tissue is carried forward to RNA isolation straight from homogenization, whereas with the large ball homogenization the tissue must be transferred into another tube for extraction. Recent findings from one study report the use of a SPEX Freezer/Mill with a modified homogenization procedure, which involved vigorous mixing and the use of needle aspiration to facilitate further homogenization, to have produce extremely high quality RNA in high quantity (Le Bleu et al., 2017). Le Bleu and colleagues (2017) utilized human osteoarthritic cartilage, further proving that their protocol was effective using cartilage undergoing degradation. However, the use of degraded tissue may have made the isolation method easier as the tissue has naturally degraded. It would be interesting to see if this protocol could work on healthy equine tissue. This is an avenue to explore in the future where a Freezer/Mill can be tested along with the aforementioned modifications. This study was effective in keeping the tissue at the temperature of liquid nitrogen at all times up until immersion into TRIzol, preventing RNA degradation processes from being initiated.

When comparing the use of two different commercial RNA isolation kits, miRNeasy (Qiagen) and mirVana (Ambion), there were no differences in RNA quantity or quality between either kit. Several studies have looked at the effects of various isolation kits on RNA quantity and quality. Peeters and colleagues (2016) showed that the use of TRIzol, a phenol and guanidine thiocyanate reagent, is significantly inferior when it comes to quality of RNA as compared to other kits that do not use phenol-guanidine thiocyanate such as RNeasy Lipid Tissue, RNeasy Fibrous Tissue, and

Aurum Total RNA. However, they reported that the TRIzol kit produced greater yields of RNA than the other kits. Mienaltowksi and colleagues (2010) utilized a phenol/chloroform extraction process followed by purification using RNeasy columns with high quality, assessed through both absorbance ratios and RIN values. Another study found that the RNAqueous kit isolation produced RNA of superior quality than the TRIzol kit (Ruettger et al., 2010). In contrast, Le Bleu and colleagues (2017) obtained high quality RNA in greater quantities than other published studies using a TRIzol extraction method. This could be explained due to their superior homogenization method and technique, which may have allowed for greater retrieval of cellular contents. In this study, the total RNA isolation protocols for both the miRNeasy and mirVana kit. The miRNeasy kit utilizes a phenol/guanidine thiocyanate reagent similar to TRIzol while the mirVana uses a phenol/chloroform reagent for the organic extraction process. The lack of a difference between these kits could be explained by the abundance of proteoglycans that may be inhibiting the kits from working efficiently, as well as preventing the binding of RNA molecules to the filter columns used to extract RNA. Additionally, the low cell yield from the disrupted tissue may prevent this study from truly observing differences between these kits, as there is not a substantial amount of RNA isolated to elucidate any major differences.

There was no significant difference with respect to RNA quantity or quality between samples that had been buffered in lysis reagent for 24 hours as compared to samples that were subjected to RNA isolation immediately after homogenization. Lysis buffer plays a role in neutralizing the cellular components that are released as a result of cell lysis, it protects the nucleic acid material from degradation and regulates acidity

(Longmire, Maltbie, & Baker, 1997). The conclusion is that there may not be any effect of the lysis buffer incubation as there is not a substantial amount of cellular contents being released due to the low cellularity and yield from homogenization. Thus, the amount of lysis buffer required is minimal and the incubation period does not provide any advantages. Perhaps if the yield of RNA material was substantially higher, it proves useful to have a lysis buffer incubation to prevent degradation. Incubation with lysis buffer is a cumbersome step to include for RNA isolation as it prolongs extraction by 24 hours, which can in turn cause degradation of molecules. Additionally, with the limitations of RNA isolation from equine articular cartilage, the lysis buffer incubation did not have any significant benefits.

There were no differences in RNA isolation yield or the quality of RNA after cleaning and concentrating the samples using the Clean & Concentrator Kit (Zymo). Currently there are no studies on the efficacy of the use of a clean and concentrator step for RNA isolation in articular cartilage. Many studies utilize a clean and concentrator step as part of their established RNA isolation protocol as it allows for low yield samples to be concentrated for effective downstream analysis (Peeters et al., 2016). The clean and concentrating step is predominantly effective at removing phenol contamination from isolated RNA samples. Due to the lack of a difference in quality between pre-cleaned and cleaned samples, there may be no phenol contamination in the samples but instead there might be proteoglycans in the sample that are affecting the quality. As a result, the decreased quality and the lack of an effect from the cleaning process may be due to proteoglycan contamination. Additionally, the use of the Clean & Concentrator Kit may be effective at concentrating the sample, but some sample may

be lost during the process, which might account for the fact that no difference in quantity was observed. Furthermore, the cleaned sample is eluted in a lower volume and thus it concentrates the sample, but importantly process does not increase the actual RNA yield.

The majority of the published data on RNA expression in chondrocytes is from chondrocytes isolated from digested cartilage. Naranda and colleagues (2017) digested human articular cartilage to isolate chondrocytes and have reported the dedifferentiation of these cells in culture within 3 days of passaging. Furthermore, Sanchez-Adams and colleagues (2011) also report that cell yield from a collagenase digestion of fibrocartilage from the knee meniscus is vastly higher than other isolation methods. In our study we report a significantly higher yield of RNA from cartilage that has been digested using collagenase type II for 24 hours as compared to RNA isolated from native articular cartilage. This can largely be explained by the break down of the extensive ECM of articular cartilage. Equine articular cartilage is extremely dense and the ECM molecules contribute greatly to its strength, but the proteoglycans and collagen fibres in the ECM also hinder the isolation process (Sophia Fox et al., 2009). The use of an enzymatic digestion procedure breaks down these ECM molecules, facilitating the isolation of chondrocytes in greater amounts than just homogenizing native tissue, allowing for a greater yield of RNA. However, this comes at a cost of compromising the integrity of expression *in vivo*.

Isolation of small RNA enriched fragments resulted in stable expression of small RNAs that fell within the range of miRNAs. Gel images and electropherograms from bioanalyzer results reveal there to be stable miRNA expression. Unfortunately, a RIN

value is not an accurate measure of miRNA integrity due to its reliance on longer stranded RNA in samples for the algorithm to work effectively. Instead miRNA expression is measured qualitatively. miRNAs are reported to be more stable than mRNA transcripts, one study reported that allowing tissue to degrade caused a progressively worse mRNA expression whereas miRNA expression was not affected (Jung et al., 2010). The stable expression of miRNA molecules allows for future NGS studies where we can investigate the potential miRNAs found *in vivo*. This will allow us to explore the role of these miRNAs and potentially to use them in culturing of MSC-derived chondrocytes to improve the chondrogenic process *in vitro*.

Traditionally, the use of the housekeeping genes such as GAPDH, 18S, or ACTB have been used, however there are several findings that claim that these genes are not stable across different samples (Suzuki, Higgins, & Crawford, 2000; Lee et al., 2002). As a result, the MIQE guidelines report was published to assist researchers in performing qPCR analysis that was meaningful and validated. They recommend validating reference gene expression and determining if it is stable across samples, tissues, or treatment groups (Bustin et al., 2009). In this study, three different softwares were utilized to assess the stability of a set of candidate reference genes.

The geNorm software produced several reference genes, when looking at both native and digested cartilage samples combined that fell below the minimum threshold for reference gene stability of 0.8. RPLP0 was the only reference gene that met this minimum cutoff. However, when assessing the pairwise variation of multiple reference genes, no combination of reference genes resulted in a V score below the cutoff set by geNorm of 0.15. When assessing solely native cartilage samples, GUSB, B2M,

GAPDH, RPLP0, SOX9, and 18S were below the minimum 0.8 M score cutoff from most to least stable. Additionally, several combinations of reference genes met the minimum pairwise variation cutoff of 0.15. Similarly, assessment of just the digested cartilage samples resulted in several stable reference genes that met the M score cutoff; 18S, RPLP0, GAPDH, B2M, and SOX9 all were deemed stable. When comparing pairwise variations, only the combination of 18S and RPLP0 resulted in a V score below 0.15. The inefficacy of the geNorm software for the combined samples is a result of the variance in expression of the reference genes between the native and digested cartilage samples. In all analyses, COL1A2 and ACTB were both found to be unstable and not reliable reference genes. COL1A2 is a gene of interest and is expected to vary in expression and thus was used as a parameter to assess the efficacy of the software. When assessing the samples separately, only allowing for donor variation amongst samples, the results of the geNorm analysis were more conclusive. This further expands on the concept that digestion of cartilage causes a change in expression that is much different from the expression of native cartilage. Mienaltowksi and colleagues (2010) used geNorm analysis for assessment of reference genes to be used for expression analysis across cartilage repair tissue, neonatal cartilage, and cultured chondrocytes. They concluded that GUSB and B2M were the most stable reference genes to use for all their samples. In another study, when investigating the transcriptional profiles of healthy and repair tissue cartilage, they concluded that B2M and RPLP0 were most stable following geNorm analysis (Mienaltowski et al., 2008). In a study conducted on human testicular tissue, it was deemed the geNorm software was

superior to other available softwares due to its ability to account for PCR efficiencies when assessing genes (Spiegelaere et al., 2015).

The use of the BestKeeper software assessed reference genes against the BestKeeper index, which catalogues the expression levels of genes in a database and compares the inputted genes to said index. The assessment of reference genes based on standard deviation concluded that the candidate genes, from most to least stable, were GAPDH (1.20), COL1A2 (1.22), SOX9 (1.22), ACTB (1.22), RPLP0 (1.23), 18S (1.23), GUSB (1.24), and B2M (1.24). These findings were incongruent with the results from the other two softwares, as COL1A2 and ACTB were both concluded to be more stable than several other reference genes. This was contrary to the other softwares and biologically did not make sense. COL1A2 is a gene that should not be stably expressed in healthy articular cartilage and this indicated that this software might not be the most reliable assessor for reference gene stability for our samples. Furthermore, the software assessed genes based on standard deviation and upon looking at the results there was not a major difference between the standard deviation values of the genes, concluding that there were no genes that were drastically more stable.

NormFinder is another excel based software, however it differs from the other two softwares in that is allows for the input of different treatment groups. This allows the software to assess the expression stability of reference genes within each treatment group as well as across all samples. NormFinder concluded that the three most stable reference genes were 18S (0.321), B2M (0.497), and RPLP0 (0.600). However, it concluded that the combined use of both B2M + RPLP0 was more stable (0.220). NormFinder utilizes an algorithm to assign stability values to genes and further

compares differences between groups using a 2-way ANOVA. Furthermore, a study done using NormFinder without the group separation saw similar results when groups were identified, indicating that there was not a bias from identifying treatment groups (De Gregoris et al., 2009). The results from this analysis correlate with the findings of another study, that had utilized geNorm analysis and found that B2M and RPLP0 were the most stable reference genes when analyzing both native cartilage and cultured chondrocytes, which had been isolated using enzymatic digestion (Mienaltowski et al., 2008).

Digestion of cartilage is utilized to isolate a greater quantity of chondrocytes and to extract larger quantities of RNA. However, studies have shown that digestion of tissue results in a phenotypic change to the cells, altering gene expression and metabolic activity (Mitrovic, Lippiello, & Mankin, 1979). Sanchez-Adams and colleagues (2011) found that enzymatic digestion of fibrocartilage from the meniscus of bovine knee joints resulted in significantly different phenotypes as compared to RNA isolated from undigested native cartilage. SOX9, COL1, COL2, and ACAN expression was significantly increased after digestion.

In this study, no significant differences were observed in the expression of SOX9 between native and digested cartilage from both weight bearing and non-weight bearing regions of cartilage. SOX9 is a major transcription factor of chondrogenesis, regulating the expression of many genes. The findings in our study are consistent with another study that noted no difference in the expression of SOX9 (Lin et al., 2008). This may be due to the multifaceted role of SOX9 that may still be involved in regulating cellular processes despite digestion of the ECM.

COL2A1 and ACAN are both highly important components of the ECM of articular cartilage, which lend the tissue its compressive and tensile properties.This study reports a decrease in their expression between native and digested cartilage from both weight bearing and non-weight bearing regions of the joint. Many groups have reported a decrease in expression of COL2A1 and ACAN, two fundamental proteins in healthy articular cartilage, upon enzymatic digestion. Mienaltowski and colleagues (2010) report a decrease in transcript abundance of both COL2A1 and ACAN in chondrocytes isolated from digested cartilage. A study that looked at the differences between osteoarthritic native and digested human cartilage also observed a decrease in COL2A1 and ACAN in digested cartilage samples (Lin et al., 2008). Hayman and colleagues (2006), also reported a decrease in COL2A1 expression in digested cartilage from adult bulls, but reported no difference in ACAN expression. The decrease in COL2A1 and ACAN expression correlates with what is occurring biologically, it has long been reported that digestion of cartilage and subsequent culturing of chondrocytes shows a dedifferentiation (Cheng et al., 2011; Saris et al., 2008). This dedifferentiation causes an upregulation of collagen type I, which contributes to a fibrocartilage phenotype. However, the expression of COL2A1 and ACAN was downregulated, both of which give articular cartilage its distinct characteristics. Digestion of cartilage facilitates the loss of integrin-mediated signalling between the ECM molecules and chondrocytes, which may be causing cytoskeletal reorganization and ultimately a decrease in gene expression (Ruoslahti, 1991).

The decreases in COL2A1 and ACAN expression are consistent with a chondrocyte phenotype transition towards a fibrocartilage phenotype. However,

COL1A2, a marker of fibrocartilage, had significantly decreased in expression in digested cartilage from both weight bearing and non-weight bearing regions. This result was not in correspondence with the literature, as other groups have reported an increase in COL1A2 expression after digestion of cartilage (Naranda et al., 2017; Lin et al., 2008). The findings in this study may be explained due to the short time before analysis as RNA was isolated 24 hours after cartilage was digested. This may not have allowed the cells to respond and upregulate the expression of COL1A2 to a significant level. Many studies that report COL1A2 upregulation look at the expression profile after 7 days (Naranda et al., 2017) or after multiple passages (Lin et al., 2008). Additionally, Hering and colleagues (1994) found that culturing chondrocytes that had been isolated through enzymatic digestion resulted in minimal expression of COL1A2 in the beginning, but became increasingly abundant after several days of culture. This length of time may have allowed for complete dedifferentiation of the isolated chondrocytes, permitting the upregulation of the fibrocartilage marker COL1A2. Investigating the differences in expression at several time points or after passaging of cells may be of interest for culturing purposes of isolated chondrocytes. However, for purposes of analyzing gene expression immediately following digestion, there is a significant decrease in COL1A2 expression in digested cartilage.

There was a significant increase in COLX expression in digested cartilage, however it was only seen in non-weight bearing cartilage. There was no significant difference in expression of COLX between digested cartilage and native cartilage in the weight-bearing region. Lin and colleagues (2008) reported an overall decrease in COLX expression after several passages of chondrocytes that were enzymatically isolated.

However, they reported an initial increase in COLX expression, which had decreased after passage 6. The increase in COLX expression in our sample could be explained because of the degradation of the ECM. Hypertrophic cartilage is associated with the upregulation of matrix metalloproteinase 13 (MMP13), which is a collagenase precursor and aids in digestion of the ECM in hypertrophic cartilage to allow for calcification (van der Kraan & van den Berg, 2012). Similarly, digestion of the ECM using collagenase in this study may have caused the chondrocytes to temporarily begin expressing COLX as a compensatory mechanism. This may have been an immediate response due to degradation of the ECM, as this is a part of the mechanism in which OA begins. A study assessing the effect of various digestion regimens on gene expression found that there was an increased expression of MMP13 1 week after isolating chondrocytes digested using pronase-collagenase and trypsin-collagenase (Hayman et al., 2006). Unfortunately, this latter study did not look at the expression of COLX. Exploring whether the expression of COLX is still upregulated at later time points or after culturing is a future step to determine if expression decreases or increases with time.

There was a significant increase in COLX expression in non-weight bearing cartilage that had been digested using collagenase in comparison to weight-bearing digested cartilage. Eggli and colleagues (1988) reported a decrease in numerical volume density of cells in weight bearing regions of articular cartilage in rabbits compared to less weight bearing regions. The high physiological load resulted in more cell death in the weight bearing regions. As a result, the findings in this study may be reflecting a discrepancy in cell density between weight bearing and non-weight bearing

regions, causing a significantly increased expression in non-weight bearing cartilage due to the increased cell content.

This study has provided insight into the effects of enzymatic digestion of equine articular cartilage. Particularly, there is a significant difference in the expression profile of native cartilage and digested cartilage. Thus, the use of enzymatically digested cartilage, as a benchmark for comparison, is erroneous and invalid.

CONCLUSIONS

The large ball steel canister homogenization method had higher RNA yield compared to small balls in plastic tubes, but had no impact on quality. The assessment of multiple RNA isolation kits did not yield a superior kit, as both had no effect on quality or quantity. Additionally, the use of a lysis buffer incubation or clean and concentrator kit did not have any particular advantage for RNA isolation.

Attempts at utilizing recently published methods for high quality RNA isolation from Le Bleu and colleagues (2017) resulted in unsuccessful RNA extraction and thus this method in it's current form was not suitable for equine articular cartilage.

Using the protocol that was optimized in this study, miRNA-enriched samples were extracted from native articular cartilage that showed strong and stable expression of RNA molecules within the miRNA nucleotide range. This permits future studies to move forward and test these samples using other assessment parameters such as qPCR and ultimately NGS to further investigate the microenvironment present *in vivo*.

RPLP0 and B2M were identified as the two most stable reference genes to utilize in assessing chondrocyte-associated mRNA expression when comparing RNA isolated from both digested and undigested cartilage.

Enzymatic digestion of equine articular cartilage changes the chondrocyte mRNA expression profile. Benchmarks for normal and diseased cartilage mRNA expression should therefore be based on RNA isolated directly from cartilage tissue and without the use of enzymes.

LIMITATIONS

One of the fundamental limitations of this study was the variance in equine articular cartilage samples that were analyzed. In both the first and second objectives, samples were collected from cadaver limbs upon availability. This resulted in a great diversity of horses of different ages, different sexes, and breeds. This variation may have contributed to some of the variance seen in both the ability to isolate RNA as well as the expression profiles assessed. Cartilage remodelling occurs over the course of a horse's life, similar to human remodelling and deterioration because of injury or daily activities. Thus older horses may have an altered gene expression compared to a younger horse. Furthermore, articular cartilage from younger horses is easier to homogenize and leads to a greater retrieval of chondrocytes and subsequent RNA yield. Additionally, the variance in breeds and the daily activity of the horses may have contributed a degree of variance. Racehorses and horses with a higher activity level may possess cartilage defects at an earlier age due to the degree of force being placed on their joints. This study could be improved using a controlled cohort of horses, either collecting cartilage from horses of the same age or that have been under similar degrees of activity/stress. This may help to diminish the variance in biological samples.

Another limitation is the lack of a highly efficient RNA isolation protocol for native articular cartilage. Due to the low cellularity and high proteoglycan content of articular cartilage, extracting high-quality RNA in high concentrations is extremely tough. This study sought out many methods to improve the established lab protocol that was previously being used. However, this optimized protocol results in a very low yield of RNA from native cartilage, which has implications in downstream analyses. Additionally,

the integrity and quality of RNA from native tissue is still not ideal and may have implications on downstream analysis. Degraded RNA can affect qPCR results, as the expression observed may not be accurate. Isolation of higher quality RNA would thus strengthen findings. Lastly, in addition to establishing controlled cohorts, the use of larger sample sizes will obviously strengthen the findings reported in this thesis.

FUTURE DIRECTIONS

This study has elucidated many potential projects that can be pursued. Firstly, the use of collagenase type II as a method of digestion was explored in this study. Future studies may explore the effects of various other digestion methods. Although literature has shown that collagenase type II digestion has the least effect on fibrocartilage gene expression changes, there are other digestion methods that could be explored to determine their effects on articular cartilage. Different enzymes digest the ECM in different manners and it would be interesting to understand their effects. Furthermore, looking at the long-term expression effects is of value for culturing purposes. Chondrocytes from digested cartilage are currently being used in monolayer culture. Thus, looking at the expression at later passages is important.

Secondly, preliminary work in this study has shown that the chondrocytes in articular cartilage upon enzymatic digestion have an altered phenotype, but it will be important to further confirm these findings by examining surface protein and transitional markers. Digestion has caused chondrocytes to adopt an altered phenotype in which upregulation of hypertrophic genes was observed, but it would be of great interest to see whether the chondrocytes exhibit the surface markers of this phenotype such as MMP-9/Gelatinase B or CD73+ as reported in the literature (Campbell & Pei, 2012; Vu et al., 1998). Furthermore, looking at a greater panel of genes that have been indicated to play a role in hypertrophic cartilage, such as matrix metalloproteinases and Runx2 which are associated with the expression of COLX in hypertrophic chondrocytes (Ding et al., 2012), as well as certain cytokines such as IL-β, which is commonly associated with early and late-stage OA would be important to explore (Goldring, 2012).

Additionally, as mentioned in the limitations, the lack of a highly effective RNA isolation protocol is an area that requires further refinement. This study established a working protocol, but there are many other kits and homogenization techniques that could be explored. Recently, Le Bleu and colleagues (2017) published a technique they had developed for effectively isolating high-quality RNA from human articular cartilage. This work did preliminary investigations using this technique (please see appendix). Unfortunately, we were unsuccessful at reproducing the results published by Le Bleu and colleagues. Future work should look at modifying their technique and optimizing it to produce optimal results using equine articular cartilage.

Lastly, as part of optimizing an effective RNA isolation protocol, isolation of miRNAs from native equine cartilage was successfully attempted. Stable expression of miRNAs, suggestive of minimal degradation, was confirmed by gel electrophoresis and electropherograms. This provides the foundation to explore the role of miRNAs in articular cartilage and help to elucidate which miRNAs are present *in vivo.* This information will be important in improving accurate culturing of MSC-derived chondrocytes. In addition, miRNAs in diseased cartilage can be explored as a biomarker for cartilage defects and/or post-traumatic OA.

REFERENCES

Aigner, T., Stoss, H., Weseloh, G., Zeiler, G., & von der Mark, K. (1992). Activation of collagen type II expression in osteoarthritic and rheumatoid cartilage. *Virchows Archiv B Cell Pathol,* 62, 337-45.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell,* 136(2), 215-233.

Basser, P.J., Schneiderman, R., Bank, R.A., Wachtel, E., & Maroudas, A. (1998). Mechanical Properties of the Collagen Network in Human Articular Cartilage as Measured by Osmotic Stress Technique. *Archives of Biochemistry and Biophysics,* 351(2), 207-219.

Behjati, S. & Tarpey, P.S. (2013). What is next generation sequencing? *Archives of Disease in Childhood Education and Practice Edition,* 98(6), 236-238.

Benya, P. & Shaffer, J.D. (1982). Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell,* 30, 215-24.

Benya, P.D., Brown, P.D., & Padilla, S.R. (1988). Microfilament modification by dihydrocytochalasin-B causes retinoic acid-modulated chondrocytes to reexpress the differentiated collagen phenotype without a change in shape. *J Cell Biol,* 106, 161-70.

Berezikov, E. (2011). Evolution of microRNA diversity and regulation in animals. *Nature Reviews,* 12(12), 846-860.

Bhosale, A.M. Richardson, J.B. (2008). Articular cartilage: structure, injuries and review of management. *British Medical Bulletin,* 87(1), 77-95.

Bi, W., Deng, J.M, Zhang, Z., Behringer, R.R., & de Crombrugghe, B. (1999). Sox9 is required for cartilage formation. *Nature Genetics,* 22(1), 85-89.

Bodo, G., Hangody, L., Modis, L., & Hurtig, M. (2004). Autologous osteochondral grafting (mosaic arthroplasty) for treatment of subchondral cystic lesions in the equine stifle and fetlock joints. *Veterinary Surgery,* 33(6), 588–596.

Bogenhagen, D.F. & Brown, D.D. (1981). Nucleotide sequences in Xenopus 5S DNA required for transcription termination. *Cell,* 24(1), 261-270.

Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O., & Peterson, L. (1994). Treatment of Deep Cartilage Defects in the Knee with Autologous Chondrocyte Transplantation. *New England Journal of Medicine,* 331(14), 889–895.

Brittberg, M., Peterson, L., Sjorgen-Jansson, E., Tallheden, T., & Lindahl, A. (2003). Articular cartilage engineering with autologous chondrocyte transplantation. A review of recent developments. *Journal of Bone and Joint Surgery (American Volume),* 85, S109-115.

Broom, N.D. & Poole, C.A. (1983). Articular cartilage collagen and proteoglycans their functional interdependency. *Arthritis & Rheumatology,* 26(9), 1111-1119.

Buckwalter, J.A., Hunziker, E.B., Rosenberg, L.C., et al. (1988). Articular cartilage: Composition and structure. In S.L Woo & J.A. Buckwalter (Eds.) *Injury and Repair of the Musculoskeletal Soft Tissues* (p.405-425). Park Ridge, IL: American Academy of Orthopaedic Surgeons. 405-425.

Buckwalter J.A., Mow V.C., & Ratcliffe A. (1994). Restoration of injured or degenerated articular cartilage. *Journal of the American Academy of Orthopaedic Surgeons,* 2(4), 192-201.

Buckwalter, J.A. & Mankin, H.J. (1998). Articular cartilage: tissue design and chondrocyte-matrix interactions. *Journal of Bone and Joint Surgery,* 79(4), 600-611.

Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Kibenge FS, Olsvik PA, Penning LC, Toegel S. MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Molecular Biology*. 2010;11:74.

Cancedda, R., Dozin, B., Giannoni, P., & Quarto, R. (2003). Tissue engineering and cell therapy of cartilage and bone. *Matrix Biology,* 22(1), 81–91.

Chen, Y., Pelekanos, R. A., Ellis, R. L., Horne, R., Wolvetang, E. J., & Fisk, Nicholas, M. (2012). Small molecule mesengenic induction of human induced pluripotent stem cells to generate mesenchymal stem/stromal cells. *Stem Cells Translational Medicine,* 83–95.

Cheng, N-C., Estes, B.T., Young, T-H., & Guilak, F. (2011). Engineered cartilage using primary chondrocytes cultured in a porous cartilage-derived matrix. *Regenerative Medicine,* 6(1), 81–93.

Chu, C. R., Szczodry, M., & Bruno, S. (2010). Animal models for cartilage regeneration and repair. *Tissue Eng Part B Rev,* 16(1), 105–115.

Clark, J. M. (1985). The organisation of collagen in cryofractured rabbit articular cartilage: a scanning electron microscopic study. *Journal of Orthopaedic Research,* 3(1), 17–29.

Co, C., Vickaryous, M.K., & Koch, T.G. (2014). Membrane culture and reduced oxygen tension enhances cartilage matrix formation from equine cord blood mesenchymal stromal cells in vitro. *Osteoarthritis and Cartilage / OARS, Osteoarthritis Research Society,* 22(3), 472–80.

Cohen, N.P, Foster, R.J., & Mow, V.C. (1998). Composition and Dynamic of Articular Cartilage: Structure, Function, and Maintaining Healthy State. *Journal of Orthopaedic & Sports Physical Therapy*, 28(4), 203-215.

Damsky, C.H. & Werb, Z. (1992). Signal transduction by integrin receptors for extracellular matrix: cooperative processing of extracellular information. *Curr Opin Cell Biol,* 4, 772-81.

de Crombrugghe, B., Lefebvre, V., Behringer, R.R., Bi, W., Murakami, S., Huang, W. (2000). Transcriptional mechanisms of chondrocyte differentiation. *Matrix Biol,* 19, 389-394.

De Gregoris, T.B., Borra, M., Biffali, E., Bekel, T., Burgess, J.G., Kirby, R.R., & Clare, A.S. (2009). Construction of an adult barnacle (Balanus Amphitrite) cDNA library and selection of reference genes for quantitative RT-qPCR studies. *BMC Molecular Biology,* 10(62).

Dozin, B., Malpeli, M., Cancedda, R., Bruzzi, P., Calcagno, S., Molfetta, L., … Marcacci, M. (2005). Comparative evaluation of autologous chondrocyte implantation and mosaicplasty: a multicentered randomized clinical trial. *Clinical Journal of Sport Medicine : Official Journal of the Canadian Academy of Sport Medicine,* 15(4), 220–6.

Dragoo, J.L., Choi, J.Y., Lieberman, J.R., Huang, J., Zuk, P.A., Zhang, J., Hedrick, M.H., & Benhaim, P. (2003). Bone induction by BMP-2 transduced stem cells derived from human fat. *Journal of Orthopaedic Research,* 21(4), 622-629.

Eggli P.S., Herrmann W., Hunziker E.B., & Schenk R.K. (1985). Matrix compartments in the growth place of the proximal tibia of rats. *The Anatomical Record,* 211(3), 246-257.

Esquisatto, M. a, Pimentel, E. R., & Gomes, L. (1997). Extracellular matrix composition of different regions of the knee joint cartilage in cattle. *Annals of Anatomy = Anatomischer Anzeiger : Official Organ of the Anatomische Gesellschaft,* 179(5), 433–7.

Evans, V. (2010). 2010 Canadian Equine Industry Profile Study: The State of the Industry, 3, 2010–2011.

Exiqon (2015). A guide to the microRNA and small RNA Sequencing Service – Guidelines v2.1.

Eyre, D. (1987). Collagen cross-linking amino acids. *Methods in Enzymology,* 144(7), 115-139.

Frisbie, D.D., Bowman, S.M., Colhoun, H.A., DiCarlo, E.F., Kawcak, C.E., & McIlwraith, C.W. (2008). Evaluation of autologous chondrocyte transplantation via a collagen membrane in equine articular defects - results at 12 and 18 months. *Osteoarthritis and Cartilage,* 16(6), 667–679.

Frisbie, D.D., Cross, M.W., & McIlwraith, C.W. (2006). A comparative study of articular cartilage thickness in the stifle of animal species used in human pre-clinical studies compared to articular cartilage thickness in the human knee. *Veterinary and Comparative Orthopaedics and Traumatology : V.C.O.T,* 19(3), 142–6.

Frisbie, D.D., Trotter, G.W., Powers, B.E., Rodkey, W.G., Steadman, J.R., Howard, R.D., & McIlwraith, C. W. (1999). Arthroscopic subchondral bone plate microfracture technique augments healing of large chondral defects in the radial carpal bone and medial femoral condyle of horses. *Veterinary Surgery : VS : The Official Journal of the American College of Veterinary Surgeons,* 28(4), 242–255.

Geyer, M., Grassel, S., Straub, R.H., Schett, G., Dinser, R., Grifka, J., Gay, S., Neumann, E., & Muller-Ladner, U. (2009). Differential transcriptome analysis of intraarticular lesional vs intact cartilage reveals new candidate genes in osteoarthritis pathophysiology. *Osteoarthritis and Cartilage,* 17, 328-335.

Ha, M. & Kim, V.N. (2014). Regulation of microRNA biogenesis. *Nature Reviews (Molecular Cell Biology),* 15(8), 509-524.

Hangody, L. & Fules, P. (2003). Autologous osteochondral mosaicplasty for the treatment of full-thickness defects of weight-bearing joints: ten years of experimental and clinical experience. *Journal of Bone and Joint Surgery (American Volume),* 85-A(S2), 25-32.

Hangody, L., Vasarhelyi, G., Hangody, L. R., Sukosd, Z., Tibay, G., Bartha, L., & Bodo, G. (2008). Autologous osteochondral grafting-Technique and long-term results. *Injury,* 39(1), S32–39.

Hardingham, T.E. & Fosang, A.J. (1992). Proteoglycans: many forms and many functions. *The FASEB Journal,* 6(3), 861-870.

Hayman, D.M., Blumberg, T.J., Scott, C., & Athanasiou, K.A. (2006). The effects of isolation on chondrocyte gene expression. *Tissue Engineering,* 12(9), 2573-2581.

Hering, T.M., Kollar, J., Huynh, T.D., Varelas, J.B., & Sandell, L.J. (1994). Modulation of extracellular matrix gene expression in bovine high-density chondrocyte cultures by ascorbic acid and enzymatic resuspension. *Archives of Biochemistry and Biophysics,* 314(1), 90-98.

Horas, U., Pelinkovic, D., Herr, G., Aigner, T., & Schnettler, R. (2003). Autologous chondrocyte implantation and osteochondral cylinder transplantation in cartilage repair of the knee joint. A prospective, comparative trial. *The Journal of Bone and Joint Surgery (American Volume),* 85–A(2), 185–92.

Horwitz, A., Duggan, K., Greggs, R., Decker, C., & Buck, C. (1985). The cell substrate attachment (CSAT) antigen has properties of a receptor for laminin and fibronectin. *Journal of Cell Biology,* 101, 2134-2144.

Hu, J. C., & Athanasiou, K. A. (2006). A self-assembling process in articular cartilage tissue engineering. *Tissue Engineering,* 12(4), 969–79.

Hu, J. C., & Athanasiou, K. A. (2006). The effects of intermittent hydrostatic pressure on selfassembled articular cartilage constructs. *Tissue Engineering,* 12(5), 1337–44

Huntzinger, E. & Izaurralde, E. (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nature Reviews Genetics,* 12, 99-110.

Imbeaud, S., Graudens, E., Boulanger, V., Barlet, X., Zaborski, P., Eveno, E., Mueller, O., Schroeder, A., & Auffray, C. (2005). Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. *Nucleic Acids Research,* 33(6), e56.

Iqbal J., Bird, J.L., Hollander, A.P., & Bayliss, M.T. (2004). Effect of matrix depleting agents on the expression of chondrocyte metabolism by equine chondrocytes. *Research in Veterinary Science,* 77(3), 249–256.

Jung, M., Schaefer, A., Steiner, I., Kempkensteffen, C., Stephan, C., Erbersdobler, A., & Jung, K., (2010). Robust microRNA stability in degraded RNA preparations from human tissue and cell samples. *Clinical Chemistry,* 56(6), 998-1006.

Kempson, G.E. (1980). The mechanical properties of articular cartilage. In L. Sokoloff (Eds.) *The Joints and Synovial Fluid, Vol. II* (p. 177-238). New York, NY: Academic Press.

Kiani, C., Chen, L., Wu, Y. J., Yee, A. J., & Yang, B. B. (2002). Structure and function of aggrecan. *Cell Research,* 12(1), 19–32.

Kidd, J.A., Fuller, C., & Barr, A.R.S. (2010). Osteoarthritis in the horse. *Equine Veterinary Education,* 13(3), 160–168.

Knutsen, G. (2007). A Randomized Trial Comparing Autologous Chondrocyte Implantation with Microfracture. *The Journal of Bone and Joint Surgery (American),* 89(10), 2105.

Knutsen, G., Engebretsen, L., Ludvigsen, T.C., Drogset, J.O., Grontvedt, T., Solheim, T., Strand, T., Roberts, S., Isaksen, V., & Johansen, O. (2004). Autologous chondrocyte implantation compared with microfracture in the knee. A randomized trial. *Journal of Bone and Joint Surgery (American Volume),* 86-A(3), 455-464.

Korhonen, R. K., Laasanen, M. S., Toyras, J., Lappalainen, R., Helminen, H. J., & Jurvelin, J. S. (2003). Fibril reinforced poroelastic model predicts specifically mechanical behavior of normal, proteoglycan depleted and collagen degraded articular cartilage. *Journal of Biomechanics*, 36(9), 1373–1379.

Lacourt, M., Gao, C., Li, A., Girard, C., Beauchamp, G., Henderson, J. E., & Laverty, S. (2012). Relationship between cartilage and subchondral bone lesions in repetitive impact traumainduced equine osteoarthritis. *Osteoarthritis and Cartilage,* 20(6), 572–583.

Le Bleu, H.K., Kamal, F.A., Kelly, M., Ketz, J.P., Zuscik, M.J., Elbarbary, R.A. (2017). Extraction of high-quality RNA from human articular cartilage. *Analytical Biochemistry,* 1(518), 134-138.

Lee, C.R., Grodzinsky, A.J., Hsu, H-P., Martin, S.D., & Spector, M. (2000). Effects of harvest and selected cartilage repair procedures on the physical and biochemical properties of articular cartilage in the canine knee. *Journal of Orthopaedic Research,* 18, 790-799.

Lee, D.A., Bentley, G., & Archer, C.W. (1994). Proteoglycan depletion alone is not sufficient to stimulate proteoglycan synthesis in cultured bovine cartilage explants. *Osteoarthritis Cartilage,* 2, 175-85.

Lee, P.D., Sladek, R., Greenwood, C.M.T., & Hudson, T.J. (2002). Control Genes and Variability: Absence of Ubiquitous Reference Transcripts in Diverse Mammalian Expression Studies. *Genome Research,* 12(2), 292-297.

Lefebvre, V., Li, P., & de Crombrugghe, B. (1998). A new long form of Sox-5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. *The EMBO Journal,* 17(19), 5718-5733.

Lin, Z., Fitzgerald, J.B., Xu, J., Willers, C., Wood, D., Grodzinsky, A.J., & Zheng, M.H. (2008). Gene Expression Profiles of Human Chondrocytes during Passaged Monolayer Cultivation. *Journal of Orthopaedic Research,* 26(9), 1230-1237.

Longmire, J.L., Maltbie, M., & Baker, R.J. (1997). Use of “Lysis Buffer” in DNA Isolation and its Implications for Museum Collections. *Occasional Papers (Museum of Texas Tech University),* 163.

Mankin H.J., Mow V.C., Buckwalter, J.A., & Iannotti, J.P. (1994) Form and function of articular cartilage. In S.R. Simon (Eds.), *Orthopaedic Basic Science* (p. 1-44). Rosemont, IL: American Academy of Orthopaedic Surgeons.

Mardis, E.R. (2007). The impact of next-generation sequencing technology on genetics. *Trends in Genetics,* 24(3), 133-141.

Maroudas A (1979). Physicochemical properties of articular cartilage. In: Adult articular cartilage. Freeman MAR, editor. Kent, UK: Pitman Medical, pp. 215-290.

Maroudas A, Wachtel E, Grushko G, et al. (1991). The effects of osmotic and mechanical pressure on water and partitioning in articular cartilage. *Biochem Biophys Acta*, 1073, 285-294.

Martel-Pelletier, J., Boileau, C., Pelletier, J.P., & Roughley, P.J. (2008). Cartilage in normal and osteoarthritis conditions. *Best Practice & Research in Clinical Rheumatology,* 22(2), 351-384.

McIlwraith, C.W., Fortier, L.A., Frisbie, D.D., & Nixon, A.J. (2011). Equine Models of Articular Cartilage Repair. *Cartilage,* 2(4), 317-326.

McIlwraith, C.W., Frisbie, D.D., & Kawcak, C.E. (2012). The horse as a model of naturally occurring osteoarthritis. *Bone and Joint Research,* 1(11), 297–309.

McKenna, L.A., Gehrsitz, A., Soder, S., Eger, W., Kirchner, T., & Aigner, T. (2000). Effective isolation of high-quality total RNA from human adult articular cartilage. *Analytical Biochemistry,* 286(1), 80-85.

Mendrick, D.L. (2011). Transcriptional profiling to identify biomarkers of disease and drug response. *Pharmacogenomics,* 12(2), 235-249.

Metzker, M.L. (2010). Sequencing technologies – the next generation. *Nature Reviews – Genetics,* 11, 31-46.

Mienaltowski, M.J., Huang, L., Stromberg, A.J., & MacLeod, J.N. (2008). Differential gene expression associated with postnatal equine articular cartilage maturation. *BMC Muskuloskeletal Disorders,* 9(149).

Mienaltowski, M.J., Huang, L., Stromberg, A.J., Bathke, A.C., & MacLeod, J.N. (2010). Transcriptional comparisons between equine articular repair tissue, neonatal cartilage, cultured chodnrocytes and mesenchymal stromal cells. *Brief Functional Genomics,* 9(3), 238-250.

Mithoefer, K., McAdams, T., Williams, R.J., Kreuz, P.C., & Mendelbaum, B.R. (2009). Clinical efficacy of the microfracture technique for articular cartilage repair in the knee: an evidence-based systematic analysis. *American Journal of Sports Medicine,* 37(10), 2053-2063.

Mitrovic, D., Lippiello, L., & Mankin, H.J. (1979). Use of enzymatically isolated chondrocytes or short term metabolic studies. *J Rheumatol,* 60, 124-130.

Mow, V. C., Holmes, M. H., & Michael Lai, W. (1984). Fluid transport and mechanical properties of articular cartilage: A review. *Journal of Biomechanics,* 17(5), 377–394.

Mow, V.C. & Guo X.E. (2002). Mechano-electrochemical properties of articular cartilage: their inhomogeneities and anisotropies. *Annual Review of Biomedical Engineering,* 4, 175-209.

Mueller, O., Hahnenberger, K., Dittmann, M., Yee, H., Dubrow, R., Nagle, R., & Isley D. (2000). A microfluidic system for high-speed reproducible DNA sizing and quantitation. *Electrophoresis,* 21, 128-134.

Muir H. (1995). The chondrocyte, the architect of cartilage: biomechanics, structure, function and molecular biology of cartilage matrix molecules. *Bioessays,* 17(12), 1039-1048

Nagy, K., Sung, H.-K., Zhang, P., Laflamme, S., Vincent, P., Agha-Mohammadi, S., … Nagy, A. (2011). Induced pluripotent stem cell lines derived from equine fibroblasts. *Stem Cell Reviews and Reports,* 7(3), 546–546.

Naranda, J., Gradisnik, L., Gorenjak, M., Vogrin, M., & Maver, U. (2017). Isolation and characterization of human articular chondrocytes from surgical waste after total knee arthroplasty (TKA). *PeerJ,* 5, e3079.

Nixon, A.J., Begum, L., Mohammed, H.O., Huibregtse, B., O'Callaghan, M.M., & Matthews, G.L. (2011). Autologous chondrocyte implantation drives early chondrogenesis and organized repair in extensive full- and partial-thickness cartilage defects in an equine model. *Journal of Orthopaedic Research,* 29(7), 1121–1130.

Pascual-Garrido, C., Slabaugh, M.A., L'Heureux, D.R., Friel, N.A., & Cole, B.J. (2009). Recommendations and Treatment Outcomes for Patellofemoral Articular Cartilage Defects with Autologous Chondrocyte Implantation. *The American Orthopaedic Society for Sports Medicine,* 37(1), S33-41.

Peeters, M., Huang, C.L., Vonk, L.A., Lu, Z.F., Bank, R.A., Helder, M.N., & Doulabi, B.Z. (2016). Optimisation of high-quality total ribonucleic acid isolation from cartilaginous tissues for real-time polymerase chain reaction analysis. *Bone & Joint Research,* 5(11), 560-568.

Poole, A.R. (1993). Cartilage in health and disease. In D.J. McCarty (Eds.) *Arthritis and Allied Conditions: A Textbook of Rheumatology* (p. 279-333). Philadelphia, PA: Lea & Febiger.

Romero, I.G., Pai, A.A., & Gilad, Y. (2014). RNA-seq: impact of RNA degradation on transcript quantification. *BMC Biology,* 12(42).

Ruettger, A., Neumann, S., Wiederanders, B., & Huber, R. (2010). Comparison of different methods for preparation and characterization of total RNA from cartilage samples to uncover osteoarthritis *in vivo*. *BMC Research Notes,* 3(7), 1-5.

Ruoslahti, E. (1991). Integrins. *J Clin Invest,* 87, 1-5.

Sambrook, J., Fritsch, E., & Maniatis, T. (1989) Molecular Cloning, a laboratory manual 2nd edition. New York, NY: Cold Spring Harbor Laboratory Press.

Sanchez-Adams, J. & Athanasiou, K.A. (2012). Regional effects of enzymatic digestion on knee meniscus cell yield and phenotype for tissue engineering. *Tissue Engineering,* 18(3), 235-243.

Sandell, L.J. & Aigner, T. (2001). Articular cartilage and changes in arthritis An introduction: cell biology of osteoarthritis. *Arthritis Research* 3(2), 107-113.

Saris D.B.F., Vanlauwe, J., Victor, J., Haspl, M., Bohnsack, M., Fortems, Y., Vandekerckhove, B., Almqvist, K.F., Claes, T., Handelberg, F., Lagae, K., van der Bauwhede, J., Vandenneucker, H., Yang, K.G., Jelic, M., Verdonk, R., Veulemans, N., Bellemans, J., & Luyten, F.P. (2008) Characterized chondrocyte implantation results in better structural repair when treating symptomatic cartilage defects of the knee in a randomized controlled trial versus microfracture. *American Journal of Sports Medicine,* 36(2), 235-46.

Schroeder, A., Mueller, O., Stocker, S., Salowsky, R., Leiber, M., Gassmann, M., Lightfoot, S., Menzel, W., Granzow, M., & Ragg, T. (2006). The RIN: an RNA

integrity number for assigning integrity values to RNA measurements. *BMC Molecular Biology,* 7(3), 1-14.

Sophia Fox, A.J., Bedi, A., & Rodeo, S.A. (2009). The Basic Science of Articular Cartilage: Structure, Compositions, and Function. *Sports Health,* 1(6), 461-468.

Spiegelaere, W.D., Dern-Wieloch, J., Weigel, R., Schumacher, V., Schorle, H., Nettersheim, D., Bergmann, M., Brehm, R., Kliesch, S., Vandekerckhove, L., & Fink, C. (2015). Reference Gene Validation for RT-qPCR, a Note on Different Available Software Packages. *PLOS ONE,* 10(3), e0122515.

Steadmann, J.R., Rodkey, W.G., & Briggs, K.K. (2002). Microfracture to treat full-thickness chondral defects: surgical technique, rehabilitation, and outcomes. *The Journal of Knee Surgery,* 15(3), 170-176.

Steadmann, J.R., Rodkey, W.G., & Rodrigo, J.J. (2001). Microfracture: surgical technique and rehabilitation to treat chondral defects. *Clinical Orthopaedics and Related Research,* 391, S362-369.

Strauss, E.J. & Galos, D.K. (2013). The evaluation and management of cartilage lesions affecting the patellofemoral joint. *Current Reviews in Muskuloskeletal Medicine,* 6(2), 141-149.

Strauss, E.J., Fonseca, L.E., Shah, M.R., & Youm, T. (2011). Management of focal cartilage defects in the knee: is ACI the answer? *Bulletin of the NYU Hospital for Joint Disease,* 69(1), 63-72.

Suzuki, T., Higgins, P.J., & Crawford, D.R. (2000). Control selection for RNA quantitation. *Biotechniques,* 29(2), 332-337.

Szirmai, J.A. (1969). Structure of cartilage. In A. Engel & T. Larsson (Eds.) *Aging of Connective and Skeletal Tissue* (p. 163-200). Stockholm, Sweden: Nordiska.

Takahashi, I., Nuckolls, G. H., Takahashi, K., Tanaka, O., Semba, I., Dashner, R., … Slavkin, H. C. (1998). Compressive force promotes Sox9 , type II collagen and aggrecan and inhibits IL-1 β expression resulting in chondrogenesis in mouse embryonic limb bud mesenchymal cells. *Journal of Cell Science,* 111, 2067–2076.

Takigawa, M., Takano, T., Shirai, E., & Suzuki, F. (1984). Cytoskeleton and differentiation: effects of cytochalasin B and colchicine on expression of the differentiated phenotype of rabbit costal chondrocytes in culture. *Cell Differ,* 14, 197-204.

Tuli, R., Li, W-J., & Tuan, R.S. (2003). Current state of cartilage tissue engineering. *Arthritis Research & Therapy,* 5(5), 235-238.

van der Kraan, P.M. & van den Berg, W.B. (2012). Chondrocyte hypertrophy and osteoarthritisL role in initiation and progression of cartilage degeneration? *Osteoarthritis Cartilage,* 20(3), 223-232.

Vandamme, T.F. (2014). Use of rodents as models of human diseases. *Journal of Pharmacy & BioAllied Sciences,* 6(1), 2-9.

von der Mark, K. & von der Mark, H. (1977). Immunological and biochemical studies of collagen type transition during in vitro chondrogenesis of chick limb mesodermal cells. *Journal of Cell Biology,* 73, 736-747.

von der Mark, K. (1986). Differentiation, modulation and dedifferentiation of chondrocytes. *Rheumatology,* 10, 272-315.

Wahid, F., Shehzad, A., Khan, T., & Kim, Y.Y. (2010). MiRNAs: Synthesis, mechanism, function, and recent clinical trials. *Biochimica et Biophysica Acta,* 1803, 1231-1243.

Wang, Y., Ding, C., Wluka, A.E., Davis, S., Ebeling, P.R., Jones, G., & Cicuttini, F.M. (2006). Factors affecting progression of knee cartilage defects in normal subjects over 2 years. *Rheumatology*, 45(1), 79-84.

Werb, Z., Tremble, P.M., Behrendtsen, O., Crowley, E., & Damsky, C.H. (1989). Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *Journal of Cell Biology,* 109, 877-89.

Wieczorek, D., Delauriere, L., & Schagat, T. (2012). Methods of RNA Quality Assessment. *Open Journal of Animal Sciences,* 7(2).

Wilfinger, W.W., Mackey, K., & Chomczynski, P. (1997). Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *Biotechniques,* 22(3), 474-476, 478-481.

Williams, G.M., Chan, E.F., Temple-Wong, M.M., Bae, W.C., Masuda, K., Bugbee, W.D., & Sah, R.L. (2010). Shape, loading, and motion in the bioengineering design, fabrication, and testing of personalized synovial joints. *Journal of Biomechanics*, 43(1), 156-165.

Williams, R.J. & Harnly, H.W. (2007). Microfracture: indications, techniques, and results. *Instructional Course Lectures,* 56, 419-428.

Yanagashita, M. (1993). Function of proteoglycans in the extracellular matrix. *Pathology International,* 43(6), 283-293.

Zanetti, N.C. & M. Solursh, M. (1989). Effect of cell shape on cartilage differentiation. In W.D., Stein & F., Bonner (Eds.) *Cell Shape: Determinants, Regulation and Regulatory Role.* New York, NY: Academic Press.

APPENDIX

RNA Extraction Results Using Method from Le Bleu et al., 2017

Materials & Methods

Native equine articular cartilage shavings from fetlock joints of 3 independent horse donors that had been snap-frozen and stored at -80 were utilized for this study. Shavings were weighed and 100mg of tissue was used for each sample. Samples were then subjected to the protocol as outlined in the article by Le Bleu and colleagues (2017).

Figure 19: SPEX Freezer Mill utilized to disrupt equine articular cartilage samples prior to RNA isolation.

Results

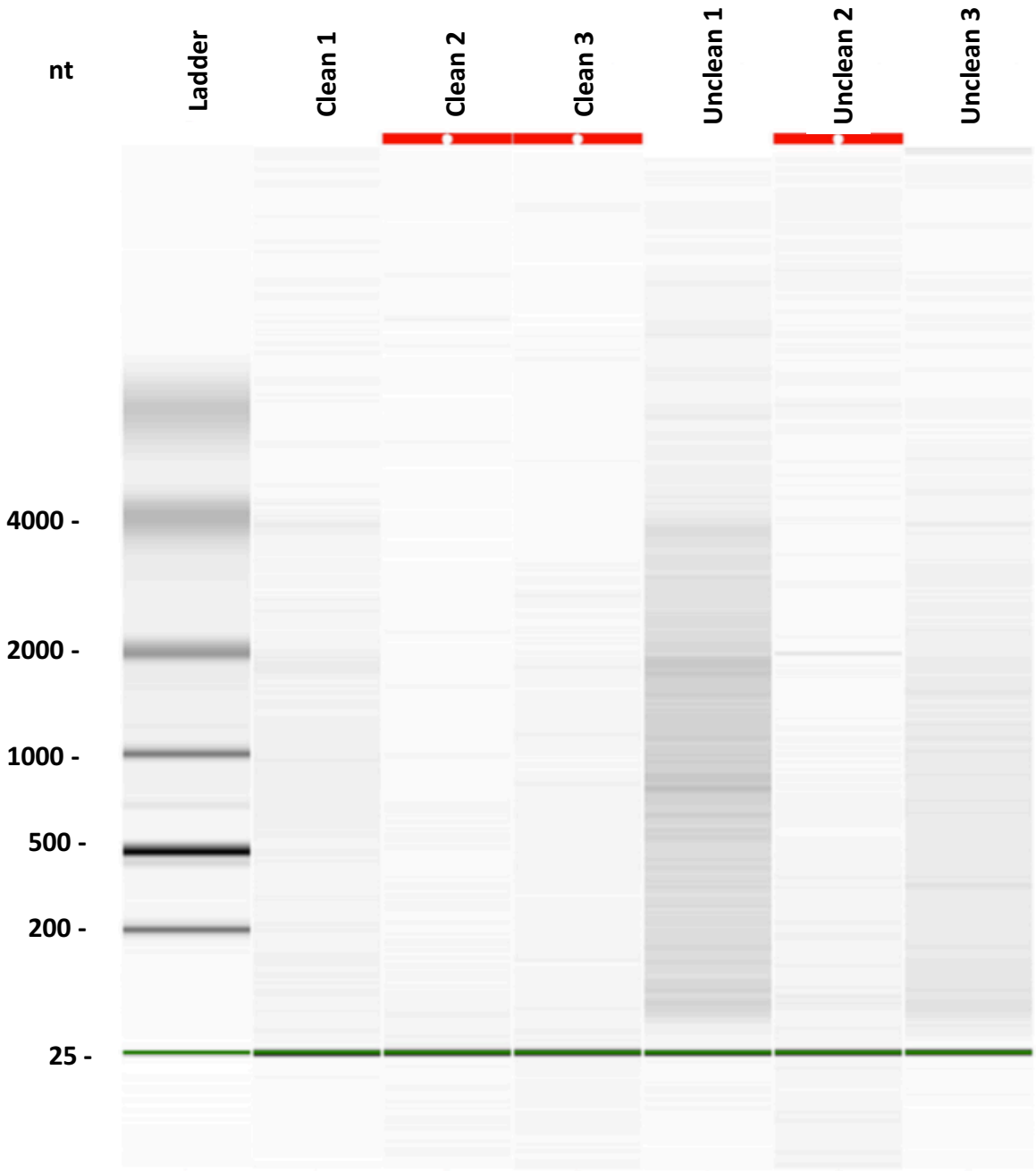

Figure 20: Gel electrophoresis image for samples extracted using methods published in *Le Bleu et al., 2017*. Lanes 1-3 are samples that were extracted and cleaned using the Clean & Concentrator Kit™ whereas lanes 4-6 are uncleaned samples analyzed immediately after extraction.

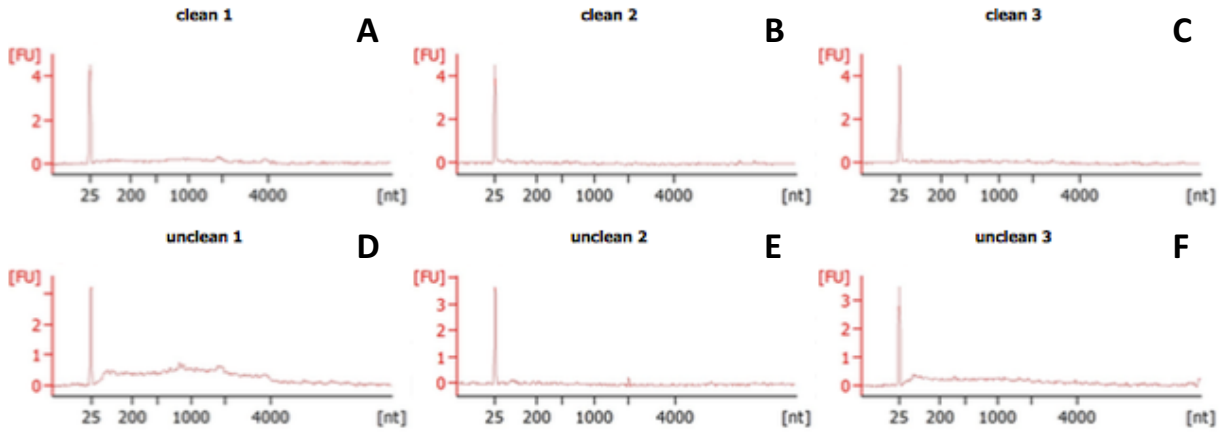

Figure 21: Electropherograms of RNA samples extracted using methods published in *Le Bleu et al., 2017*. A-C represent samples that were extracted and subsequently cleaned using the Clean & Concentrator Kit™. D-F represent uncleaned samples that were analyzed immediately following extraction.

Discussion

Le Bleu and colleagues (2017) recently published a method for isolation of high quality RNA from human articular cartilage. Osteoarthritic human articular cartilage was harvested from patients undergoing total knee replacement surgery and immediately snap frozen and stored for subsequent isolation. Isolation with a TRIzol protocol followed with salt precipitation was utilized. Le Bleu and colleagues (2017) reported RIN values of 7.9 and 8.4 with gel images showing intact RNA with little degradation.

Preliminary efforts undertaken to test this method using equine articular cartilage did not produce similar results as published by Le Bleu and colleagues. This method was followed exactly as outlined including the use of a freezer mill (Figure 19), however RIN values were either not available or fairly low (~2.0). Furthermore, upon viewing gel electrophoresis images the samples showed substantial degradation and there were no detectable bands of RNA at 28S and 18S. Additionally, when testing the samples using

the Nanodrop Spectrophotometer 2000c, high contamination was detected which was evident in the low 260/230 ratio values. Therefore, samples were also cleaned using the Clean & Concentrator Kit™ to remove contamination. However, cleaned samples also showed high degradation and RIN values were either low or not available.

Our findings may be explained due to the low abundance of chondrocytes that is found in cartilage tissue, for example in human chondrocytes comprise approximately 1% of articular cartilage tissue (Bhosale & Richardson, 2008). This would result in less RNA that could be extracted and ultimately affect the results obtained. Furthermore, Le Bleu and colleagues (2017) were able to snap freeze their articular cartilage samples immediately following harvesting during an operation, effectively minimizing degradation of tissue and preserving the tissue as close to in vivo conditions as possible. The equine samples were retrieved within 24 hours of euthanasia of the horses and not immediate upon euthanasia of the horse. This delay in retrieval and subsequent harvest of tissues could have increased degradation and affected RNA isolation procedures. However, a similar protocol for cartilage procurement was used in the main studies presented in this thesis without such signs of degradation making the cartilage procurement less likely as a main contributor to these findings. Lastly, Le Bleu and colleagues (2017) utilized osteoarthritic cartilage tissue to isolate RNA from. This tissue is already going through degradation as a result of the disease, which causes the extracellular matrix to decompose and the tissue to become less compressible and shock absorbent (Sandell & Aigner, 2001). Thus, decomposing tissue may facilitate easier retrieval of chondrocytes and subsequently make isolation of RNA more effective.

Ultimately, the method utilized by Le Bleu and colleagues (2017) was not successful using equine articular cartilage. Although these are preliminary findings, future work should attempt to optimize and modify this method and determine its efficacy with equine articular cartilage.