

A Comparison of the Histochemical and Image-Derived Proteoglycan Content of Articular Cartilage

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Abstract

There are several methods for determining the proteoglycan content of cartilage in biomechanics experiments. Many of these include assay-based methods and the histochemistry or spectrophotometry protocol where quantification is biochemically determined. More recently a method based on extracting data to quantify proteoglycan content has emerged using the image processing algorithms, e.g., in ImageJ, to process histological micrographs, with advantages including time saving and low cost. However, it is unknown whether or not this image analysis method produces results that are comparable to those obtained from the biochemical methodology. This paper compares the results of a well-established chemical method to those obtained using image analysis to determine the proteoglycan content of visually normal ($n=33$) and their progressively degraded counterparts with the protocols. The results reveal a strong linear relationship with a regression coefficient (R^2) = 0.9928, leading to the conclusion that the image analysis methodology is a viable alternative to the spectrophotometry.

Keywords: Articular cartilage; Proteoglycans quantification; Spectrophotometry; Image analysis

Introduction

Biochemical assay analyses have been commonly applied to determine the proteoglycan quantity of articular cartilage in laboratory and clinical research [1,2]. While these methods have produced reliable results over the years, they all include the destruction of the entire sample and time-consuming laboratory work. Recently, image analysis-based protocols have been suggested and are gaining increased usage in biomechanical experiments [3-7] since they only require a very small biopsy rather than destruction of the entire sample, enabling their capacity to support experiments where progressive degradation of cartilage specimens is required. In this paper we observe that while this image analysis methodology [7] can deliver benefits over and above the biochemical or assay-based methods, there still remains the necessity to establish its accuracy and relationship to the well-established and extensively tested protocols [4,5]. To this extent we propose to determine whether or not there is a relationship between the data from image analysis and a standard protein (proteoglycan) quantification method.

Several methods are available for protein quantification, including HPLC-based assay, carbazole assay, and colorimetric assays techniques such as Alcian Blue assay, BCA, and Bradford. However, these methods are either limited in their sensitivity, time-consuming, or expensive [8,9]. Direct micro-volume quantification of protein via UV-Vis absorbance was employed in this study using the NanoDrop™ spectrophotometer. This method was adopted because it requires very small sample volume (1 – 2 μ L), has high degree of reproducibility and accuracy, and the speed of analysis is extremely fast [10,11].

Visually normal articular cartilage samples taken from the same joints are known to display varying quantities of their microconstituents, namely, collagen, proteoglycans and cells [7], where proteoglycans and cell are extractable from the matrix by enzymatic and biochemical manipulations. We exploited the proteoglycan removal protocol in this paper and determined the remaining content in the tissue as cartilage specimens were progressively degraded. Proteoglycan quantification

was conducted at every stage with both spectrophotometric and image analysis methods. The experimental data were then subjected to statistical analysis to determine the correlation between the results of image analysis and spectrophotometry/histochemical quantification of the proteoglycan content in the normal intact and progressively enzymatically modified cartilage.

Materials and Methods

Sample preparation

Visually intact normal bovine patellae samples ($n = 12$), harvested from prime oxen within 24 hr of slaughter, were used in this study. The patellae were wrapped in 0.15M saline soaked towels and stored at -20°C until required for testing. Prior to testing, the intact patellae were thawed in 0.15M saline at room temperature for 6 hours, then cartilage-on-bone blocks ($n = 33$, $l \times b \times h = 10 \times 10 \times 5$ mm) were extracted from the patellae. All tests were conducted with the specimens fully hydrated in 0.15M saline.

Proteoglycan-depletion protocol

After extraction of an initial 7 μ m lateral surface-to-bone osteochondral slice, each normal sample was incubated in trypsin (T4667, Sigma Aldrich, Sydney, Australia) of 1 mg/ml concentration in 0.15 M phosphate buffered saline solution at 37°C to remove proteoglycans. The proteoglycan depletion program was performed for

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4 hrs, with 7 µm lateral surface-to-bone osteochondral slices extracted at intervals of 1hr. The slices from normal and at each time interval were prepared for histology and proteoglycan content evaluation (Figure 1). The gradual progression of enzymatic action from surface to bone closely resembles the pattern of proteoglycan loss observed in the initial stages of osteoarthritis [12]. Trypsin is a serine protease which cleaves peptides on the C-terminal side of lysine and arginine amino acid residues, and it has also been reported to cause minor degradation to already cleaved collagen fibrils [13], which contains three possible sites for trypsin cleavage.

Histochemistry vs. Image processing

Image processing and analysis to determine the quantity of proteoglycans inside cartilage is not the “gold standard” protocol; hence, it is necessary to compare its result with accepted histochemically derived data. Kiviranta et al. [5] established a semi-quantitative histochemical estimation of glycosaminoglycans by microspectrophotometry based on the stoichiometric staining of proteoglycans. Moody et al. [7] and Afara et al. [3] adapted this method for quantifying the proteoglycan content in Safranin-O stained samples of articular cartilage by processing the stained image with ImageJ software (Wayne Rasband, National Institute of Health, USA). Safranin-O stain was used in this study because it has been previously shown to be an excellent cationic dye for histochemical quantification of cartilage matrix proteoglycans, since the staining intensity curves show high linear correlation ($r = 0.900-0.995$) with the fixed charge density curves from the adjacent cartilage [4]. Also, the stain distribution was consistently uniform across the sections, and in 1 microns-thick glycolmethacrylate sections, the Safranin O staining gradient showed almost perfect identity with the fixed-charge density curve [4].

To validate the assumption that the area under the absorbance profile of the stained tissue micrograph is highly correlated to the proteoglycan content in the tissue, we compare the parameter from image analysis with results obtained from a standard protein quantification method. We determined the quantities of proteoglycans

extracted from cartilage samples using the well-established micro-volume protein quantification method via a NanoDrop™ spectrophotometer, and stained the samples from which proteoglycans were extracted for image analysis. The quantities of proteoglycans (mg/ml) were then plotted against the relevant data from ImageJ analysis. The experimental protocol is as follows.

Image analysis and quantification of proteoglycan content:

In order to quantify proteoglycan depletion over time, a small biopsy was removed from the samples at hourly intervals (Figure 1). This was then cryosectioned and stained with Safranin-O, using a previously published staining regime [7]. Optical absorbance provided visualisation and a relative indicator of the probable amount of proteoglycan concentration and distribution. This method was based on the stoichiometric binding of Safranin-O to chondroitin 6-sulphate and keratan sulphate that has previously been validated by biochemical comparison in solution [1] and in cartilage tissue sections [4,5]. Linearity of this relationship between dye and proteoglycan was maintained through alcohol fixation to transform Safranin-O to orthochromatic form [1,4]. This was followed by absorbance profiling under monochromatic light source using a Nikon Labo-Phot light microscope to obtain micrographic images of each sections before and after staining.

Image analysis to determine the proteoglycan content was performed using a custom-written macro (plugin) in ImageJ software, version 1.45k (Wayne Rasband, NIH, USA), according to protocols observed by Moody [7] and Brown [14]. As previously established that there is a linear correlation between Safranin-O concentration and absorbance based on Beer-Lambert’s law [7], the unstained negative image was captured and subtracted from the Safranin-O stained tissue image in the exact location-orientation and processed on a per pixel basis. This determined the absorbance of Safranin-O and therefore proteoglycan concentration and distribution as an absorbance-depth profile [4,7]. The content in the depleted sample was expressed as a percentage of the amount remaining in the tissue after depletion relative to the amount in its corresponding normal tissue.

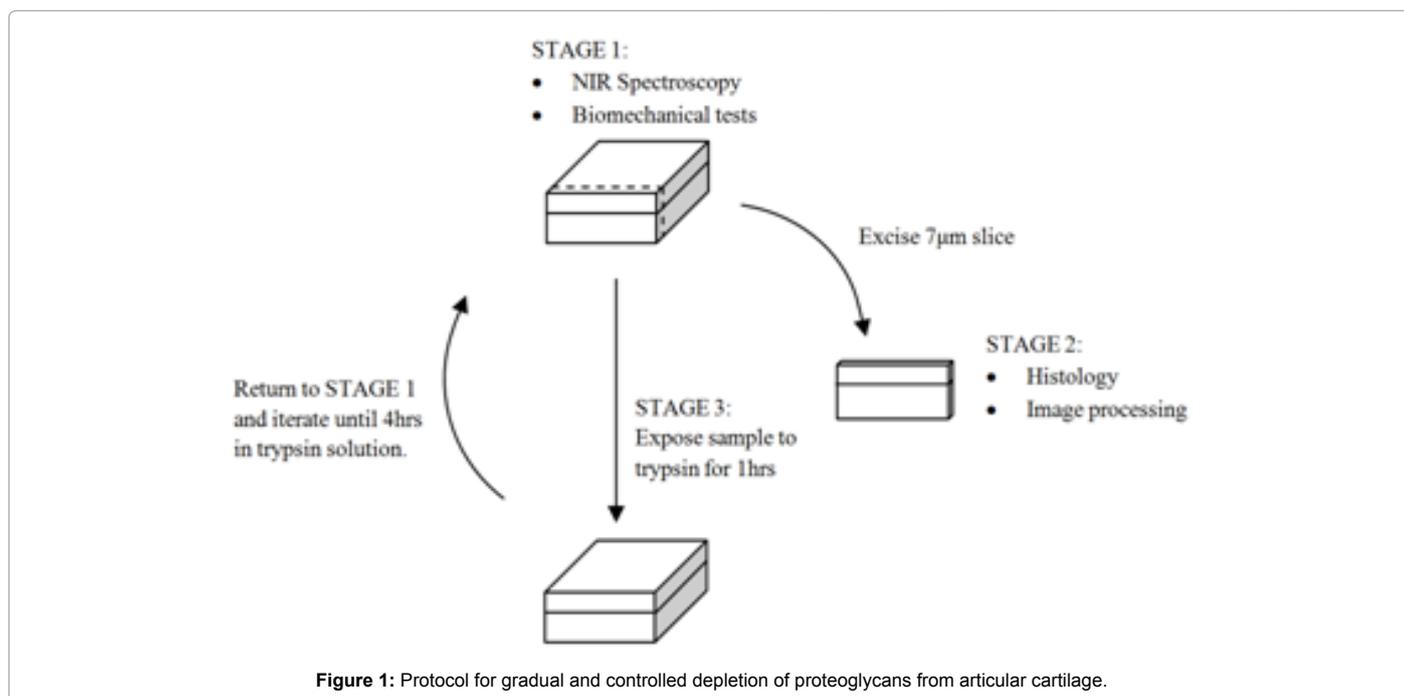


Figure 1: Protocol for gradual and controlled depletion of proteoglycans from articular cartilage.

To avoid differences in exposure time, all camera settings and variables were kept constant for all samples as they were software controlled. Lights were turned off in the room and windows blacked out so as to avoid stray lights. Furthermore, to account for variations caused by factors other than those resulting from changes in PG content, the plugin was applied to multiple regions (from surface-to-bone) per slice for several slices from the same sample, and the average of the image-analysis results was used as representative value for the sample.

Spectrophotometric quantification of proteoglycan: Ten osteochondral block samples of dimension ($l \times b \times h = 15 \times 15 \times 10$ mm) were obtained from two different patellae and subjected to the proteoglycan depletion protocol shown in (Figure 1). After incubation in trypsin solution for known amounts of time (hourly intervals for 4hrs), 7 μ m section was extracted from each sample for histological and image analysis. A minute quantity of the trypsin incubated (enzymatic) solution was also extracted for biochemical analysis to determine the concentration of proteoglycan (protein) released into the solution as a result of enzymatic digestion. For quantification of proteoglycan concentration in mg/ml, a standard 2 μ l of the extracted solution was placed in a NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE USA) and its concentration measured at an absorbance of 280nm. The use of NanoDrop spectrophotometers (Thermo Scientific) for nucleic acid quantification is well established in the life sciences, and are also capable of quantifying proteins at 280nm with high degree of accuracy and reproducibility [11]. The NanoDrop spectrophotometer was adopted in this study because it uses the UV-Vis absorbance A280 module which is based on the principle that

proteins that contain Trp, Tyr residues or Cys-Cys disulphide bonds (present in proteoglycan macromolecules) will absorb in the UV range (i.e. 280 nm) making absorbance spectroscopy a fast (approx. 5secs), convenient method for protein quantification, as opposed to other assay methods including colorimetric assays techniques such as BCA, Bradford and Lowry [10]. The method generally measures the total amount of protein in the solution that absorbs at 280nm, hence the total amount of PG in the solution. Trypsin is specific to PGs, and has minimal effect on the collagen meshwork of articular cartilage [13]. The size of sections extracted from the sample, and the solution taken at hourly intervals were chosen such that their effect on the overall degradation process was minimal or negligible.

Statistical analysis

The data obtained from both spectrophotometric and image analyses were expressed as mean \pm 95% confidence interval (CI) and were compared using one-way ANOVA, a p-value of less than 0.05 was considered to be statistically significant. The normal distribution assumption, required for the application of ANOVA, was tested and passed for the group of samples used in this study. All analyses were performed on Graphpad Prism statistics software (version 5.0).

Results

Figure 2a-e presents the Safranin-O stained histological micrographs for normal intact and progressively degraded cartilage samples, and their corresponding ImageJ image analysis results. The area under the absorbance profile was taken as an approximate measure of the proteoglycan content in the tissue. This was calculated and

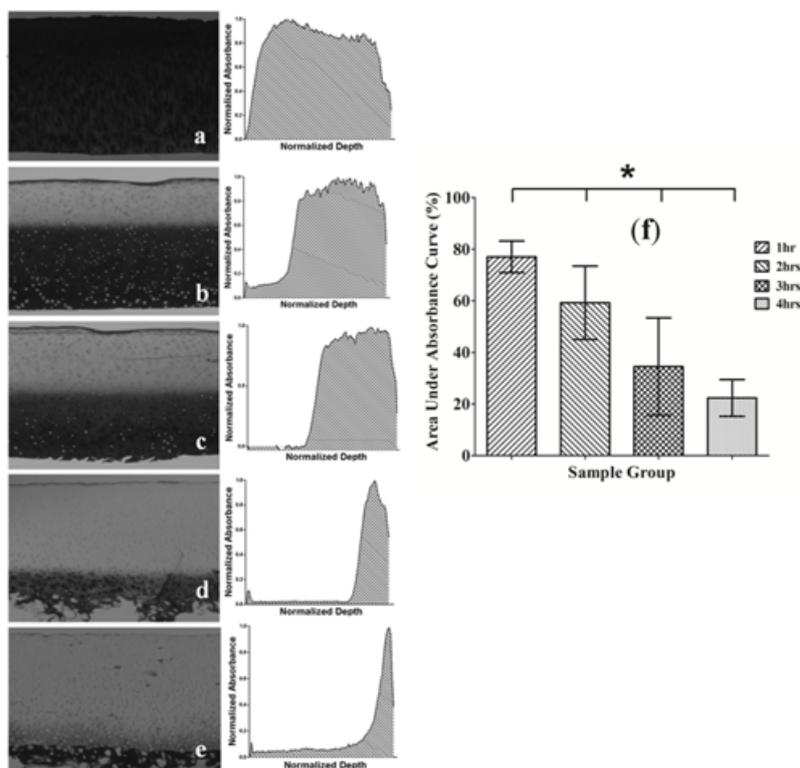


Figure 2: Safranin-O stained histological micrographs and corresponding ImageJ absorbance profiles of cartilage samples. (a) Normal, (b) 1hr trypsin treated, (c) 2hrs trypsin treated, (d) 3hrs trypsin treated, (e) 4hrs trypsin treated. The area under each curve is indicative of the proteoglycan content in the tissue. (f) Distribution of percentage area under absorbance curve for proteoglycan-depleted cartilage samples relative to their corresponding normal, taken as 100% and not shown (* p<0.0001)

expressed as percentage relative to the area under the absorbance curve for the corresponding normal tissue, thus also providing information on the relative measure of how much proteoglycan is left in the sample and how much has been removed. A distribution of the area under the absorbance profile is presented in the bar plot of Figure 2f.

Similarly, the proteoglycan content measured spectrophotometrically was obtained for each sample grouped by exposure time (Figure 3a). It is worth noting that from preliminary laboratory work, incubation of cartilage samples in trypsin solution for a minimum of 6 hrs is sufficient for complete removal of proteoglycan from the sample. Hence for complete proteoglycan removal, samples were exposed for 24 hrs. The samples tested, i.e. the osteochondral block, yielded between 5.5 and 6.7 mg/ml proteoglycans after 24 hrs of enzymatic digestion in the trypsin bath. Direct comparison of the amount of proteoglycan released into the bath solution and the size (volume) of tissue subjected to digestion yielded an average of 4.2 µg of proteoglycans in each 7 µm slice. This provides useful data for representing the amount of proteoglycans in percentage (Figure 3b).

As earlier proposed, the area under the ImageJ absorbance curves (Figure 2a-e) represents the quantity of proteoglycan remaining in a given sample, and is also related to the amount removed. To address any doubt, this was validated against the quantities derived spectrophotometrically. The percentage area under the absorbance profile obtained from image analysis (with the normal cartilage samples expressed as 100%), was then plotted against the measured proteoglycan content, both in mg/ml and percentage, as shown in (Figure 4) below. The average deviation between both methods was calculated as the difference between their percentage values for the different sample groups tested (Figure 4b).

Discussion and Conclusion

In this study, we have validated the capacity of image analysis method to accurately determine the proteoglycan content of stained histological section of articular cartilage micrographs using a well-established biochemical method of determining the protein content in solution. Spectrophotometric method to assess the proteoglycan released into the solution was adopted because of its fast, accurate and reproducible outcome. The comparison between the spectrophotometric and the image analysis methods presented a highly linear relationship (Figure 4) between the measured proteoglycan content (from the histochemical

Nanodrop tests) and the area under the absorbance profile of the stained sample micrographs (i.e. ImageJ data). We argue that this result is a categorical validation of the feasibility and efficacy of the application of image-analysis in the determination of the proteoglycan content of articular cartilage samples. This provides a means of eliminating time consuming, expensive, and sample destructive techniques in the estimation of proteoglycan content, such as those involved in assay-based quantification methods.

It is worth noting that this method was validated for Safranin-O, which is a cationic dye that binds stoichiometrically to mucopolysaccharides (GAGs in PGs), that is, one positively charged dye molecule binds to one negatively charged carboxyl or sulphate group [1,4,5,15]. Due to the inefficiency of other cationic dyes for histochemical quantification of cartilage matrix proteoglycans, the image analysis result may yield a different and less accurate correlation with spectrophotometric data. Although the proteoglycan content in this study was artificially removed, the method is arguably applicable to naturally degraded (osteoarthritic) samples since during degenerative changes, the glycosaminoglycans (GAG) and protein core are cleaved at many sites by MMP's and aggrecanases. The broken fragments may then denature or be expelled from the matrix by the efflux of water during compression. The cartilage will therefore exhibit a decrease in proteoglycan concentration, hence a decrease in the Safranin-O staining. Therefore osteoarthritic degeneration of the GAG is unlikely to affect the read-out and hence the image analysis results.

Furthermore, the correlation (Figure 4) obtained in the present research can be used to provide an approximate value of the proteoglycan content of a sample in mg/ml when this is the preferred option. The reference value of 4.2 µg of proteoglycans in a representative stained section, obtained by direct comparison of the amount of proteoglycan released and the size of tissue subjected to digestion, can be used to express in percentage (Figure 3) the proteoglycan content of sections after depletion, thereby removing the time-based relationship and leading to a direct correlation of image-analysis (absorbance) and histochemical proteoglycan content data. This correlation also presents a highly linear relationship with the image analysis results (Figure 4). The potential laboratory significance of this is that qualitative information from the stained micrograph (stain intensity and variation in the tissue) can be quantified and expressed as actual and percentage proteoglycan content in the tissue (Figure 4).

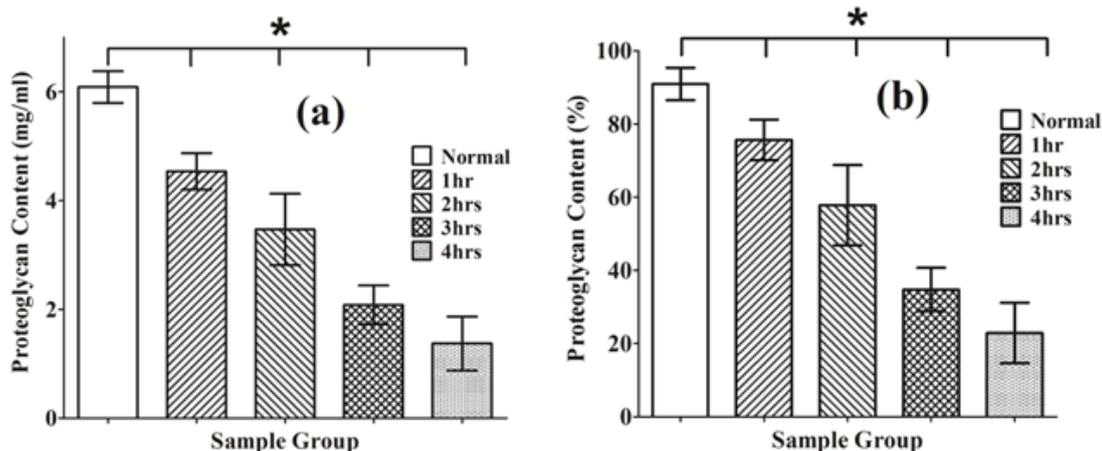


Figure 3: (a) Distribution of proteoglycan content from spectrophotometric analysis of samples expressed in (a) mg/ml, and (b) percentage (%). (* $p < 0.0001$).

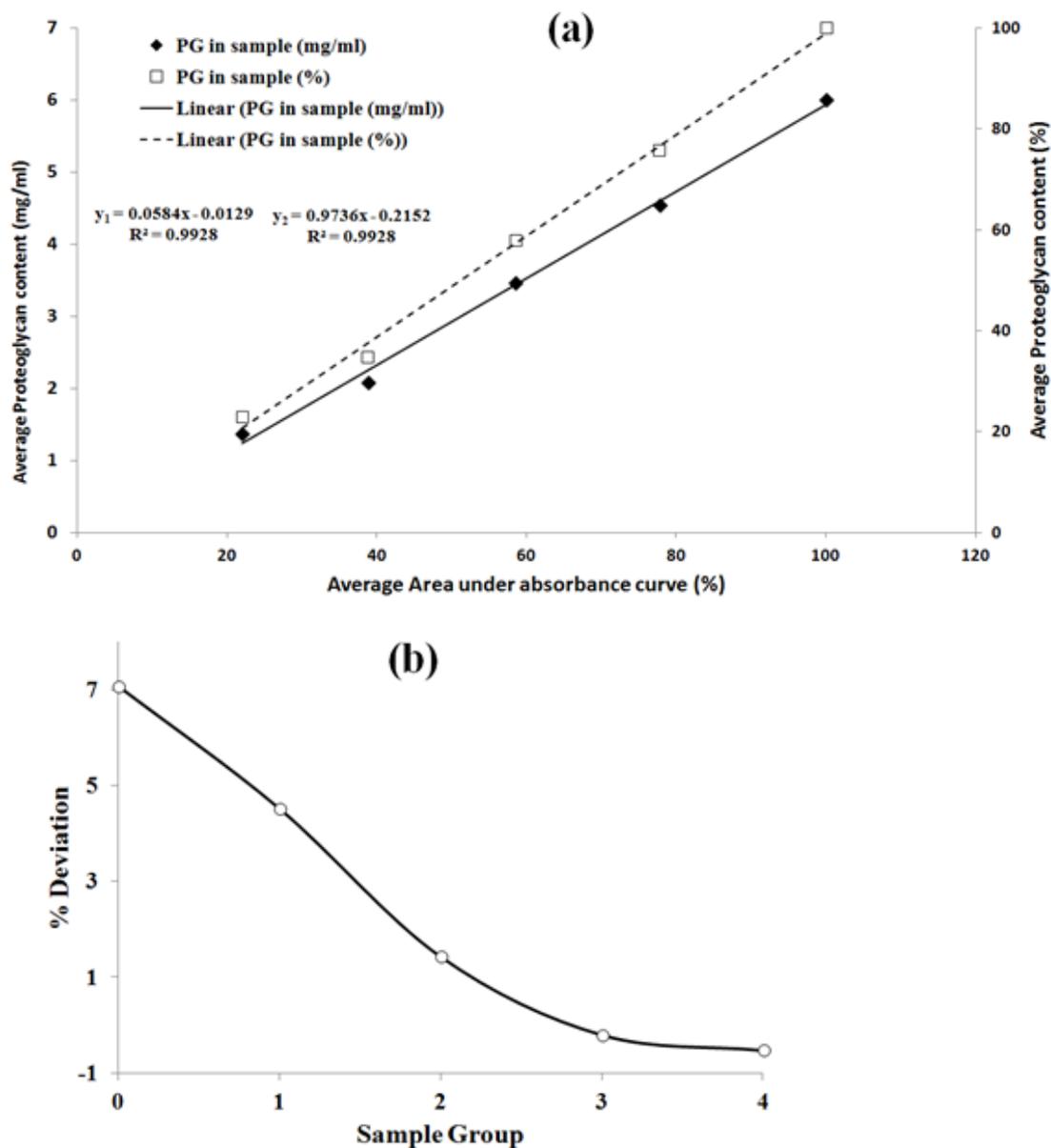


Figure 4: (a) Correlation between articular cartilage proteoglycan content (in *mg/ml* and %) measured by spectrophotometry versus area under the absorbance curve obtained from image analysis of Safranin-O stained micrographs. (b) Average percentage deviation (difference) between both methods (0 – normal, 4 – 4hrs trypsin treated samples).

The overlap observed between samples can be attributed to the differences in the initial proteoglycan content of each normal tissue sample. It is well known that samples taken from adjacent points on the same tissue is likely to have different proteoglycan content due to the wide variation of this component of the tissue across and depth-wise [7]. Hence, it is possible to find a normal sample with the same proteoglycan content as one that has been subjected to enzymatic digestion for 1hr. Nevertheless, with these inherent differences in the initial (normal) tissues, the image analysis method still correlated significantly ($R^2 = 0.9928$) with the histochemical method. The average percentage deviation between both methods (Figure 4b), which is also indicative of the sensitivity of the image analysis method, shows slightly higher deviations at higher PG levels, with significant decrease

in deviations at lower PG content. This suggests that the image-analysis methods increases in accuracy with decreasing sample PG content. This effect is arguably due to the inherent difference in the initial PG content in visually normal samples.

With the establishment of this correlation, an algorithm and software for determining the proteoglycan content in stained histological micrographs can easily be developed. This method can also be extended to determining the content and integrity of other matrix components such as collagen meshwork, Cellularity, etc. This also has the potential to facilitate the determination of established histological grading scores such as Mankin for different stained samples. Although ImageJ software was employed for image processing and analysis in this study, the analytical protocol can be adapted and implemented

in other platforms and packages such as MATLAB, FIJI, and custom programs.

In conclusion, we have demonstrated that there is significant linear relationship between image analysis results (area under absorbance profile) of stained articular cartilage sections and the gold standard method of histochemistry based on spectrophotometry, thus validating the potential of image analysis method to approximate with high accuracy the proteoglycan content of articular cartilage histological sections.

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