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Identification and Quantification of the Collagen Type I, III and V in Rabbit Patellar Tendons

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IDENTIFICATION AND QUANTIFICATION OF THE COLLAGEN TYPE I, III AND V IN RABBIT PATELLAR TENDONS

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

By
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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Mahesh Chandra Kodali ENTITLED Identification and Quantification of the Collagen Type I, III And V In Rabbit Patellar Tendons BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Kodali, Mahesh Chandra. M.S. Department of Pharmacology & Toxicology, Wright State University, 2015. Identification and Quantification of the Collagen Type I, III and V in Rabbit Patellar Tendons.

Tendon injuries pose a great clinical challenge to orthopedic surgeons. Over 200,000 patients undergo tendon repair every year in the United States alone. The role of progression of a tendon injury is multifactorial as a lot of factors come into play during and after the injury at various phases of healing process. There is a still a vast requirement for thorough elucidation and understanding of the pathophysiology and the factors involved in the progression of tendon injury. Although the degenerative role of several MMPs and ADAMTs have been reported, yet there is very less information on the actual role and function of the various types of collagen in tendons and their response to the above factors, particularly after an injury and during the healing and repair phases. Our results indicate that there is a lot of variability in the levels of collagen types I, III and V in relation to the age of the animal, injury and the healing period. This might not be any surprising as the fluctuation in the levels indicate that there might be a different mechanisms involved in progression and healing of the injuries in young and old rabbits, at various phases of healing.

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INTRODUCTION

1.1 Tendons

In the simplest description, tendons are tough belt like strips of fibrous connective tissue consisting of collagen fibers, which bridge a bone with a skeletal muscle (Figure 1). Their predominant activity is to limit, confine, absorb, withstand tension and to transmit the forces from the skeletal muscle to the bone[1]. The higher the force generated by the muscle, the more stress transmission takes place through the tendon[2]. Tendons are white to pale colored anatomical structures present connecting muscles to bones[3]. They offer resistance to mechanical loads[4], and aid in joint movement, serving as rigid and stable fibro-elastic structures and conveying the muscular contraction forces with the least possible energy scattering[5,6].

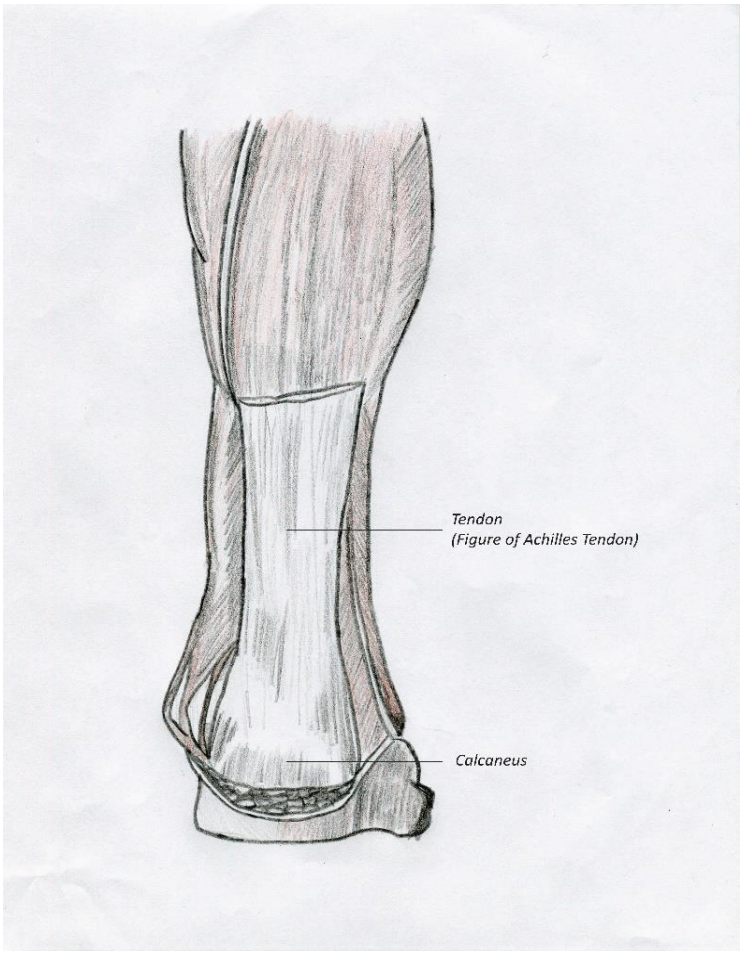


FIGURE 1: Drawing of a tendon bridging the calf muscle with the heel. Adapted from Gray's Anatomy.

1.2 Composition of tendons:

Tenoblasts are the primary cells in tendons. More than 90% (90-95%) of the tendon's cells are tenoblasts[4,7]. A tenocyte is a less metabolically active form of a tenoblast[8]. In comparison with tenocyte, tenoblasts have a larger rough endoplasmic reticulum, a well-organized mature Golgi-apparatus and an increased number of mitochondria. These important phenotypic differences are required in order to synthesize and secrete collagen fibers, ground substance, cytokines, enzymes, and other proteins necessary for the uninterrupted generation of extracellular components[9,10]. The other major components of mammalian tendons include extracellular collagen fibrils enclosed in a proteoglycan/ water extracellular matrix[4,7,11-13]. The tendon is a perfect example of a remarkably structured extracellular matrix where-in the molecular chains of collagen protein cluster into filamentous collagen fibrils, constituted by microfibrils[14-18] which package together to make up the main structural components i.e. collagen fibers[12,19-21]. The main extracellular component in tendons are collagen fibers and they make up 70-80% of the dry weight [9,22,23] and 30% of the wet weight[22]. In the extracellular matrix, the larger part (95%) is constituted by the presence of type I collagen, nonetheless, 5% of mature adult tendon also has some of other types of collagen, of which type III and type V are prominently observed[24]. The total dry weight of the tendon is comprised of 70-80% of collagen I and the rest 20 to 30% of other minor collagens along with proteoglycans, glycolipids, elastin and matrix[25-27].

Ground substance is also synthesized by tenoblasts and serves as a vehicle through which the gases, nutrients, minerals and metabolic by-products are diffused from the active tenoblasts[22]. It is a viscous watery and jelly-like liquid comprised of

glycosaminoglycans, proteoglycans and glycoproteins [9]. Glycosaminoglycans are the straight chain polysaccharides grouped by a continuous sub-units of disaccharides[8,9]. These include chondroitin-6 and dermatan sulphate[9]. These are highly hydrophilic, and despite these accounting for only 1% of the dry weight, they combine with water and make up to 65-75% of the wet weight of the tendon[23]. Proteoglycans include a larger amount of decorin[28,29], and also smaller amounts of hyaluronan, biglycan, fibromodulin, lumican, epiphycan and keratocan[30,31] and are joined to the collagen fibers in orthogonal fashion. These make up less than 1% of the dry weight of tendon[32]. Glycosaminoglycans and proteoglycans together determine the thickness of the collagen fibril and also control the inter-fibrillar and inter-fiber space[9]. Fibronectin is the predominant glycoprotein and aids in the attachment of tenoblasts to the collagen fibers[22]. Elastin is the other fiber type which includes for just 1-2% of the dry weight[4], under 2% of the wet weight and plays a vital role in maintaining the elasticity of the tendon[22].

1.3 Structural Organization of Tendon

The predominant extracellular building block of tendons is collagen and is the protein most abundantly present in mammalians[33,34]. Tropocollagen is the structural unit of collagen[22]. The precursor of tropocollagen is procollagen. Tropocollagen is synthesized when the non-helical N- and C- terminal propeptide units from procollagen molecule are cleaved enzymatically by matrix metalloproteinase C- proteinase and N- proteinase after it comes out from the tenoblast[22,35]. Each tropocollagen molecule is characterized by the presence of three helically ordered polypeptide chains of alpha chains[9]. Amino acid residues make up the poly peptide chains. Tropocollagen has a glycine amino acid

residue at every third position[9,22]. The other main amino acid residues of the alpha chains include proline (12%), hydroxyproline (10%), lysine and hydroxylysine[9,22]. A group of

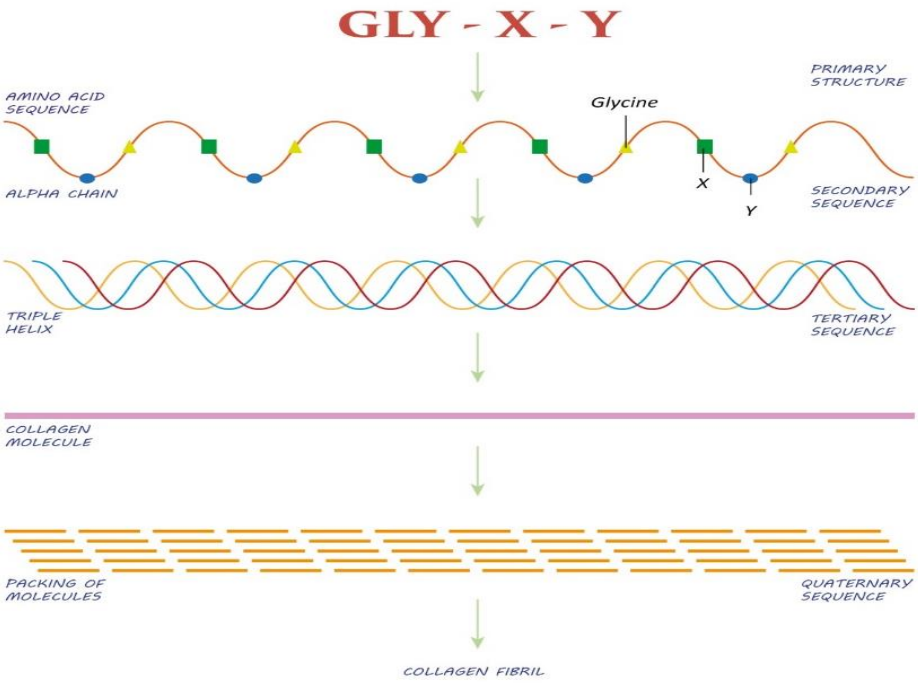


FIGURE 2: Organization of a Collagen Fibril, Adapted from Peter A et al, "Tendon Injury - A Review"

four to five tropocollagen molecules join together to form collagen microfibrils[9,23,36]. These and aggregate to form a collagen fibril[37]. Fibrils again group to form parallel fibers, which further aggregate to form fascicles[38]. Some of the tendons are covered by a loose connective tissue sheath known as the para-tenon. It predominantly consists of collagen I and collagen III fibrils[39]. Beneath the para-tenon is the epi-tenon, made of fine connective tissue. The collagen fibers are again covered by loose areolar connective tissue sheaths, at various levels of hierarchy[40]. There is also the presence of endo-tenon under the epitenon which carries blood vessels, lymph vessels and nerves to the inner areas of the tendon[23,41].

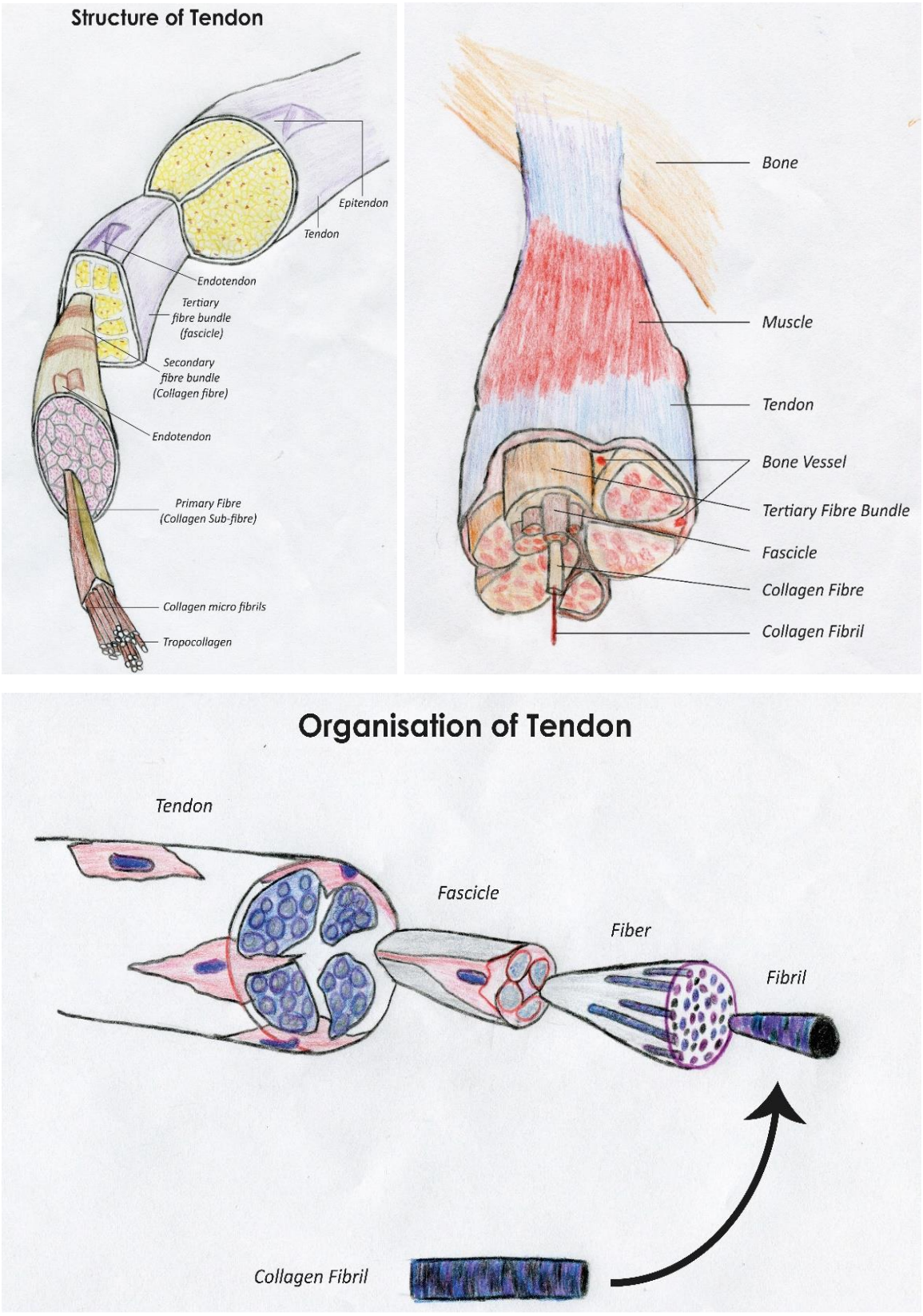


FIGURE 3: Various diagrammatic representations of the Structural Organization of Tendon

1.4 Tendon Injuries

Tendon injuries pose a great clinical challenge to orthopaedic surgeons[42]. Over 200,000 patients undergo tendon repair every year in the United States alone[43]. Overuse tendon injuries are approximated to be responsible for more than 30 to 50% of the sports linked injuries in the US[38,44]. Overuse tendon injuries along with tendinopathies serve as a source for approximately 7% of all injury associated doctor visits in the US[45].

1.5 Etiology of tendon injuries

The basic mechanism underlying a tendon injury is simply explained by the development a rupture in the tendon followed by disarrangement of collagen fibrils leading to its failure. This teardown primarily happens due to the overall increase in the stress limit or mechanical load on the tendon, due to increasing age[44,46] or overuse[23]. Chronic tendon injury develops due to failure in the healing of initially occurred ruptures, and the rate of healing to be far less when compared to the rate of accruing new injuries over time, which may be due to lack of rest or overuse[23]. The tendon injury primarily originates because of the action of increased forces on the tendon, which are far greater than the limit which it is designed to effectively withstand, combat and compensate for[10]. In addition, the tendon might undergo structural, cellular or molecular transformations, due to genetic disorders, vascular modifications, endocrine effects like hormonal imbalances, deficiencies in nutrition, inactivity, immobilization and exercise related causes, which ultimately progress towards injury even due to the action of normal forces[22,23,44,46-48]. Even antibiotics like fluoroquinolones are shown to induce apoptosis[49], oxidative damages[50] and breakdown of collagen i.e. collagenolysis[51] in tendon.

1.6 Pathophysiology of tendon injury:

Tendon overuse causes the release of pro-inflammatory mediators[52], which in-turn stimulate metalloproteinases, leading to collagenolysis ultimately[51,53]. The histopathological characterization of tendon injuries have depicted disarranged collagen fibril organization and alignment along with an increase in the content of proteoglycan, ground substance and neovascularization (figures x and y)[54,55]. The levels of collagen I and III were also found to be increased in the matrix. Collagen V is involved in the regulation of collagen I diameter during the fibrillogenesis[56,57]. The levels of collagen V were also found to increase during healing, and this increased level of collagen V might help the synthesis of smaller collagen I fibrils, and ultimately lead to decrease in mechanical strength[57,58]. The levels of fibronectin, Tenacin C were also found to be increased. Glycosaminoglycans were also found to be on the higher side in injured tendons[51,59].

1.7 Tendon Injury Healing:

The healing process of tendon injury can be explained in three distinct yet mutually coinciding processes. The inflammatory stage, the repair stage and then the remodelling phase[60]. Initially, different cells like erythrocytes and neutrophils approach the site of injury and secrete vasoactive and chemotactic factors, which cause the tenoblasts to actively proliferate and move to the site and start the repair process. These tenoblasts synthesize large amounts of collagen and other constituents of extracellular matrix which regenerate the repaired tissue[60].

MATERIALS AND METHODS

2.1 Animals:

Rabbits 1 year (young) and 4 year (old) had full length patellar tendon injuries created in the middle 3rd of the tendon. Tendon injuries were made on one side and the contralateral tendon was used as an unoperated control. They were allowed to heal for 6, 12 and 26 weeks and later harvested for analysis of the proximal, distal and middle portion of the tendon. The tissues were stored at -80 until extraction and analysis.

2.2 Methods:

2.2.1 Extraction of the collagens I, III and V from the tissues – Method development.

Collagen was extracted from the tissues using both acid and enzyme soluble extraction methods[61]. In order to extract the maximal amounts of the types of collagen from the tissues, these two methods were combined. The procedure mainly included the extraction of ground tendon tissue in organic acid (0.5M CH3COOH) in the presence of 5mM EDTA and pepsin with concentration of 0.0025 mg per 5 mg of tissue, pH = 2.5-3.0 for 48 -96 h at 4°C. The collagen isolation was carried out in the following steps: Initially, the ground tissue pieces were washed thoroughly with distilled water. This was followed by washing with 0.05M Na_2HPO_4, at pH 8.7 – 9.1 at 4°C so as to extract the non-collagenous proteins. The tissue was then dissolved in 0.5M CH3COOH with 5mM

EDTA. Pepsin was then added directly into the solution of collagen in acetic acid at a ratio of 50 mg of pepsin per 1g of tendons, at 4°C. Addition of pepsin was repeated once more after 12 hours.

The entire procedure was repeated 3 times and each time, the supernatants of the extracted solutions were collected after centrifugation at 3000 X g for 45 min at 4°C. The three extracts were combined and the collagen salted out by adding NaCl at an initial concentration of 0.8M and then increasing to a final concentration of 4M[62], so as to precipitate each of the three collagen types (I, III and V). The obtained precipitate contained collagen that was dissolved in 400 μl of 1M acetic acid. Then the solution was finally dialyzed against 0.02M Na2HPO4 for 72 hours at 4°C to remove the salt. The dialysis solution was changed every 24 hours.

2.2.2 Determination of the total collagen by modified Bradford assay

The collagen extracts were then analyzed for determining the total collagen content. For this, the modified Bradford assay was employed which more accurately detects the amount of collagen in the solution[63,64]. Bradford reagent was initially diluted four fold with water and 0.0035% of SDS was added to it. The Bradford assay was carried out, and the plates were read at 380nm on the Spectramax Plus 384 UV/VIS spectrophotometer (Molecular devices, Sunnyvale, CA) and the concentrations of the extracted proteins were calculated.

2.2.3 Poly-Acrylamide Gel Electrophoresis

Native PAGE: To detect collagen I, V and actin in western blotting, native PAGE was performed. Non-denaturing and non-reducing conditions were employed so as to

maintain the original fold and confirmation of the proteins in the samples. The samples with equal amount of protein were mixed with equal amounts of native loading buffer and then, loaded and separated on Criterion Tris HCL 10% – 20% precast gels.

SDS – PAGE: SDS-PAGE was performed to detect collagen III in western blotting. The samples with equal amount of protein were mixed with equal amounts of Laemmli sample buffer and heated at 95°C in water bath for 7 minutes. Denaturing and reducing conditions were followed. The samples were then loaded and run on Criterion Tris HCL 10% – 20% precast gels.

2.2.4 Western Blotting

The proteins were then transferred overnight to PVDF membranes and then blocked in 3% non-fat dry milk in 1X PBS containing 0.3% Tween 20 for an hour and washed subsequently with the wash buffer (1X PBS containing 0.3% Tween 20). They were then incubated in the primary antibody (Collagen I, III, V and actin) at a dilution of 1:500, for about an hour. The membranes were then washed for 15 minutes and incubated with HRP-conjugated anti mouse secondary antibody for an hour. The membranes were finally washed for 15 minutes and incubated in the West-Femto substrate and imaged on Bio-Rad Chemidoc MP imaging system using image-lab software.

2.2.5 Band densitometry:

The band density corresponding to each of collagen I, III, V and actin were quantified using the image-lab software, and later the levels of Collagen I, III and V were normalized with respect to the level of actin for each sample.

2.2.6 Statistical analysis

Statistica 10.0 was used for the statistical analysis of the data. All data were expressed as mean ± SD (Standard deviations), and the statistical differences among different groups were assessed by one-way ANOVA. Tukeys post hoc test was used for comparing three groups. The level of significance was considered as $P < 0.05$ which indicated a significant difference between the groups.

RESULTS

3.1 Development of procedure for extracting collagen from tendons.

The main goal of this project was to compare types of collagen from tendons following damage. To achieve this goal, we first needed to develp a method for extracting the collagen from the tendons. Collagen is a very insoluble protein complex that is difficult to extract and work with, therefore, we spent some time to learn and adapt previously described protocols[61,62,65,66]. In figure 4, we show that the protocol required steps for cleaning, digestion with enzyme, precipitation with salts, and dialysis to remove salts.

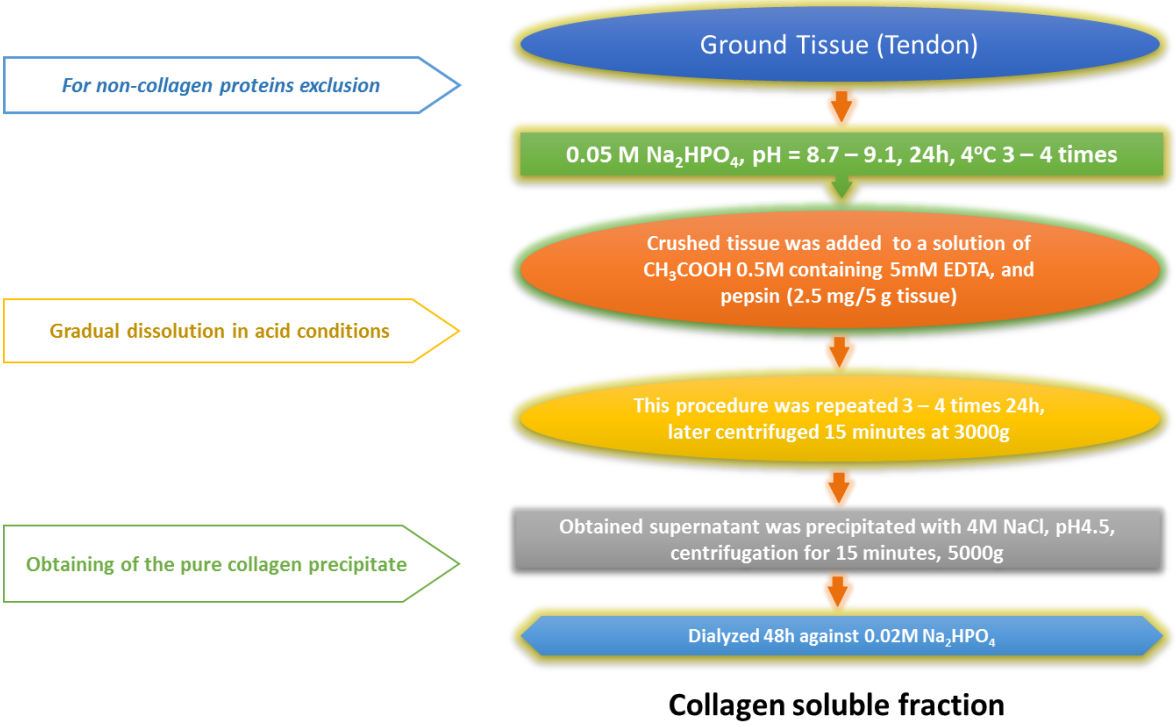


FIGURE 4: Protocol for extraction of Collagen type I, III and V from Rabbit tendons, Adapted from Mocan et al, "Aspects of Collagen Isolation Procedure".

From this protocol we were able to observe multiple bands putatively identified as collagen on a coomassie stained gel (Figure 5). The number of bands suggested that collagen was not the only protein extracted.

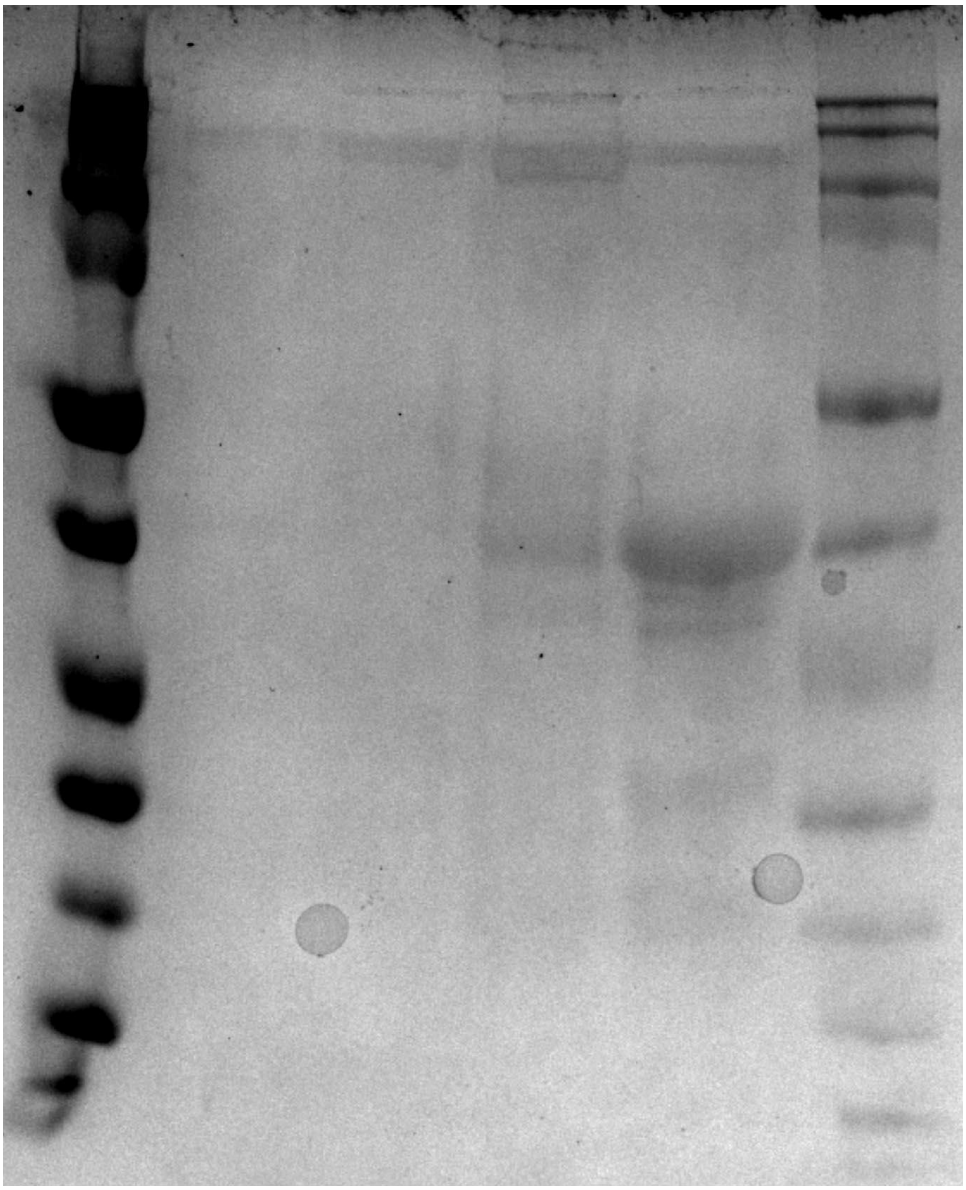


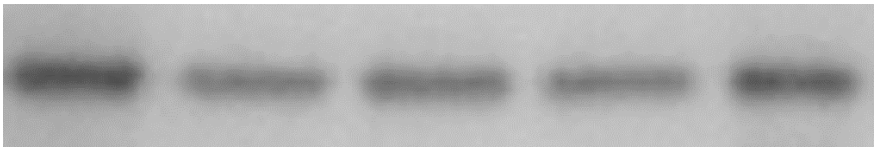
FIGURE 5: Denaturing SDS-PAGE of Collagen Extraction from 4 tendon samples.

The coomassie stained gel was loaded with standard marker (Bio-Rad) in lanes 1 and 6. Lanes 2-5 contained an aliquot of proteins extracted from four different tendons as described above.

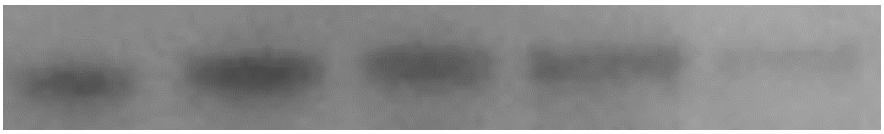
To analyze these gels further, western blots were probed for collagen I, III, V and actin were (figure 6).

The following are the images of the western blot. The bands were seen at 42 kda for actin, at 45 kda for collagens I, III and V.

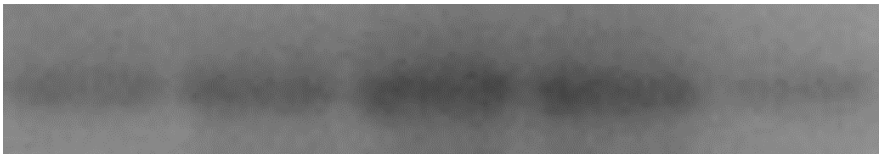
1. **Collagen I**



2. **Collagen III**



3. **Collagen V**



4. **Actin**

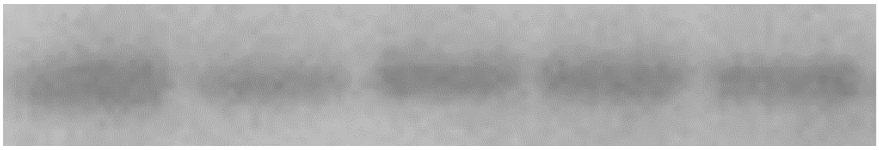


FIGURE 6: WESTERN BLOT

3.2 Levels of collagen I, III and V in control and open rabbits

3.2.1 Levels of Collagen I, III and V in young rabbits.

The first analysis was to determine if the age of the rabbit had an effect on the type of collagen expressed in the wound. In young rabbits, at 6 weeks of healing, the levels of collagen I III, and V were not significantly different in control rabbits compared to open rabbits (Figure 7).

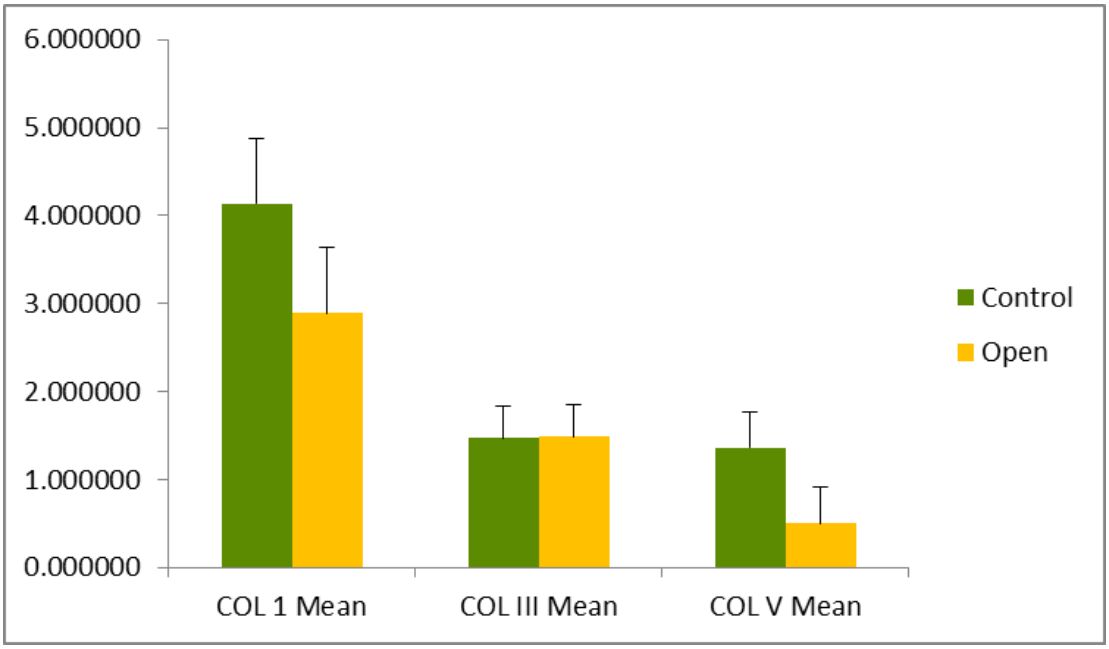


FIGURE 7: Levels of Collagen I, III and V in young control rabbits vs young open rabbits at 6 weeks of healing. The bars represent the mean ± SD values of normalized levels of Collagen I, III and V in young control (green) and in young open (yellow) rabbits at 6 weeks of healing. In young rabbits, at 6 weeks of healing, the levels of collagen I, III and V were not significantly different in open rabbits compared to control rabbits.

By western blot, the ratio of I:III:V appeared to be ~3:1:1 in controls and 2:1:0.4 in Open. However, at 12 weeks of healing, there was still no significant change in the types of collagens III and V, though there was a slight 1.4 fold increase in collagen I in open which was significant (Figure 8). In contrast to 6 weeks, there appeared to be no

difference in the ratios of any of the collagens with each being ~1:1:1, representative of a decrease in collagen I (Figure 8). At 26 weeks, there was no difference in open or control levels of collagen I and V, but there was a significant decrease in the levels of collagen III (Figure 9) in the open compared to control. Also, the overall levels were considerably lower. However, there did appear to be a significant decrease in the ratio of collagen I to III, i.e., ~0.67% (Figure 9).

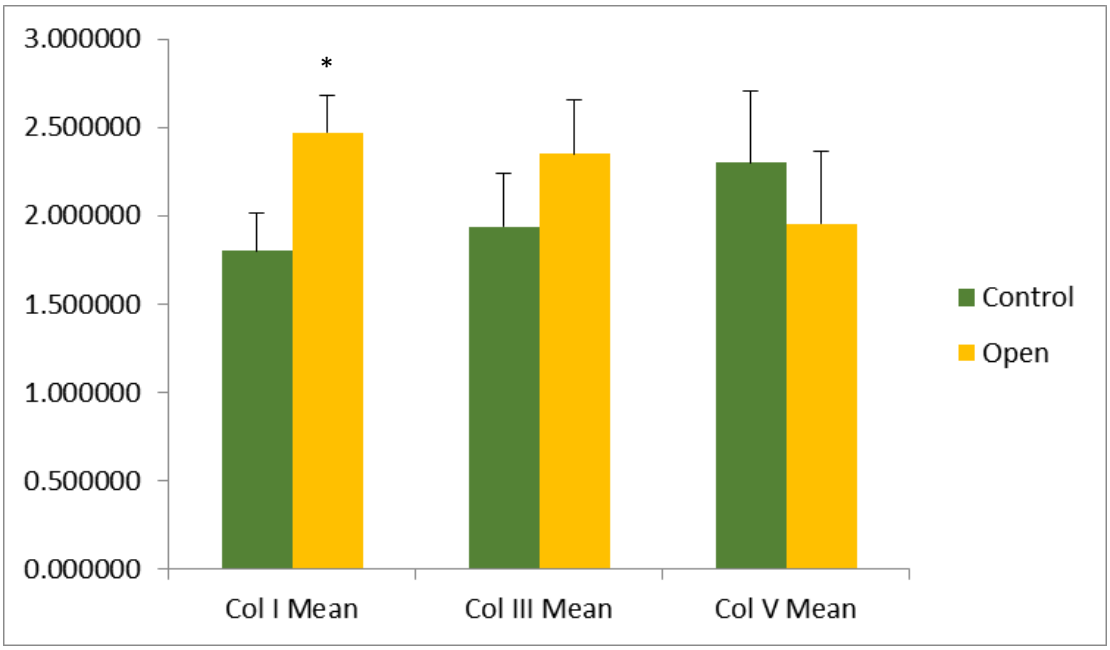


FIGURE 8: Levels of Collagen I, III and V in young control rabbits vs young open rabbits at 12 weeks of healing. The bars represent the mean ± SD values of normalized levels of Collagen I, III and V in young control (green) and in young open (yellow) rabbits at 12 weeks of healing. In young rabbits, at 12 weeks of healing, the levels of collagen I were significantly higher (indicated by *, $P < 0.05$, N = 18, n (control) = 9, n (open) = 9)) in open rabbits compared to control rabbits. The levels of collagen III and V were not significantly different.

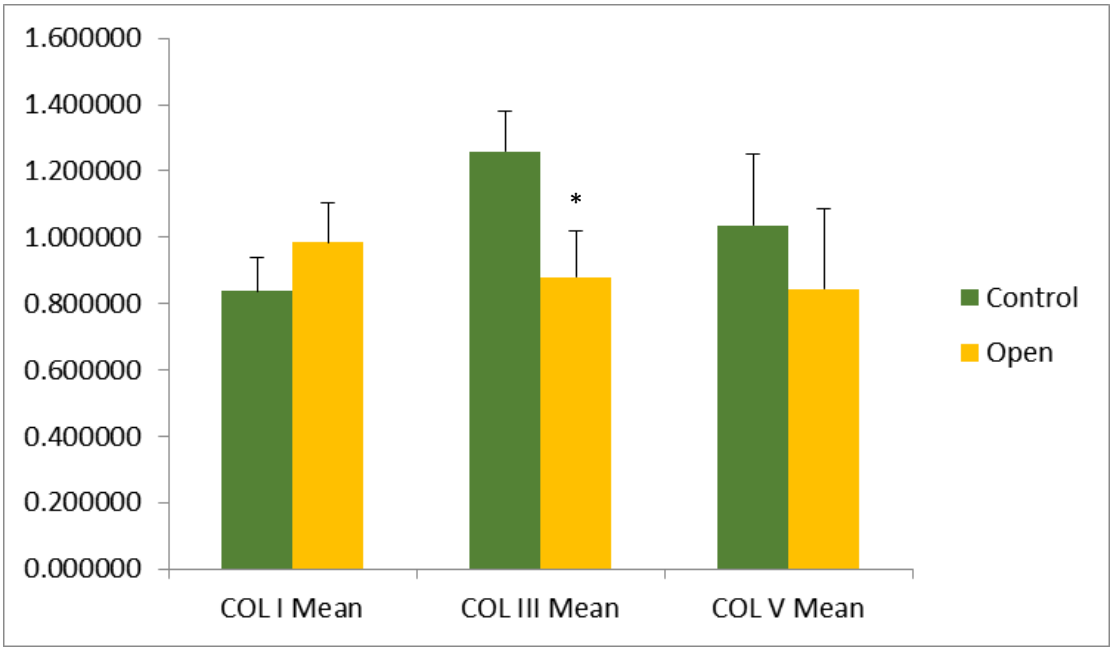


FIGURE 9: Levels of Collagen I, III and V in young control rabbits vs young open rabbits at 26 weeks of healing. The bars represent the mean ± SD values of normalized levels of Collagen I, III and V in young control (green) and in young open (yellow) rabbits at 26 weeks of healing. In young rabbits, at 26 weeks of healing, the levels of collagen III were significantly higher (indicated by *, $P < 0.05$, $N = 32$, n (control) = 18, n (open) = 14)) in open rabbits compared to control rabbits. The levels of collagen III and V were not significantly different.

When each collagen from the Young Open was compared between 6, 12 and 26 weeks, the levels of collagen I appeared to show a significant decrease at 26 weeks compared to 6 weeks and 12 weeks, while the levels of collagen III appear to be increased at 12 followed by a significant decrease at 26 weeks compared to 12 weeks. Collagen V showed a significant increase at 12 compared to 6 weeks followed by a decrease to nearly the same 6 week levels (Figure 10). In control animals, there was a significant decrease in collagen I at 12 and 26 weeks, but no change in any of the other collagens.

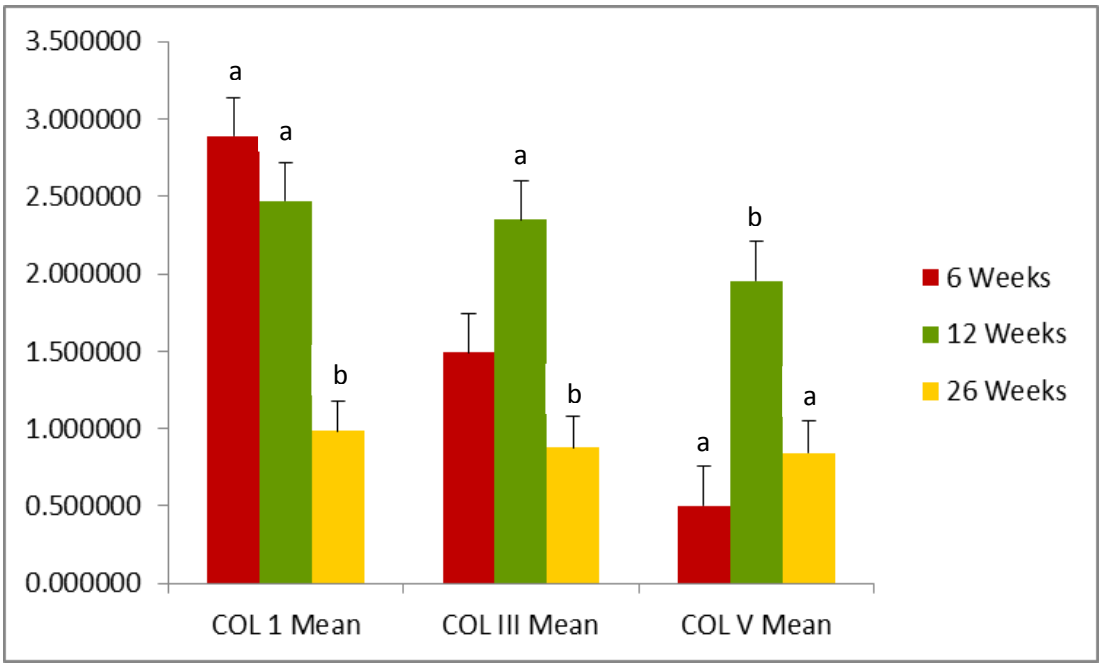


FIGURE 10: Levels of Collagen I, III and V in young open rabbits at 6, 12 and 26 weeks of healing. The bars represent the mean ± SD values of normalized levels of Collagen I, III and V in young open rabbits at 6 weeks (red), 12 weeks (green) and 26 weeks (yellow) of healing. In young open rabbits, the levels of collagen I were significantly lower at 26 weeks compared to the levels at 6 weeks ($P < 0.05$, N = 23, n (26 weeks) = 14, n (6 weeks) = 9) and the levels at 12 weeks ($P < 0.05$, N = 23, n (26 weeks) = 14, n (12 weeks) = 9)). The levels of collagen III were significantly lower at 26 weeks compared to the levels at 12 weeks ($P < 0.05$, N = 23, n (26 weeks) = 14, n (12 weeks) = 9). The levels of collagen V were significantly higher at 12 weeks compared to the levels at 6 weeks ($P < 0.05$, N = 18, n (12 weeks) = 9, n (6 weeks) = 9) and significantly lower at 26 weeks compared to the levels at 12 weeks ($P < 0.05$, N = 23, n (26 weeks) = 14, n (12 weeks) = 9))

3.2.2 Levels of Collagen I, III and V in Old Rabbits.

The levels of collagen, I, III, and V were analyzed in tendons from older rabbits, i.e., at 4 years of age. In old rabbits, at 6 weeks of healing, the levels of collagen I, III, and V were no different in control rabbits compared to open rabbits (Figure 11). There appeared to be a difference between control and open for both collagen I and III, though the variability made these non-significant. At 12 weeks of healing, the levels of collagen

I, III, and V were found to be the same between control and open. The ratio of collagen I to III to V were also the same for both open and control (Figure 12).

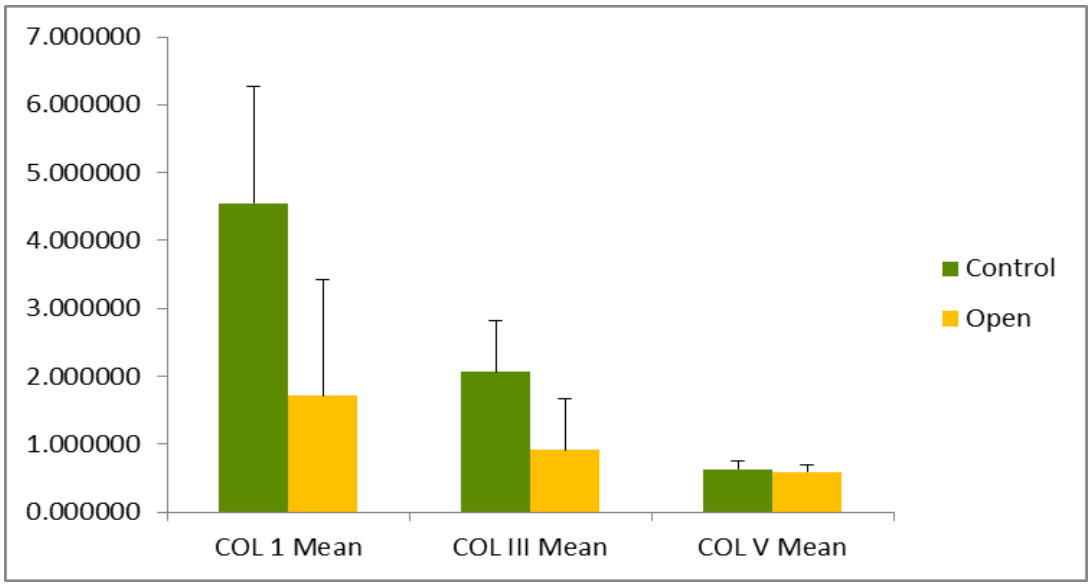


FIGURE 11: Levels of Collagen I, III and V in old control rabbits vs old open rabbits at 6 weeks of healing. The bars represent the mean ± SD values of normalized levels of Collagen I, III and V in old control (green) and in old open (yellow) rabbits at 6 weeks of healing. In old rabbits, at 6 weeks of healing, the levels of collagen I, III and V were not significantly different in open rabbits compared to control rabbits.

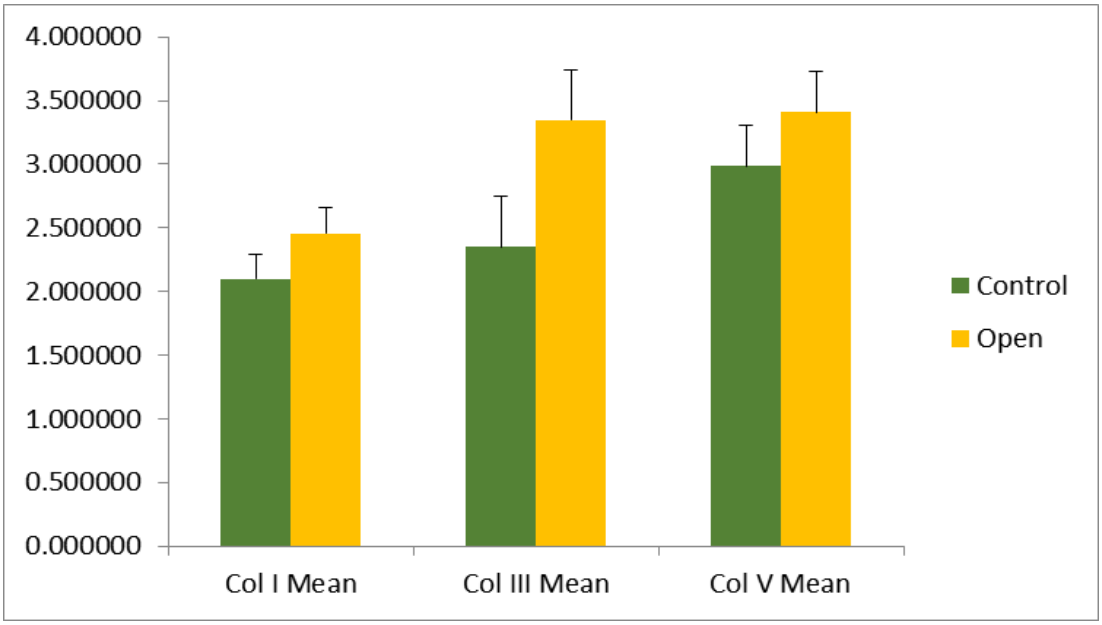


FIGURE 12: Levels of Collagen I, III and V in old control rabbits vs old open rabbits at 12 weeks of healing. The bars represent the mean ± SD values of normalized levels of Collagen I, III and V in old control (green) and in old open (yellow) rabbits at

12 weeks of healing. In old rabbits, at 12 weeks of healing, the levels of collagen I, III and V were not significantly different in open rabbits compared to control rabbits.

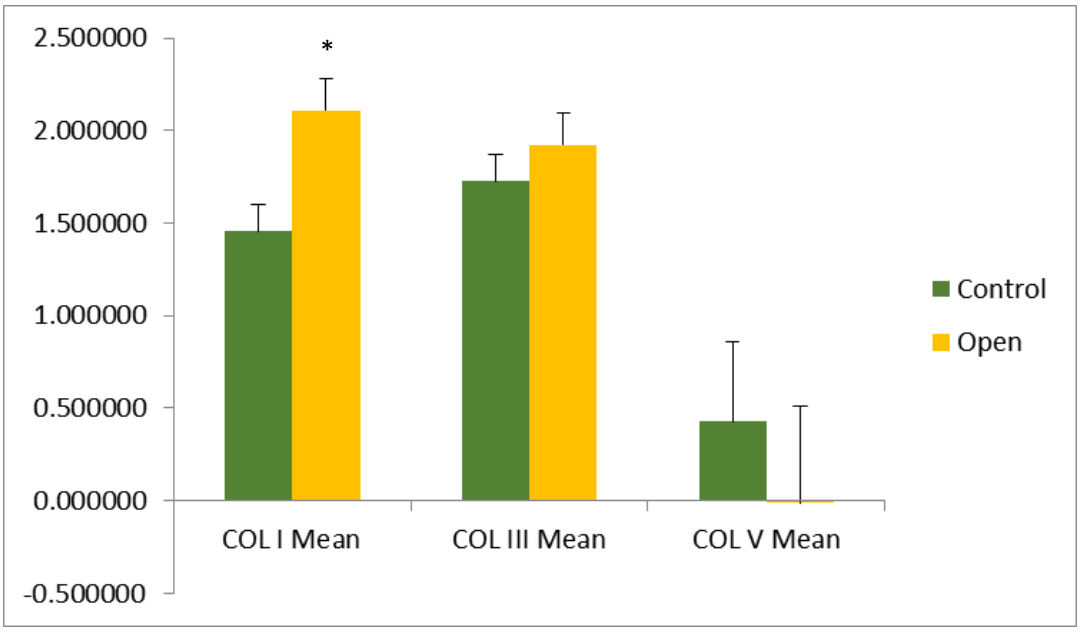


FIGURE 13: Levels of Collagen I, III and V in old control rabbits vs old open rabbits at 26 weeks of healing. The bars represent the mean ± SD values of normalized levels of Collagen I, III and V in old control (green) and in old open (yellow) rabbits at 26 weeks of healing. In young rabbits, at 26 weeks of healing, the levels of collagen I were significantly higher (indicated by *, $P < 0.05$, $N = 10$, n (control) = 6, n (open) = 4)) in open rabbits compared to control rabbits. The levels of collagen III and V were not significantly different.

At 26 weeks, the levels of collagen I appeared to be significantly different but not in III or V (Figure 13). The ratios of collagen I:III:V were shifted in these to a 1:1:0.3 for controls and 1:1:0 for open, suggesting decrease in collagen V with age (Figure 13).

When the age of the rabbits was taken into consideration, there appeared to be significant shifts in collagen production in open rabbits, especially collagen III and V (Figure 13). Most notably, there was a significant increase in each of these at 12 weeks, but followed by a significant decrease in the level of collagen V at 26 weeks (figure 14).

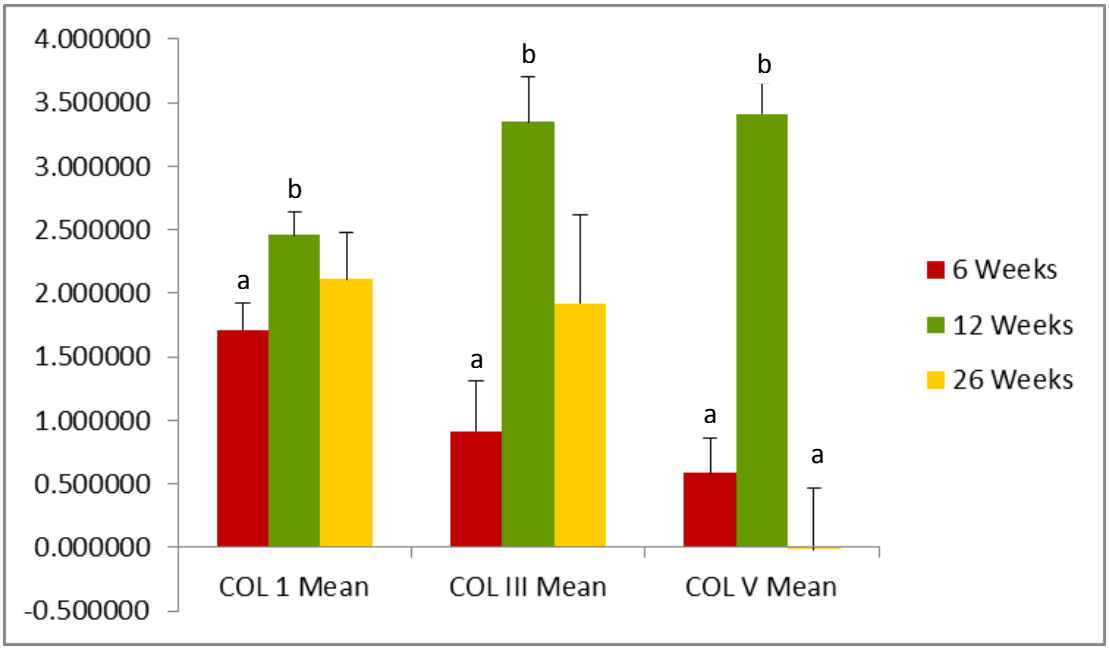


FIGURE 14: Levels of Collagen I, III and V in old open rabbits at 6, 12 and 26 weeks of healing. In old open rabbits, the levels of collagen I were significantly higher at 12 weeks compared to the levels at 6 weeks ($P < 0.05$, $N = 27$, n (12 weeks) = 15, n (6 weeks) = 12). The levels of collagen III were significantly higher at 12 weeks compared to the levels at 6 weeks ($P < 0.05$, $N = 27$, n (12 weeks) = 15, n (6 weeks) = 12). The levels of collagen V were significantly higher at 12 weeks compared to the levels at 6 weeks ($P < 0.05$, $N = 27$, n (12 weeks) = 15, n (6 weeks) = 12) and significantly lower at 26 weeks compared to the levels at 12 weeks ($P < 0.05$, $N = 19$, n (26 weeks) = 4, n (12 weeks) = 15))

DISCUSSION

After the development of tenocytes, i.e. the tendon progenitor cells, they aggregate to form characteristic tendon units and initiate the synthesis of collagenous matrix as small diameter fibrils[67]. This is the initial phase of hierarchical association of collagen, and is called fibrillogenesis[68]. This fibrillogenesis lasts on and progresses after birth, with the aggregation of collagen I molecules, leading to lateral and linear growth[24]. Thus the collagen molecules aggregate in a three step process to form the hierarchical structure of tendon. Collagen I, III and V are the major fiber forming collagens[69]. The collagen molecules initially aggregate to form immature fibril intermediates. These collagen fibril intermediates then join end to end to produce longer fibrils. Finally the lateral association takes place to increase the diameter of the fibril[70]. These two processes lead to a varying distribution of fibril diameters in adult tendons. Recent evidence suggests collagen – collagen interactions produce and regulate the architecture of the fibrils and/or fibers[69]. This is due to the association of collagen I and III[71], I and V[72], and I, III, V[73]. Collagen III is found to have a role in the initiation of fibril assembly[74]and also affects the linear growth of the fibrils and causes less fibrillar organization[75]. The association of collagen V with collagen I results in smaller fibril size[57,58]. This co-assembling explains the mechanism of regulation in the diameter and linear length of the fibers, which further characterize the mechanical strength of the tendon[69].

It is already established that the levels of collagen I and III increase during tendon healing, but there is no information on the activity of collagen V[56,57]. Our experiments were designed primarily to identify the levels of collagen I, III and V in comparison to control (normal)

and injured (open) tendons. The injured tendons were allowed to heal naturally without any pharmacological intervention for a period of 6, 12 and 26 weeks.

The decreased levels of collagen I and V in open young rabbits at 6 weeks of healing might be primarily due to the injury and pathogenesis. As the injury begins to heal new collagen molecules would be synthesized by the tenocytes so as to repair and rebuild the injured tissue. The levels of collagen I and III start to increase at 12 weeks and the levels of collagen V decrease. Collagen III is found to have a role in the initiation of fibril assembly[74], and hence, the levels of collagen III should be increased so as to favor the synthesis of new collagen fibers. As the new fibrils had formed, the levels of collagen III begin to decrease. The decrease in the levels of collagen V might be explained in young rabbits, as its association with collagen I results in smaller fibril size[57,58]. This ultimately suggests that there would be a better healing response in younger rabbits, due to the decrease in levels of collagen V, which causes no reduction in the length and diameter of collagen fibrils.

The decrease in levels of collagen I and III in old open rabbits at 6 weeks suggests the changes following the injury. The levels of collagen I, III and V start to increase in old open rabbits at 12 weeks. Collagen V levels decline at 26 weeks again. The increase in collagen I and III in old rabbits suggests a slow healing response as collagen III is associated post-injury fibrillogenesis[74,76]. The decrease in the levels of collagen V suggest the tissue adaptation to prevent the formation of small collagen fibrils as collagen V is associated with formation of smaller fibrils[57,58].

In open rabbits, the increased levels of collagen III in old rabbits compared to young suggest a delayed and ongoing fibrillogenesis and hence forth the delayed healing process. In open young as well as old rabbits, the increase in levels of collagen III and V at 12 weeks followed by a decrease at 26 weeks suggest us of the initiation of fibrillogenesis and a healing response, which is similar in both. The decrease in the level of collagen I is surprising though.

This unusual pattern in the decreasing levels of collagen I and III that totally differs and contradicts with earlier studies can be possibly due to their degradation due to prolonged storage of the harvested tissues. Additional bands were visualized in the western blots which were not accounted for, and possibly might be degradation products.

The differences in the levels of collagen in old rabbits can also be partially attributed to their poor solubility due to the Amadori re-arrangement leading to the formation of AGEs i.e advanced glycation end products like pentosidine[77-79]. This could decrease the solubility of collagen in acetic acid during our initial extraction, and might have caused to an overall lower level of extracted collagen.

The wide variability in the levels of collagen in control rabbits suggest us of the fact that there is a continuous remodeling of the tendon matrix, due to the activity of matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs), transforming growth factor- β (TGF- β) which is associated with the production of collagen, influence the proteoglycans as well as collagen[80,81]. This might also due to the increased mechanical load on the tendon because of the normal functioning of only one of the legs, the other being injured[82].

LIMITATIONS

The following are some of the limitations associated with the extraction procedure followed for quantifying the collagen types I, III and V.

Although the protocol followed for the extraction of the collagen is believed to extract all the three types of collagen i.e. I, III and V, the corresponding bands for the collagen types I, III and V in the western blots did not appear at their respective predicted sizes. The actual predicted bands of collagen I, III, and V were reported at 130, 135, and 185 kilodaltons daltons respectively[83-85]. But the bands which were visualized in our study were around 45 kilodaltons. The protocol followed for the extraction of the collagen types I, III and V needs to be further tested on pure samples of collagen I, III and V individually and together so as to identify the corresponding control bands for the collagen types I, III and V. By this, the collagen I, III and V controls can be compared with the extracted samples of collagen I, III and V, and thereby the variation in the molecular weights of these can be identified.

The collagen type I content is expected to be ~90-95% of the total collagen content, and the collagen III and V account for approximately less than 10% of the total[25-27]. In our study, there appeared to be almost identical proportions of all of the three types of collagen extracted. This might be due to the differences in the extraction levels of collagen I, III and V. The amount of the type of collagen extracted is of debate as there might be a chance that the extraction of collagen I was more efficient compared to types

III and V. This is because the protocol was primarily designed to extract collagen type I, and was optimized to extract the other minor collagens too. Collagens I and V were precipitated by a salt concentration of 0.8 M and III was precipitated at 4 M salt concentration. This differential precipitation might have led to differences in the levels of extraction of the collagens I, III and V. Since we did not run a standard curve for the western blots, or an ELISA, the amount cannot be accurately determined. In addition, the antibodies used might have been more sensitive for one type over the others. Again, these problems could result in a skewed relative determination.

The structure of collagens I, III and V are very similar to each other, having a high number of glycine, arginine, and proline residues, and hence a specific antibody for a particular type of collagen might have cross reactivity with another. The antibodies that we used to detect the collagens I, III and V were purchased to be specific to the type of collagen but the cross reactivity cannot be ruled out completely. So, the bands which appeared at 45 kilodaltons for all of the collagens I, III and V might be due to non-specific immunostaining. Alternatively, it is possible that there could be a subunit of collagen that is cleaved to 45 kDa, and which may be similar in all three types of collagen. While the pepsin used in the experiment is not supposed to cleave collagen, it might contain impurities that cause non-specific cleavage. Testing with pure collagen would give us answers to this question.

Furthermore, it is difficult to identify and quantitate the collagens effectively in tissues like tendons. As there is a lack of a reliable method to accurately identify and quantify full-length collagen molecules. Therefore, mass spectrometric analysis might help to achieve better characterization of the collagen types I, III and V, such that these

can be quantified accordingly. The problem is that even then, there might be so much overlap in sequence that specific regions might not be isolated and adequately identified. This has been particularly difficult in past studies and we did not have success during the course of this project. One problem with the digestion of the collagen is that the types of enzymes used to remove other proteins and glycosylation etc, could have been impure and caused non-specific cleavage.

The tendon tissues were dissected and excised after the treatment. There is a possibility that dissection of the surrounding tissue which might not be the actual damaged tendon tissue, but rather the undamaged surrounding tissue. This inclusion of the surrounding tissue could lead to a skewed presence of a particular type of collagen over another and explain the wide variability in the levels of collagen I, III and V. Likewise, since the injured tendon was compared with the uninjured tendon from the opposite leg, there may have been some compensation in the other leg and lack of use in the injured, causing a non-standard healing to occur.

The thermal denaturation of collagen results in the formation of gelatin molecules[86]. The tendon tissues used for this study were stored in the freezer for more than a decade. There may have been improper storage of the tendons or a thaw on the tissue at some point, which could have compromised its integrity. While this is thought to be unlikely, nothing can be certain over such an extended period of time.

CONCLUSION

Our results indicate that there is a lot of variability in the levels of collagen types I, III and V in relation to the age of the animal, injury and the healing period. However, there is no difference in the healing patterns of young and old rabbits. Yet, a reliable method for quantifying and analyzing the different types of collagen in tendons needs to be developed so as to unravel the various pathophysiological events and healing responses occurring in the tendons, for which the types of collagen are thought to be responsible and accounted for.

The fluctuation in the levels indicate that there might be a different mechanisms involved in progression and healing of the injuries in young and old rabbits. Also there might be various other factors which come into play like the MMPs and ADAMTs, during the various phases of healing which explains the wide differences in the collagen levels at different healing points.

The role of progression of a tendon injury is multifactorial as many pathways and processes come into play during and after the injury at various phases of healing process. There is a still a vast requirement for thorough elucidation and understanding of the pathophysiology and the factors involved in the tendon injury. Although the degenerative role of several MMPs and ADAMTs have been reported, yet there is very less information on the actual role and function of the various types of collagen in tendons and their response to the above factors, particularly during the healing and repair phases. Future work needs to be targeted on the identification and characterization of all the types of collagen present and involved during the pathogenesis, as well as in healing, repair and regenerative phases of tendinopathy. This information can be used to clearly understand and well establish the pathophysiology of tendinopathy, can lead to better

prognostic approach and help to device an effective pharmacological strategy so as to treat the tendon injuries.

APPENDIX - I

List of Samples and their corresponding Tissues

Sample Label	Tissue No	Age	Treatment	Weeks	Tendon Type
1	58	Old	Control	6 Weeks	Patellar
2	58	Old	Control	6 Weeks	Medial
3	58	Old	Control	6 Weeks	Tibial
4	58	Old	Open	6 Weeks	Patellar
5	58	Old	Open	6 Weeks	Medial
6	58	Old	Open	6 Weeks	Tibial
7	59	Old	Open	6 Weeks	Patellar
8	59	Old	Open	6 Weeks	Medial
9	59	Old	Open	6 Weeks	Tibial
10	59	Old	Control	6 Weeks	Patellar
11	59	Old	Control	6 Weeks	Medial
12	59	Old	Control	6 Weeks	Tibial
13	60	Old	Control	6 Weeks	Patellar
14	60	Old	Control	6 Weeks	Medial
15	60	Old	Control	6 Weeks	Tibial
16	60	Old	Open	6 Weeks	Patellar
17	60	Old	Open	6 Weeks	Medial
18	60	Old	Open	6 Weeks	Tibial
19	62	Young	Open	6 Weeks	Patellar
20	62	Young	Open	6 Weeks	Medial
21	62	Young	Open	6 Weeks	Tibial
22	62	Young	Control	6 Weeks	Patellar
23	62	Young	Control	6 Weeks	Medial
24	62	Young	Control	6 Weeks	Tibial
25	490	Old	Control	6 Weeks	Patellar
26	490	Old	Control	6 Weeks	Medial
27	490	Old	Control	6 Weeks	Tibial
28	490	Old	Open	6 Weeks	Patellar
29	490	Old	Open	6 Weeks	Medial
30	490	Old	Open	6 Weeks	Tibial
31	1461	Young	Control	6 Weeks	Patellar
32	1461	Young	Control	6 Weeks	Medial
33	1461	Young	Control	6 Weeks	Tibial

34	1461	Young	Open	6 Weeks	Patellar
35	1461	Young	Open	6 Weeks	Medial
36	1461	Young	Open	6 Weeks	Tibial
37	1462	Young	Open	6 Weeks	Patellar
38	1462	Young	Open	6 Weeks	Medial
39	1462	Young	Open	6 Weeks	Tibial
40	1462	Young	Control	6 Weeks	Patellar
41	1462	Young	Control	6 Weeks	Medial
42	1462	Young	Control	6 Weeks	Tibial
43	463	Young	Open	12 Weeks	Patellar
44	463	Young	Open	12 Weeks	Medial
45	463	Young	Open	12 Weeks	Tibial
46	463	Young	Control	12 Weeks	Patellar
47	463	Young	Control	12 Weeks	Medial
48	463	Young	Control	12 Weeks	Tibial
49	464	Young	Open	12 Weeks	Patellar
50	464	Young	Open	12 Weeks	Medial
51	464	Young	Open	12 Weeks	Tibial
52	464	Young	Control	12 Weeks	Patellar
53	464	Young	Control	12 Weeks	Medial
54	464	Young	Control	12 Weeks	Tibial
55	465	Young	Control	12 Weeks	Patellar
56	465	Young	Control	12 Weeks	Medial
57	465	Young	Control	12 Weeks	Tibial
58	465	Young	Open	12 Weeks	Patellar
59	465	Young	Open	12 Weeks	Medial
60	465	Young	Open	12 Weeks	Tibial
61	493	Old	Open	12 Weeks	Patellar
62	493	Old	Open	12 Weeks	Medial
63	493	Old	Open	12 Weeks	Tibial
64	493	Old	Control	12 Weeks	Patellar
65	493	Old	Control	12 Weeks	Medial
66	493	Old	Control	12 Weeks	Tibial
67	494	Old	Control	12 Weeks	Patellar
68	494	Old	Control	12 Weeks	Medial
69	494	Old	Control	12 Weeks	Tibial
70	494	Old	Open	12 Weeks	Patellar
71	494	Old	Open	12 Weeks	Medial
72	494	Old	Open	12 Weeks	Tibial
73	498	Old	Open	12 Weeks	Patellar
74	498	Old	Open	12 Weeks	Medial
75	498	Old	Open	12 Weeks	Tibial
76	498	Old	Control	12 Weeks	Patellar

77	498	Old	Control	12 Weeks	Medial
78	498	Old	Control	12 Weeks	Tibial
79	499	Old	Open	12 Weeks	Patellar
80	499	Old	Open	12 Weeks	Medial
81	499	Old	Open	12 Weeks	Tibial
82	499	Old	Control	12 Weeks	Patellar
83	499	Old	Control	12 Weeks	Medial
84	499	Old	Control	12 Weeks	Tibial
85	500	Old	Control	12 Weeks	Patellar
86	500	Old	Control	12 Weeks	Medial
87	500	Old	Control	12 Weeks	Tibial
88	500	Old	Open	12 Weeks	Patellar
89	500	Old	Open	12 Weeks	Medial
90	500	Old	Open	12 Weeks	Tibial
91	414	Young	Control	26 Weeks	Patellar
92	414	Young	Control	26 Weeks	Medial
93	414	Young	Control	26 Weeks	Tibial
94	414	Young	Open	26 Weeks	Patellar
95	414	Young	Open	26 Weeks	Tibial
96	415	Young	Open	26 Weeks	Patellar
97	415	Young	Open	26 Weeks	Tibial
98	415	Young	Control	26 Weeks	Patellar
99	415	Young	Control	26 Weeks	Medial
100	415	Young	Control	26 Weeks	Tibial
101	418	Young	Open	26 Weeks	Patellar
102	418	Young	Open	26 Weeks	Tibial
103	418	Young	Control	26 Weeks	Patellar
104	418	Young	Control	26 Weeks	Medial
105	418	Young	Control	26 Weeks	Tibial
106	420	Young	Control	26 Weeks	Patellar
107	420	Young	Control	26 Weeks	Medial
108	420	Young	Control	26 Weeks	Tibial
109	420	Young	Open	26 Weeks	Patellar
110	420	Young	Open	26 Weeks	Medial
111	420	Young	Open	26 Weeks	Tibial
112	422	Young	Open	26 Weeks	Patellar
113	422	Young	Open	26 Weeks	Medial
114	422	Young	Open	26 Weeks	Tibial
115	422	Young	Control	26 Weeks	Patellar
116	422	Young	Control	26 Weeks	Medial
117	422	Young	Control	26 Weeks	Tibial
118	450	Young	Control	26 Weeks	Patellar
119	450	Young	Control	26 Weeks	Medial

120	450	Young	Control	26 Weeks	Tibial
121	450	Young	Open	26 Weeks	Patellar
122	450	Young	Open	26 Weeks	Tibial
123	477	Old	Control	26 Weeks	Patellar
124	477	Old	Control	26 Weeks	Medial
125	477	Old	Control	26 Weeks	Tibial
126	477	Old	Open	26 Weeks	Patellar
127	477	Old	Open	26 Weeks	Tibial
128	483	Old	Control	26 Weeks	Patellar
129	483	Old	Control	26 Weeks	Medial
130	483	Old	Control	26 Weeks	Tibial
131	483	Old	Open	26 Weeks	Patellar
132	483	Old	Open	26 Weeks	Tibial

APPENDIX II

List of materials used

1. Reagents and chemicals:

S.No	Name	Company	Catalogue No.
1	Acetic acid	Fisher Scientific	UN2789
2	Porcine Pepsin	Sigma Chemical Company	P-7012
3	EDTA	Invitrogen Corporation	15575-038
4	Na_2HPO_4	Sigma Chemical Company	S-0876
5	NaCl	Fisher Scientific	M-11624
6	Trizma HCL	Sigma Chemical Company	T-3253
7	Tris Base	Sigma Chemical Company	T01503
8	Glycine	Research Products International Corp.	G36050-5000
9	Methanol	Fisher Scientific	A452-4
10	Tween 20	Sigma Chemical Company	P-9416
11	SDS	Bio-Rad Laboratories Inc.	161-0302
12	Blotting Grade Blocker Non-fat dry milk	Bio-Rad Laboratories Inc.	170-6404
13	Bradford reagent	Bio-Rad Laboratories Inc.	500-0006

2. Electrophoresis materials:

S.No	Name	Company	Catalogue No.
1	Criterion Tris HCL 10% – 20% precast gels	Bio-Rad Laboratories Inc.	345-0042
2	Laemmli sample buffer	Bio-Rad Laboratories Inc.	161-0737
3	Precision Plus Protein Dual Xtra Standards	Bio-Rad Laboratories Inc.	161-0377

3. Western Blot materials:

S.No	Name	Company	Catalogue No.
1	Immun-Blot PVDF Membrane Sandwiches	Bio-Rad Laboratories Inc.	162-0239
2	Collagen I Antibody	Abcam Inc.	AB6308
3	Collagen III Antibody	Novus Biologicals	NB600-594
4	Collagen V Antibody	Novus Biologicals	NBP1-05118
5	Actin Antibody	Santa Cruz Biotechnology	H-300
6	Secondary Anti-Rabbit Antibody	Thermo Scientific	32260
7	Secondary Anti-Mouse Antibody	Pierce	31430
8	Western blot substrate (West Femto)	Pierce	34080

APPENDIX III

Total Collagen Concentrations of samples

Sample Label	OD Values	Mean Total Collagen Conc. per 10ul
1	0.134	0.176
2	0.131	-0.047
3	0.136	0.291
4	0.149	1.188
5	0.145	0.925
6	0.141	0.682
7	0.141	0.628
8	0.145	0.904
9	0.137	0.372
10	0.136	0.324
11	0.135	0.25
12	0.137	0.385
13	0.136	0.331
14	0.142	0.702
15	0.143	0.763
16	0.139	0.533
17	0.145	0.945
18	0.143	0.823
19	0.142	0.749
20	0.147	1.087
21	0.14	0.594
22	0.14	0.621
23	0.141	0.648
24	0.134	0.183
25	0.135	0.237
26	0.138	0.432
27	0.136	0.351
28	0.148	1.107
29	0.147	1.046
30	0.147	1.06
31	0.134	0.189
32	0.136	0.338
33	0.14	0.587
34	0.138	0.439

35	0.153	1.437
36	0.142	0.743
37	0.141	0.675
38	0.159	1.883
39	0.156	1.64
40	0.157	1.721
41	0.147	1.06
42	0.152	1.41
43	0.151	1.019
44	0.164	1.944
45	0.173	2.575
46	0.144	0.553
47	0.142	0.395
48	0.147	0.747
49	0.145	0.611
50	0.213	5.442
51	0.159	1.614
52	0.139	0.173
53	0.137	0.051
54	0.157	1.442
55	0.145	0.582
56	0.14	0.216
57	0.163	1.901
58	0.167	2.152
59	0.171	2.438
60	0.151	1.041
61	0.183	3.292
62	0.273	9.736
63	0.173	2.596
64	0.144	0.524
65	0.14	0.209
66	0.138	0.123
67	0.134	-0.207
68	0.138	0.109
69	0.138	0.123
70	0.158	1.492
71	0.153	1.141
72	0.152	1.076
73	0.159	1.571
74	0.167	2.173
75	0.171	2.446
76	0.139	0.173
77	0.143	0.424

78	0.141	0.317
79	0.168	2.238
80	0.158	1.499
81	0.227	6.474
82	0.144	0.496
83	0.147	0.711
84	0.139	0.18
85	0.137	0.008
86	0.146	0.661
87	0.14	0.209
88	0.163	1.858
89	0.159	1.6
90	0.163	1.908
91	0.138	0.032
92	0.143	0.548
93	0.149	1.174
94	0.148	1.055
95	0.158	1.988
96	0.154	1.591
97	0.153	1.521
98	0.143	0.548
99	0.141	0.32
100	0.146	0.836
101	0.157	1.948
102	0.161	2.315
103	0.153	1.491
104	0.148	1.015
105	0.155	1.7
106	0.142	0.409
107	0.142	0.39
108	0.15	1.243
109	0.158	2.017
110	0.156	1.829
111	0.162	2.395
112	0.161	2.335
113	0.153	1.571
114	0.147	0.926
115	0.153	1.491
116	0.148	0.995
117	0.172	3.457
118	0.159	2.087
119	0.17	3.238
120	0.18	4.191

121	0.153	1.511
122	0.156	1.819
123	0.151	1.342
124	0.143	0.519
125	0.153	1.561
126	0.158	1.998
127	0.157	1.888
128	0.142	0.419
129	0.142	0.479
130	0.143	0.509
131	0.195	5.66
132	0.167	2.931

APPENDIX IV

Densitometry Quantification Values

Sample Label	ACTIN	COL I	COL III	COL V
1	-479,898.67	-614,845.33	-74,869.33	-717,418.67
2	732,018.67	1,582,209.33	1,223,349.33	764,072.00
3	502,436.00	995,394.67	695,893.33	409,178.67
4	591,066.67	920,205.33	672,148.00	556,558.67
5	619,454.67	1,051,217.33	797,745.33	514,154.67
6	734,448.00	1,767,666.67	639,794.67	500,126.67
7	764,940.00	1,461,802.67	450,400.00	268,110.67
8	723,325.33	848,446.67	284,933.33	339,049.33
9	1,068,222.67	1,320,400.00	734,925.33	524,322.67
10	788,930.67	1,307,800.00	559,092.00	545,638.67
11	598,642.67	591,976.00	297,876.00	205,881.33
12	176,806.67	666,669.33	404,872.00	211,492.00
13	72,685.33	2,250,842.67	993,874.67	-26,989.33
14	281,190.67	1,022,113.33	184,528.00	263,616.00
15	470,594.67	945,352.00	433,381.33	131,094.67
16	698,748.00	1,475,080.00	457,993.33	630,300.00
17	848,241.33	981,153.33	655,990.67	427,742.67
18	882,893.33	1,278,706.67	596,148.00	353,409.33
19	1,019,622.67	2,048,338.67	510,689.33	684,772.00
20	1,020,274.67	1,938,053.33	685,738.67	506,917.33
21	743,868.00	1,955,786.67	771,278.67	127,794.00
22	788,637.33	1,803,237.33	335,484.00	436,545.33
23	644,576.00	1,514,141.33	538,033.33	277,937.33
24	-119,894.67	87,377.33	-48,608.00	-518,360.00
25	895,873.33	796,856.00	999,769.33	149,277.33
26	444,142.67	1,393,194.67	254,409.33	223,496.00
27	961,374.67	2,050,598.67	1,091,230.67	450,430.67
28	1,293,518.67	1,344,437.33	824,404.00	369,656.00
29	880,297.33	1,688,036.00	1,273,477.33	345,576.00
30	497,677.33	1,440,144.00	906,181.33	395,012.00
31	498,682.67	1,820,068.00	988,230.67	318,761.33
32	83,310.67	533,480.00	320,657.33	-24,864.00
33	238,602.67	1,216,754.67	695,549.33	187,822.67
34	637,669.33	2,319,844.00	1,136,393.33	448,305.33

35	605,316.00	2,470,000.00	1,242,009.33	303,872.00
36	778,934.67	2,111,405.33	1,459,225.33	2,549.33
37	568,757.33	1,209,946.67	1,000,048.00	115,901.33
38	594,350.67	1,413,241.33	1,066,154.67	385,169.33
39	374,688.00	1,706,170.67	727,596.00	428,244.00
40	19,389.33	184,454.67	41,277.33	84,585.33
41	30,918.67	191,125.33	-16,724.00	24,408.00
42	493,681.33	1,204,692.00	624,066.67	323,928.33
43	593,570.67	1,955,188.00	1,049,098.67	251,828.00
44	705,880.00	2,346,084.00	1,278,106.67	607,472.00
45	690,560.00	1,941,204.00	930,345.33	345,142.67
46	104,302.67	312,274.67	169,325.33	165,172.33
47	476,956.00	709,697.33	712,568.00	244,754.67
48	481,100.00	773,200.00	645,482.67	117,390.67
49	412,437.33	964,509.33	2,080,028.00	901,021.33
50	333,142.67	841,190.67	741,396.00	859,822.67
51	355,241.33	614,588.00	573,688.00	879,282.67
52	366,949.33	810,804.00	803,648.00	1,177,933.33
53	160,104.00	282,225.33	361,226.67	675,268.00
54	424,205.33	699,294.67	757,453.33	1,383,376.00
55	352,589.33	590,918.67	879,420.00	944,377.33
56	356,901.33	571,018.67	955,674.67	1,120,698.67
57	550,130.67	662,502.67	879,677.33	1,011,652.00
58	596,693.33	786,560.00	911,229.33	1,266,993.33
59	346,914.67	615,354.67	837,569.33	1,033,609.33
60	216,313.33	679,353.33	738,176.00	747,062.67
61	602,085.33	889,045.33	1,838,580.00	1,522,478.33
62	621,880.00	1,172,806.67	1,486,442.67	2,164,034.00
63	353,633.33	594,180.00	1,065,497.33	1,213,718.67
64	340,757.33	726,554.67	773,096.00	835,804.00
65	314,620.00	372,201.33	539,268.00	781,648.00
66	416,060.00	659,478.67	787,597.33	1,439,678.67
67	99,805.33	348,465.33	470,077.33	428,809.33
68	199,852.00	476,038.67	499,706.67	676,273.33
69	231,038.67	470,862.67	699,294.67	637,046.67
70	490,637.33	968,482.67	1,429,218.67	1,239,568.00
71	448,750.67	1,017,349.33	1,662,010.67	1,373,232.00
72	185,124.00	673,529.33	1,058,454.67	1,050,982.67
73	184,881.33	515,958.67	1,639,158.67	1,084,376.33
74	474,854.67	1,266,912.00	1,418,766.67	1,309,736.00
75	342,074.67	1,241,704.00	1,581,102.67	1,160,968.00
76	431,344.00	658,188.00	550,530.67	1,009,850.67
77	399,776.00	841,102.67	669,686.67	1,139,548.00

78	347,610.67	740,664.00	520,944.00	1,059,052.00
79	475,049.33	1,210,141.33	757,653.33	1,531,792.00
80	474,489.33	1,372,222.67	715,718.67	1,726,925.33
81	520,229.33	2,238,320.00	1,028,677.33	1,992,462.67
82	380,702.67	892,809.33	614,917.33	1,228,994.67
83	276,004.00	696,708.00	603,816.00	1,419,170.67
84	242,948.00	748,428.00	359,577.33	1,405,902.67
85	300,182.67	515,129.33	1,191,237.33	39,438.67
86	243,429.33	314,212.00	644,976.00	379,882.67
87	453,974.67	882,096.00	1,280,277.33	835,405.33
88	374,288.00	850,237.33	1,297,482.67	879,418.67
89	253,616.00	382,762.67	690,326.67	859,348.00
90	511,122.67	669,768.00	845,366.67	1,021,469.33
91	184,832.00	313,162.67	483,516.00	775,992.00
92	383,376.00	519,112.00	724,592.00	914,474.67
93	337,624.00	515,928.00	619,768.00	993,752.00
94	430,210.67	662,454.67	749,334.67	1,002,048.00
95	484,494.67	784,158.67	691,194.67	924,609.33
96	357,064.00	789,753.33	23,413.33	676,352.00
97	2,306,412.00	969,738.67	2,354,344.00	902,549.33
98	636,834.67	407,176.00	1,112,781.33	417,409.33
99	1,196,380.00	393,486.67	1,067,649.33	601,229.33
100	1,675,602.67	841,934.67	1,381,625.33	805,058.67
101	1,955,074.67	996,889.33	1,728,340.00	997,788.00
102	1,392,437.33	1,439,692.00	1,576,070.67	980,670.67
103	387,122.67	445,612.00	691,088.00	522,992.00
104	829,938.67	556,756.00	519,668.00	430,788.00
105	1,182,552.00	867,428.00	1,421,660.00	625,093.33
106	1,160,841.33	1,259,949.33	1,571,598.67	822,264.00
107	1,039,345.33	718,858.67	1,256,080.00	628,196.00
108	1,091,746.67	605,468.00	1,708,868.00	461,900.00
109	1,253,406.67	569,529.33	1,263,438.67	544,442.67
110	1,370,204.00	1,218,300.00	769,472.00	859,552.00
111	1,138,882.67	912,070.67	536,829.33	793,580.00
112	1,013,766.67	1,029,568.00	652,676.00	748,730.67
113	792,741.33	716,186.67	491,389.33	531,004.00
114	1,052,257.33	869,094.67	544,482.67	560,133.33
115	685,122.67	319,389.33	690,749.33	227,941.33
116	603,362.67	384,929.33	350,424.00	234,061.33
117	660,601.33	403,282.67	975,932.00	383,089.33
118	1,092,217.33	1,022,422.67	804,968.00	593,012.00
119	976,196.00	801,512.00	660,289.33	731,285.33
120	966,776.00	638,740.00	625,601.33	724,797.33

121	548,641.33	265,210.67	496,337.33	101,509.33
122	666,249.33	727,714.67	873,988.00	125,300.00
123	198,389.33	183,738.67	372,892.00	70,521.33
124	498,837.33	812,222.67	666,896.00	206,165.33
125	249,234.67	331,998.67	556,142.67	39,616.00
126	552,281.33	1,234,245.33	1,135,684.00	418,456.00
127	600,849.33	1,382,412.00	1,407,977.33	727,977.33
128	594,008.00	964,920.00	1,043,028.00	366,898.67
129	484,461.33	707,130.67	724,502.67	215,022.67
130	668,458.67	1,179,816.00	1,112,232.00	388,957.33
131	39,765.33	59,388.00	75,598.67	-101,193.33
132	777,066.67	1,873,604.00	1,079,477.33	404,696.00

APPENDIX V

Normalized Collagen Values

Sample Label	Normalized COL 1	Normalized COL III	Normalized COL V
1	1.281198	0.156011	1.494938
2	2.161433	1.6712	1.043788
3	1.981137	1.385039	0.81439
4	1.556855	1.137178	0.941617
5	1.697004	1.287819	0.830012
6	2.406796	0.871123	0.680956
7	1.911003	0.588804	0.350499
8	1.172981	0.393921	0.468737
9	1.236072	0.687989	0.490836
10	1.657687	0.708671	0.691618
11	0.988864	0.497586	0.343914
12	3.770612	2.289914	1.196177
13	30.96695	13.67366	-0.37132
14	3.634948	0.656238	0.937499
15	2.008846	0.920923	0.278572
16	2.111033	0.655449	0.902042
17	1.156691	0.773354	0.50427
18	1.448314	0.675221	0.400285
19	2.008918	0.500861	0.671594
20	1.899541	0.672112	0.496844
21	2.629212	1.036849	0.171797
22	2.286523	0.425397	0.553544
23	2.34905	0.834709	0.431194
24	-0.72878	0.405423	4.323462
25	0.889474	1.115972	0.166628
26	3.136818	0.57281	0.503208
27	2.132986	1.135073	0.468528
28	1.039364	0.637334	0.285776
29	1.917575	1.446645	0.392567
30	2.89373	1.820821	0.793711
31	3.649752	1.981682	0.639207
32	6.403501	3.848935	-0.29845
33	5.099501	2.915094	0.787178
34	3.638005	1.782104	0.703037

35	4.080513	2.051836	0.502006
36	2.710632	1.87336	0.003273
37	2.127351	1.758303	0.20378
38	2.37779	1.793814	0.648051
39	4.553577	1.941872	1.142935
40	9.513205	2.128868	4.362468
41	6.181551	-0.5409	0.789426
42	2.440222	1.264108	0.656149
43	3.293943	1.767437	0.42426
44	3.32363	1.810657	0.860588
45	2.811058	1.347233	0.499801
46	2.993928	1.623404	1.583587
47	1.487972	1.493991	0.51316
48	1.60715	1.341681	0.244005
49	2.33856	5.043258	2.184626
50	2.525016	2.225461	2.580944
51	1.730058	1.614925	2.47517
52	2.20958	2.190079	3.210071
53	1.762763	2.2562	4.217684
54	1.648482	1.785582	3.2611
55	1.67594	2.494176	2.678406
56	1.599934	2.6777	3.14008
57	1.204264	1.599033	1.83893
58	1.318198	1.527132	2.123358
59	1.773793	2.414338	2.979434
60	3.140599	3.412531	3.453614
61	1.47661	3.053687	2.528675
62	1.885905	2.39024	3.479826
63	1.680215	3.013	3.432139
64	2.132176	2.268758	2.452784
65	1.183019	1.71403	2.484419
66	1.585057	1.89299	3.460267
67	3.49145	4.709942	4.296457
68	2.381956	2.500384	3.383871
69	2.038025	3.026743	2.757316
70	1.973928	2.912984	2.526445
71	2.26707	3.703639	3.060122
72	3.63826	5.717544	5.677182
73	2.790756	8.866004	5.865256
74	2.667999	2.987791	2.758183
75	3.629921	4.622098	3.393902
76	1.5259	1.276315	2.341172
77	2.103935	1.675155	2.850466

78	2.130729	1.498642	3.046661
79	2.547401	1.594894	3.22449
80	2.891999	1.508398	3.639545
81	4.302564	1.977354	3.82997
82	2.345162	1.615217	3.228227
83	2.524268	2.187707	5.141848
84	3.08061	1.480059	5.786846
85	1.716053	3.968375	0.131382
86	1.290773	2.649541	1.560546
87	1.943051	2.820151	1.840203
88	2.271613	3.466536	2.349578
89	1.509221	2.721937	3.388382
90	1.310386	1.653941	1.998482
91	1.69431	2.615976	4.198364
92	1.354055	1.89003	2.385321
93	1.528114	1.835675	2.943369
94	1.539838	1.741785	2.329203
95	1.618508	1.42663	1.908399
96	2.211798	0.065572	1.894204
97	0.420453	1.020782	0.391322
98	0.639375	1.747363	0.655444
99	0.328898	0.8924	0.50254
100	0.502467	0.824554	0.480459
101	0.509898	0.884028	0.510358
102	1.033937	1.131879	0.704284
103	1.151087	1.785191	1.350972
104	0.67084	0.626152	0.51906
105	0.733522	1.202197	0.528597
106	1.085376	1.353845	0.708335
107	0.691646	1.20853	0.604415
108	0.554587	1.565261	0.423083
109	0.454385	1.008004	0.43437
110	0.889138	0.561575	0.627317
111	0.800847	0.471365	0.696806
112	1.015587	0.643813	0.738563
113	0.90343	0.619861	0.669833
114	0.825934	0.517443	0.532316
115	0.466178	1.008213	0.332701
116	0.637973	0.580785	0.387928
117	0.610478	1.477339	0.57991
118	0.936098	0.737004	0.542943
119	0.821056	0.67639	0.749117
120	0.660691	0.647101	0.749706

121	0.483395	0.904666	0.185019
122	1.092256	1.311803	0.188068
123	0.926152	1.879597	0.355469
124	1.628232	1.336901	0.413292
125	1.332073	2.231402	0.158951
126	2.234813	2.056351	0.757686
127	2.300763	2.343312	1.21158
128	1.624423	1.755916	0.617666
129	1.459623	1.495481	0.443839
130	1.76498	1.663875	0.581872
131	1.493462	1.90112	-2.54476
132	2.411124	1.38917	0.5208

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