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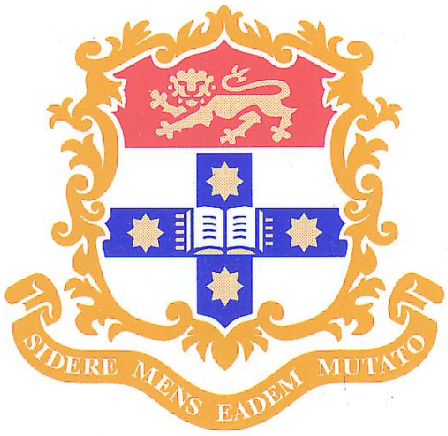

The effects of combined glucosamine sulfate and chondroitin sulfate supplements on condylar cartilage remodeling during functional appliance therapy. A Micro-CT study.

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Abstract

Glucosamine and chondroitin sulphate supplementation is used to prevent the degeneration of articular surfaces and also to enhance repair and regeneration of cartilage. The ability for adaptation of condylar cartilage to mandibular forward positioning is what constitutes the fundamental rationale for orthodontic functional therapy, which partially contributes to the correction of jaw discrepancies in growing Skeletal II mandibular retrusive patients. The purpose of this study was to qualitatively and quantitatively analyse the effect of Glucosamine sulphate (GS) and Chondroitin sulphate (CS) supplementation on condylar remodeling with functional appliance therapy in rats.

One hundred and forty 3-week-old female Sprague-Dawley rats were randomly divided into 4 groups consisting of; baseline controls, supplementation only, functional appliances only and those receiving both supplements and functional appliances. Supplements were preloaded for a period of 2 weeks prior to the placement of functional appliances at five weeks of age. The animals were sacrificed at days 0, 7 and 21 after appliance placement. The appliances were removed in the remaining experimental animals on day 21 with sacrifice on day 28 to analyse post growth modification changes. Condylar samples were then soaked in 0.2 M Gadolinium Chloride ($GdCl_3$) (aq) for 6 days and analyzed using micro-computed tomography (μCT) for morphological characteristics and linear and volumetric measurements of the mandibular condyle.

The results demonstrated supplement therapy increased the volume of cartilage with and without functional appliance therapy. Functional appliance therapy alone resulted in increases in cartilage volume over untreated animals, with peak volume increases occurring by day 7 of appliance wear followed with decreases as endochondral ossification ensued. Supplement therapy was found to enhance the normal biological response to functional appliance therapy in the rat model.

Keywords: Condylar cartilage, Glucosamine, Chondroitin Sulphate, Micro-CT, Gadolinium.

Introduction

Functional appliances have been widely used in the treatment of Class II malocclusions in orthodontic patients. Condylar cartilage proliferation and growth of the condyle induced by mandibular advancement [1-6] along with glenoid fossa remodeling [7-9] constitute the orthopedic component of the Class II correction from functional appliance (FA) therapy. Orthopedic Class II treatment with functional appliance therapy is prescribed during the peak pubertal growth period in patients to optimise the growth modification and minimise post treatment relapse[4,5,10].

The mandibular condylar cartilage (MCC) is considered a secondary cartilage and continues its growth postnatally via endochondral ossification, a process which transitions from chondrogenesis into osteogenesis [11]. The cellular and molecular mechanisms governing this growth process are only beginning to be understood. Studies have shown growth factors such as insulin-like growth factors (IGF), transforming growth factors (TGF), fibroblast growth factors (FGF), bone morphogenetic proteins (BMP), members of the hedgehog (Ihh) and Wnt gene families to be endogenously expressed in the condyle, regulating cellular proliferation, differentiation and maturation during chondrogenesis [11].

The unique biological characteristic of the MCC is its known adaptive ability to mechanical stimuli such as mandibular advancement during FA therapy [2,6,12]or during functional retrusion [13] . Recent studies investigating the molecular activations which occur within the MCC during mandibular advancement have illustrated increases in IGF-1 and FGF-2 [14] as well as IGF-2 [15], collagen type II [6,16], collagen type X [17] and vascular growth endothelial factor to occur. It is proposed that the reciprocal soft tissue forces that occur due to stretching of the bilaminar retrodiskal elastic band between the condyle and glenoid fossa [8] act as a stimulus for cellular proliferation and differentiation, mediated

by mechanotransductors such as Indian hedgehog (Ihh) [18]. In addition , the increased expression of Sox9 during mandibular protrusion is thought to accelerate the differentiation of mesenchymal cells into chondrocytes, leading to an earlier formation and increase in the amount of cartilage matrix [6].

The concept of altering condylar cartilage growth with exogenous substance administration is not a new one. Researchers have had many attempts at biochemically stimulating condylar cartilage growth, trialing compounds such as bisphosphonate [19], growth hormone [4,20], anabolic steroids [21], IGF- 1 [22-24] and FGF-2 [24,25] in animal models in the pursuit of pharmaceutical modulation of condylar growth. Most results proved promising, illustrating again perhaps that the growth of the condyle is not genetically determined but has the capacity for adaptation and stimulation, not only mechanically but biochemically. Although potentially efficacious, the clinical applicability of prescribing long term hormonal use during functional therapy in healthy adolescents is limited.

Glucosamine (2-amino-2-deoxy-D-glucose) is an amino monosaccharide precursor of the disaccharide unit of glycosaminoglycans, the building block of proteoglycans. These proteoglycans form the matrix of all connective tissues, including cartilage[26]. Glucosamine therefore is a sugar produced in the body and found in small amounts in food which plays an important role in maintaining cartilage. Glucosamine, taken in supplemental form, provides the raw material for the production of proteoglycans and glycosaminoglycans by chondrocytes [27]. Historically, glucosamine supplements have been retrieved from the extraction of chitin, a component of shellfish (shrimp, crab and lobster). Recent technological advances have led to a more efficient means of production of a vegetarian source by fermentation [28]. Glucosamine was found to effectively diffuse into the body via oral, IV and intramuscular forms [29], with the oral route being the most popular method of

administration. Glucosamine (G) may come in sulphate (GS) or a hydrochloride (GH) form however glucosamine sulphate is the one most commonly used and the most extensively studied.

Chondroitin sulphate (CS), a polymer of repeating disaccharide units (Galactosamine sulphate and glucuronic acid) is the predominant component of articular cartilage and is a natural component of several other body tissues including tendons, bones and vertebral discs [30]. The sources of chondroitin sulphate used in nutritional supplements include bovine trachea, pork by-products and shark cartilage[31]. Chondroitin sulphate is often used in combination with glucosamine in the treatment of osteoarthritis and studies have shown that the combination of glucosamine and chondroitin sulphate act synergistically in stimulating glycosaminoglycan synthesis. Evidence exists that this combination therapy is more efficacious than treatment with either agent alone[32] and that these nutraceuticals together have structure-modifying as well as symptom modifying abilities[33] in the treatment of cartilage degeneration in osteoarthritis.

Some of the proposed advantages of these medications over traditional medical therapies for the treatment of osteoarthritis include wide availability, relatively low cost, absence of known side-effects, high oral absorption rate and ready transfer into cartilage [34].

In vitro and *in vivo* studies suggest GS and CS supplementation may increase proteoglycans synthesis [35] , as well as have anticatabolic[36], chondroprotective [32,35,37,38] and mild anti-inflammatory effects [39-41] which may contribute to the beneficial results seen clinically[42]. It has been proposed these substances enhance the “protective” metabolic response of the chondrocytes, suggesting these compounds function as “biological response modifiers” in tissues under mechanical stress [35].

While the rationale for the use of these neutraceuticals is based on *in-vitro* animal and human studies as well as *in vivo* animal models, evidence of their clinical efficacy in humans is still debated. Numerous clinical trials have been conducted to assess their effectiveness in treating osteoarthritis [43-46]. Limited literature exists on the therapeutic use of GS + CS for the cartilage of the temporomandibular joint [47,48].

The concomitant prescription of the two therapies for the modification of condylar cartilage growth is investigated in this study. Micro-Ct analysis permitted three dimensional image analysis of cartilage remodeling, as well regional changes occurring in the posterior and anterior halves of the condyles. No evidence in the literature exists on utilizing this method for the analysis of morphologic and quantitative changes in condylar cartilage either in functional appliance therapy or under G and CS supplementation.

A substance which may to even be considered a promoter of growth modification, must have an excellent safety profile with minimal to no side effects, as well as FDA (USA) or TGA (Australia) approval prior to the consideration of wide spread prescription especially to adolescent patients. Perhaps orthodontists in the future may be able to prescribe not only a “functional” answer to a growing patient with mandibular retrusion but also a pharmaceutical enhancement to improve the cartilage adaptation to the functional appliance therapy.

Aims

The aim of the investigation was to quantitatively and qualitatively analyse the effects of glucosamine sulphate and chondroitin sulphate supplementation on condylar cartilage remodeling during functional appliance therapy.

Materials and Methods

Animal Grouping and Housing

One hundred and forty female Sprague-Dawley rats were used in this study. Sprague-Dawley rats were selected due to similar research conducted in the rat model in relation to bite jumping appliances [16,18,49,50] as well as G and CS supplementation [51,52], thereby providing a clear choice to combine the two elements for investigation within this model. Animals were received at 3 weeks of age to allow for an acclimatization period prior to appliance placement as well as provide a loading period for the drug administration. They were received at 3 weeks of age and divided randomly into four groups, group 1 (n=40) receiving no functional appliance treatment and no supplements, group 2 (n= 30) receiving functional appliance treatment only, group 3 (n= 40) receiving G and CS supplements only and group 4 (n= 30) receiving both functional appliance treatment and G and CS supplements (Ethics approval 07/31B Ref OA/7-2007/4674).

The appliance placement occurred at 5 weeks of age due to the coincidence of peak pubertal growth in the ensuing weeks of age [7]. Animals receiving supplementation were housed singularly in a cage for accurate monitoring of supplementation consumption per animal. Those receiving no supplementation were housed four animals to a cage.

Supplementation

A required loading period of 10 days for Sprague-Dawley rats has been documented [51]. At 3 weeks of age, the groups to receive the glucosamine and chondroitin sulfate began receiving the supplements within their food supply. The remaining groups received the same type of food without any supplementation. The food, method of drug administration and dose rate were based on a similar

study conducted by Beren *et al* 2001, investigating glucosamine and chondroitin sulphate efficacy on pubertal female Sprague-Dawley rats. The powder form of glucosamine sulphate and chondroitin sulphate was homogenized using a power mixer and combined with a commercial veterinary nutritional supplement *Nutri-gel* (the Australian equivalent of *Nutri-cal* used in the study). Nutrigel (Troy Laboratories, Australia) is a highly palatable oral supplement used in veterinary medicine to provide either partial or full nutritional support for mammals. It has been used in previous studies as mentioned above and is also used for its ability to disguise and effectively homogenize drug supplements for mammals, including rats. This feeding technique avoids the inherent stress associated with restraint and gastric tubing. Rats having no supplementation received 4 ml of Nutrigel orally every 24 hr. Rats in the supplementation groups received 4ml of Nutrigel homogenized with a dose of 1.4-1.6 g of glucosamine/ kg (250 mg of glucosamine/rat) and 1.15-1.3g of low molecular weight sodium chondroitin sulfate/kg (200mg of sodium chondroitin sulfate/rat). The mixture utilised in the Beren *et al* study involved 0.029-0.032g of manganese/kg (5mg/rat) and 0.018 – 0.021g of ascorbate kg (33mg/rat) as well however this was not incorporated into the food mixture in the present study due to the manganese present in the Nutrigel.

A loading period of 14 days with the supplementation occurred prior to the commencement of the experimental period. The same food and drug administration was used during the experiment. The paste form of the food was considered to be advantageous in the longevity and maintenance of the appliances as well as in the ease of consumption for the animals with the appliances in place. Food and drug consumption were recorded daily. Animals were permitted unrestricted access to the water supply.

Functional Appliance placement

Placement of functional appliances occurred at five weeks of age, under anesthesia with isoflurane (2%) and oxygen (2%) inhalation. The appliance placement consisted of standardized pediatric crown forms (3M™ ESPE™ Strip Crown Form Refills, USA) filled with composite resin (3M™ ESPE™ Z100 Restorative dispenser for capsule, USA) bonded to the lower incisors primed with self etching primer (3M Unitek, Monrovia, California) as seen in Figure 1. The crown forms were then smoothed with any excess composite removed. The crown forms were positioned and angled downward anteriorly to form an inclined plane which would position the mandible forward during function and rest. Animals within the control groups maintained a normal incisor relation.

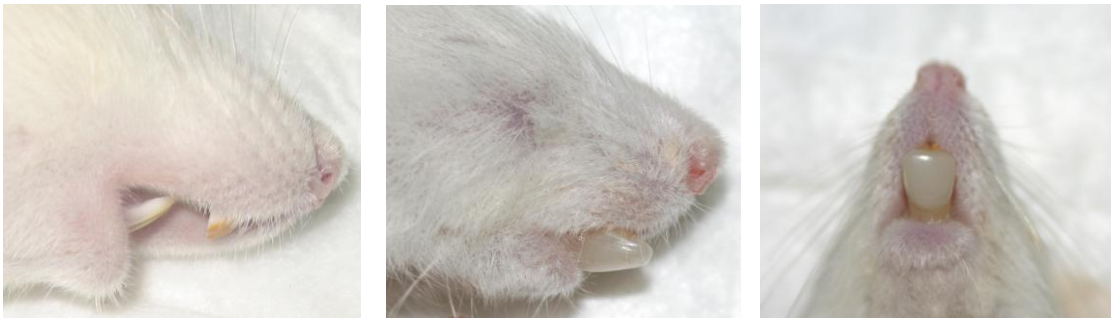


Figure 1 Functional appliance placement

a. Normal incisor relationship b. Functional appliance c. Functional appliance

On day 21 of the experimental period, animals with appliances in place were anesthetized and functional appliances were removed, utilizing rotary discs. In most cases the vibratory action would debond the appliance.

No appliances were broken or lost during the experimental period.

Monitoring samples

Intra-operative vital sign monitoring of each animal was conducted during the appliance placement, namely pulse, respiration, eye discharge and body reflexes. Post surgical monitoring and recording

occurred daily for the first week, then twice weekly subsequent to appliance placement. Animals were monitored for signs of distress and pain based on behavioral changes and eating patterns, with clinical observations recorded including changes to skin and eyes, respiratory system, and behavioral patterns. Daily monitoring of the animals occurred during feeding times. Functional adaptation to the appliances was also monitored in order to assess whether the animals were in functional protrusion.

Specimen Preparation

After the intervals of 0, 7, 21 and 28 days animals were sacrificed by CO_2 asphyxiation according to the following schedule as seen in Figure 2.

Study Criteria	Days into experiment before sacrifice	Group 1 Number of animals (no bite jumping, No G+CS)	Group 2 Number of animals (bite jumping with No G +CS)	Group 3 Number of animals (No bite jumping, With G+CS)	Group 4 Number of animals (Bite jumping with G+ CS)
Functional appliances placed	Day 0	10 (sacrificed)	0	10 (sacrificed)	0
	Day 7	10	10	10	10
Bite jumping ceased; Day 21	Day 21	10	10	10	10
	Day 28	10	10	10	10
Total sacrificed		40	30	40	30

Figure 2 - Study Design

The heads were decapitated and hemi sectioned in the median plane, then fixed separately in 10% buffered formalin. The right hemi section was utilized for this study. In order to improve the condylar cartilage visualization by X-ray absorption a staining protocol was developed involving a 6

day staining procedure of each sample in 0.2M $GdCl_3$ prior to scanning in the SkyScan 1172 high-resolution desktop x-ray microtomograph (SkyScan, Aartselaar, Belgium). A preliminary study was conducted to ascertain the ideal staining protocol for the samples to improve condylar cartilage visualization, with results indicating 6 day staining to be best. Gadolinium (Gd), a lanthanide element, has a high atomic number and therefore a higher absorption of x-rays at clinically used tube voltages when compared to elements with a lower atomic number such as Iodine [53] thereby creating a more effective contrast medium for computer tomography. A positively charged ion such as Gd^{3+}will act as an articular contrast agent due to the interaction with negatively charged groups of proteoglycans found in cartilage, with the paramagnetic cations (Gd^{3+}) diffusing from the joint fluid into the cartilage matrix [53,54]. This binding permits a higher absorption of x-rays and improved contrast for the measurement of cartilage morphology and volume. Due to its small size and charge Gd^{3+} is an effective contrast medium for 3 dimensional cartilage volumetric measurements using Micro-CT imaging[54].

Microtomographic Imaging

The X-ray microtomographic scans were acquired using the SkyScan 1172 high-resolution desktop x-ray microtomograph. After the 6 day staining period, samples were mounted utilizing a positioning jig within the x-ray unit. Three dimensional microstructural information was obtained from the digital recording of a large number of X-ray absorption radiographs whilst the specimen was rotated in small angular increments. The exposure was set at 160kV/61 µ and no filter was used. Each specimen was scanned with a rotation step of 0.39° over a total range of 360°. Preliminary scanning revealed a 7.0µm set resolution to be optimal in order to obtain the full morphology of the condylar head in the articular fossa as well as the optimal data for the histomorphometric analysis of the condylar cartilage. SkyScan's volumetric reconstruction software NRecon was used to set the

acquired angular projections and create a set of cross section slices through the sample. The program uses a modified Feldkamp algorithm [55] with automatic adaptation to the scan geometry in each micro-CT scanner. Reconstructed slices were saved in BMP data format, with the software generating a series of 985, 8 bit axial slices, each of 1024 x 1024 pixels that had Z-dimensional spacing equal to the slice pixel spacing.

The three dimensional data was subsequently rendered as 3D visualizations using the VGStudio Max software (Volume Graphics GmbH, Heidelberg, Germany).

Sample Orientations

In order to create an anatomical representation of the condyle within the glenoid fossa, as well as eliminate any errors due to sample positioning within the SkyScan Micro-CT, a reorientation step was introduced. Each scan was re-orientated in all three dimensions X, Y and Z with reference to pre-designated anatomical landmarks, using the RotateScanLine software created at the Electron Microscopy Unit (University of Sydney). Once the correct X+ Y+ Z rotation equation was established on VGStudio Max, the original BMP data set was imported into RotateScanLine software where the rotation equation was carried out. The re-orientated sample was then reintroduced into VGStudioMax for linear and volume measurements. An investigative trial into any decrease in data as a result of the RotateScanLine rotation proved there to be minimal data loss in the reorientation procedure. Condyles were re-orientated to mimic anatomical orientation *in vivo* thereby permitting a virtual sectioning of the condyle to represent anterior and posterior condylar halves. Once samples were aligned geometrically, a 15 degree rotation to the mid -sagittal plane was introduced to mimic the condylar angulation to the cranial base in the rat model. While total condylar and total cartilage volumetric measurements were taken, an anatomical representation of the anterior and posterior

halves of the condyles would more accurately represent where condylar cartilage changes were occurring.

Quantitative and Qualitative Image Analysis

Linear and volumetric measurements were taken of the condyles. The regions of interest were defined as the slices involving maximum length of the condylar head in the rostrocaudal dimension, the greatest width of the condyle represented by slices in the mediolateral dimension and the height of the condyle in the dorsoventral dimension. The dorsoventral dimension of interest was defined as those slices including the most superior portion of the condyle to a point standardized to 180 slices inferior to the lateral ridge on the lateral surface of the condyle, a clear anatomical landmark. Therefore the maximum height, width and length of each condyle was determined as well as the volume of the cube defined by these measurements.

Once the region of interest was extracted, the segmentation process and volumetric analysis were carried out eliminating superfluous data. The segmentation process in VGStudioMax required a 2D static region growing tool to select grey scale values corresponding to the cartilage which then interactively partitioned axial slices to isolate the total cartilage. This algorithm allowed regions of interest to be selected consecutively from individual sections based on a selected grayscale value and tolerance. A volumetric measurement of the isolated section could then be calculated, in this case total cartilage volume measurement. The same procedure was followed for the isolation of the condylar head from the remaining tissue in the scan, permitting a total condylar volume measurement. Total condylar volume included both bone and cartilage tissues. The rostrocaudal dimension of the condyle was then halved and reintroduced separately into the VGStudioMax Software and the process repeated for posterior condylar volume and posterior cartilage volume measurements.

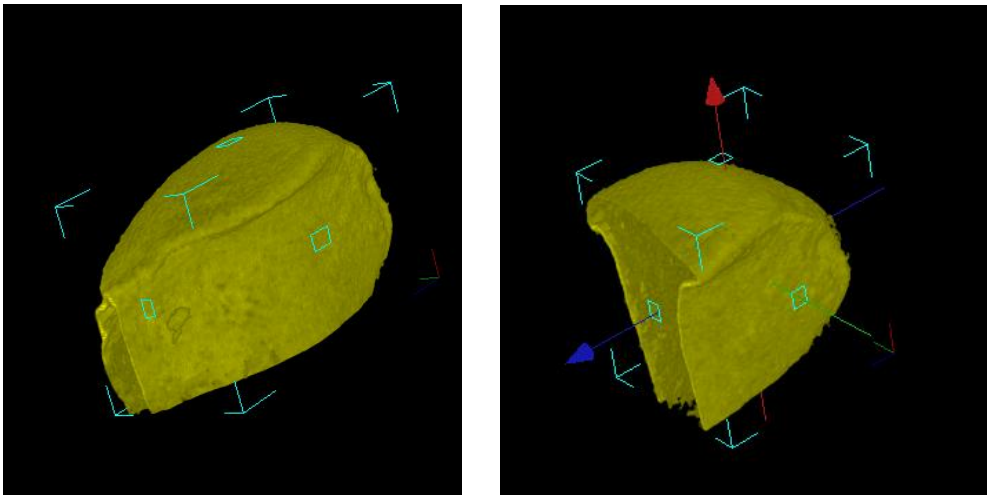


Figure 3 - Cartilage Segmentation
a. Total cartilage segmentation b. Posterior cartilage segmentation

Statistical Analysis

The data collection was carried out by a single operator and measurements repeated at two time points 2 months apart to analyse the consistency in the segmentation method. Each variable; total volume, posterior volume, total cartilage and posterior cartilage, was measured 2- 4 times and averaged for the final reading. The data were processed with SPSS® version 14 for Windows (SPSS Inc., Chicago, Illinois, USA) for ANOVA. Significance levels used were *P* values less than 0.05. The results were displayed using box plot diagrams. The box contains the middle 50% of the results; the whiskers stretch out to the maximum and minimum values, except for unusual results which are indicated separately. The median (50^{th} percentile) is marked with a bar in the box, with the top and bottom of the box representing the 75^{th} and 25^{th} percentile respectively. Error of measurement was tested by repeating each of the four measurements, three times over 23 samples. Results found a standard error of measurement of $0.0802mm^3$ for total volume, $0.0508mm^3$ for posterior volume, $0.0863mm^3$ for total cartilage, $0.0408mm^3$ for posterior cartilage and a coefficient of variation (CV) of 4%, 5%, 24% and 26% respectively.

RESULTS

Measurements including total condylar volume, posterior condylar volume, total cartilage volume and posterior cartilage volume were analyzed using a univariate analysis of variance. A covariate of total box volume was included, composed of the length, width and height measurements of each condyle. The use of the box volume as a covariate for analysis was done in order to account for individual variability in condylar size between animals. This measurement was found to be a better covariate than the weight at sacrifice. Analysis of the weights at sacrifice between groups; found average weights to be as follows, group 1 (no appliance, no supplement) 43.56g ± 0.70, group 2 (appliance only) 36.11g ± 0.73, Group 3 (supplement only) 42.50g ± 0.63 and group 4 (appliance and supplement) 42.16g ± 0.74. One way analysis of variance of the weights between groups found group 2 to be significantly different ($p < 0.001$) while no significant differences were found between groups 1, 3 and 4.

Interestingly, an individual functional adaptation to the appliance was encountered during the experimental period. Whilst some animals responded to the appliance with a functional protrusion, some adapted with a functional retrusion. The effect of functional appliance therapy with supplementation was analyzed first for statistical significance. A secondary analysis was carried out to determine outcomes based on Class; functional protrusion (termed class 3) or functional retrusion (termed class 2) and its relation to supplement therapy over the experimental period.

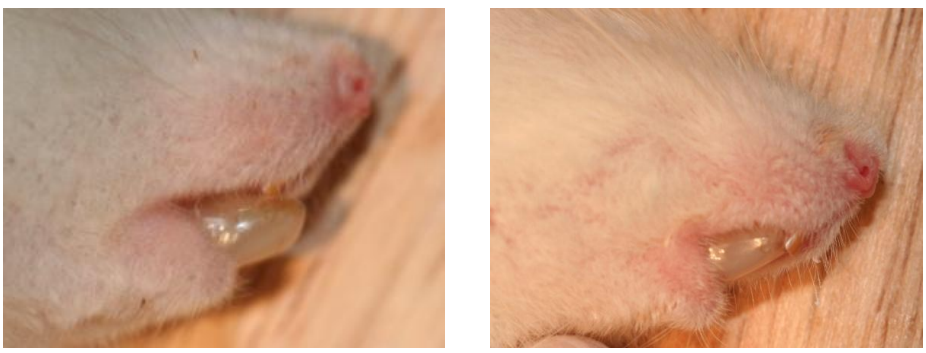
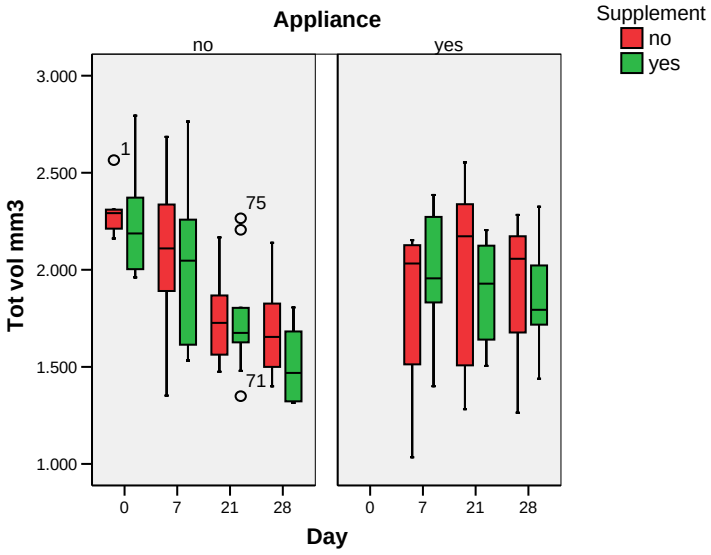


Figure 4 - Functional adaptation
a. Functional protrusion (Class 3) **b. Functional retrusion (Class 2)**

Quantitative analysis

Changes in Total Volume of the condyle

One-way analysis of variance was used to compare the total volume of the condyles between the four groups. Factors included in the analysis were day of experimental period, appliance and supplementation, with two and three way interactions between these factors and the covariate factor of box volume. Total volume changes in relation to days (p= 0.002) and class (p=0.008) as well as the interaction between day by class (p=0.034) were found to be statistically significant. Animals without functional appliances demonstrated a general decrease in condylar volume from day 0 through till day 28. The presence of a functional appliance sustained the condylar volume during this time period. However, differences in relation to class and day were found with those animals in class 2 illustrating a mild increase by day 21 followed with a decrease after appliance removal. Class 3 relations found a sustainment of condylar volume during the appliance treatment with a decrease after the removal. The presence of the supplement was found only to be of marginal significance (p= 0.065).



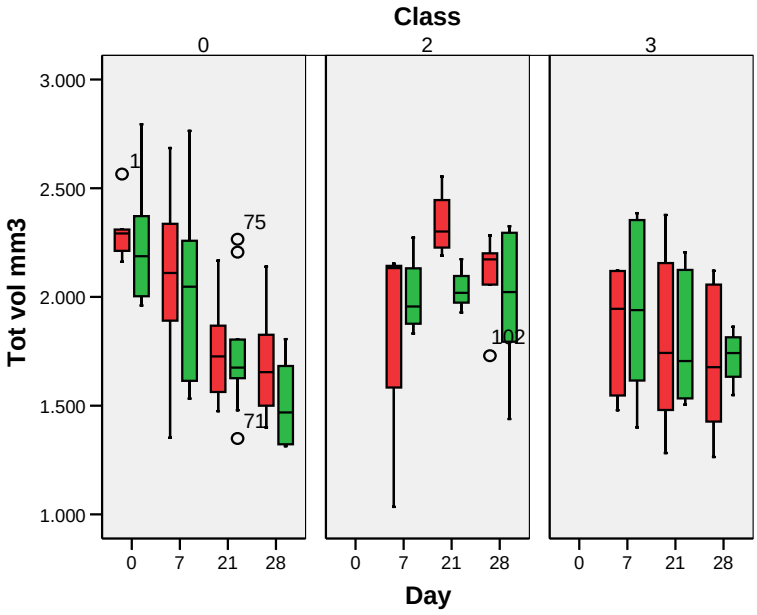
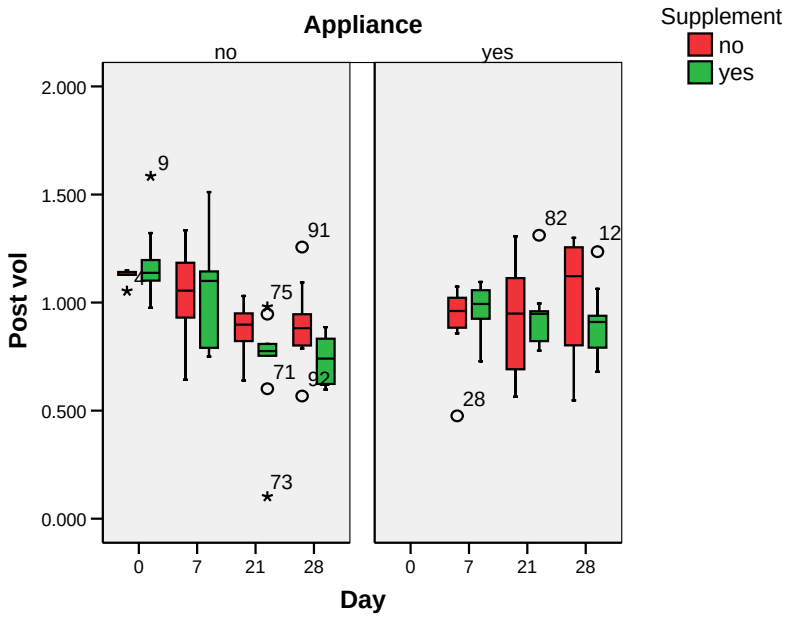


Figure 5 - Total Volume analysis

Changes in Posterior Volume of the condyle

A similar result to total volume changes were found in relation to changes in posterior volume of the condyle. An analogous trend occurred with the day by class interaction (p=0.058). Posterior volume of the condyle decreased during the time period for animals with no functional appliance, however increased slightly in those animals that functionally retruded by day 21 and decreased after appliance removal. The converse was true for the class 3 functional protrusion animals, where a reduction occurred during the appliance wear however appeared to rebound after the appliance removal.



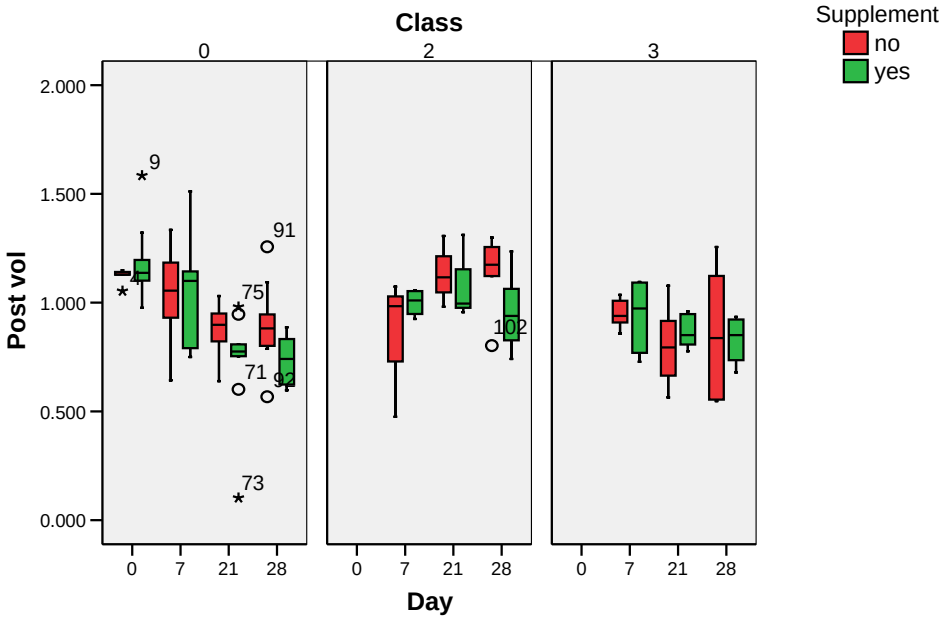


Figure 6 - Posterior Volume analysis

Changes in Total Cartilage Volume

Univariate analysis of variance was used to compare the total volume of condylar cartilage between the four groups. Factors included were day of experimental period, appliance and supplementation with two and three way interactions analyzed. A covariate of box volume was again included to account for any condylar size discrepancies in the animals.

Supplementation had a statistically significant effect on total cartilage volumes, in groups treated and untreated with the functional appliances (p=0.000). The presence of functional appliance with supplementation was also found to be statistically significant alone (p=0.014) and in relation to the different time points of investigation (p=0.009). The presence of a functional appliance had a statistically significant (p= 0.019) effect on cartilage volume as well as its effect over time (p= 0.000). A second similar analysis was completed to ascertain the effects of class on cartilage volume with a statistically significant result found in the interaction between the factors day and class (p=0.002) and class and supplement (p=0.004).

Source	Sig.
Box	0.001
Day	0.000
Appliance	0.019
Supplement	0.000
Day*Appl	0.000
Appl*Suppl	0.014
Suppl*Day	0.474
Day*Appl*Supp	0.009

Source	Sig.
Box	0.001
Day	0.000
Class	0.072
Supplement	0.000
Day*Class	0.002
Class*Suppl	0.004
Suppl*Day	0.275
Day*Class*Supp	0.099

Table 1 - Total cartilage factor analysis

a. Investigating appliance, supplement and day.

b. Investigating class, supplement and day.

A post hoc comparison of total cartilage volumes at day 21 between class 2 and class 3 failed to find a statistically significant difference, with similar results for day 28. The preloading period of supplementation resulted in an increase in cartilage volume at baseline levels (day 0). Whilst the presence of the functional appliance increased the volume of cartilage over the time periods in comparison to controls, the effect of supplementation appeared to enhance the biological response to functional appliance therapy.

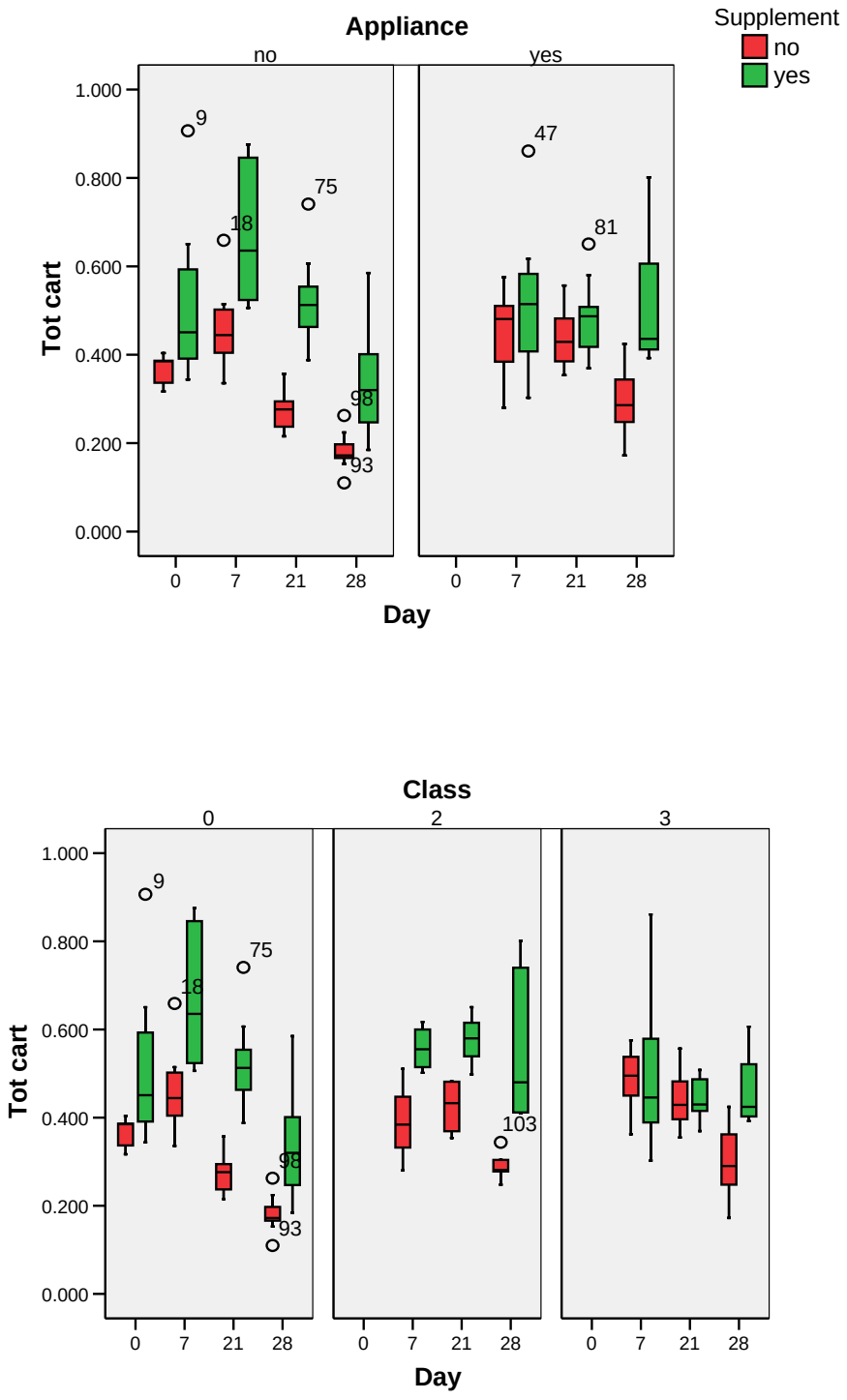
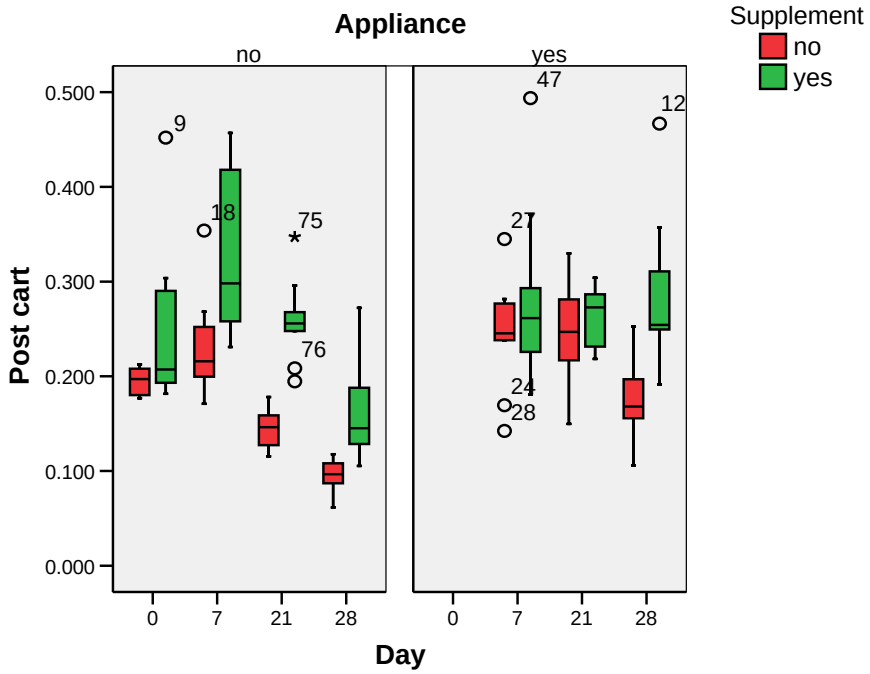


Figure 7 – Total cartilage analysis

Changes in Posterior Cartilage Volume

Similar results were yielded in response to posterior cartilage volumes in relation to the factors mentioned previously. Presence of appliance (p=0.000) and supplement (p=0.000) therapy had statistically significant effects on the posterior cartilage volume. Importantly the interaction between the factors day, appliance and supplementation were significant (p= 0.009). Results analyzed for the effects of class on posterior cartilage volume found class (p= 0.000), day by class interaction (p=0.001) as well as the day by class by supplement interaction (0= 0.031) to be statistically significant. A post hoc comparison of posterior cartilage volume at day 21 between class 2 and class 3 found statistically significant results (p <0.01), however this was no longer the case by day 28. The effect of the supplement appears to increase the biological response to functional appliance therapy particularly after the appliances are removed as seen by the changes at day 28.



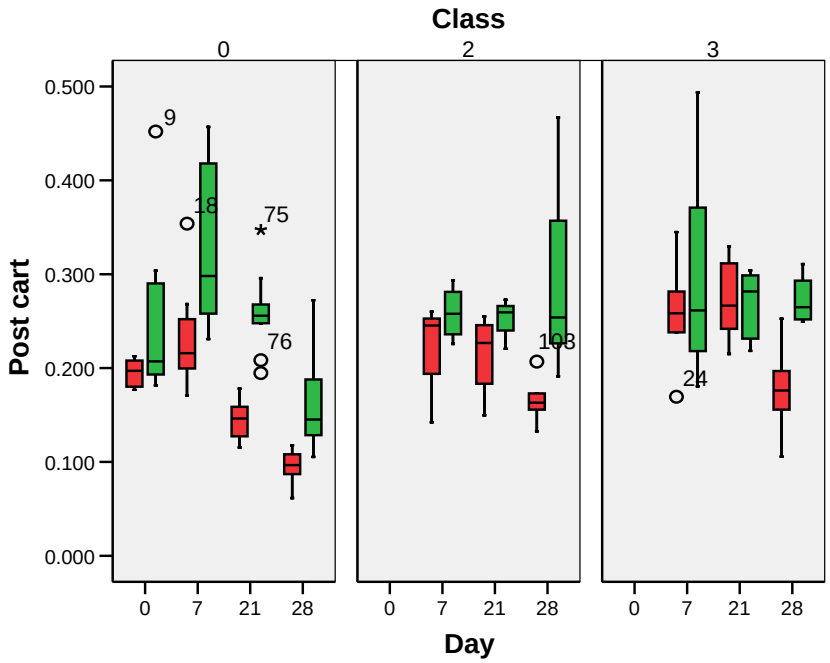


Figure 8 – Posterior cartilage analysis

Qualitative analysis

Normal growth

The shape of the condyle appeared consistent amongst groups with or without supplementation and with no functional appliances. A thicker layer of cartilage in the axial slices was observed by day 7 of the control groups with a decrease continuing into the day 28 group. Bone appeared more dense by day 28 with minimal thickness of cartilage. The overall anatomy of the condyle was smooth, with consistent anatomic characteristics as seen below.

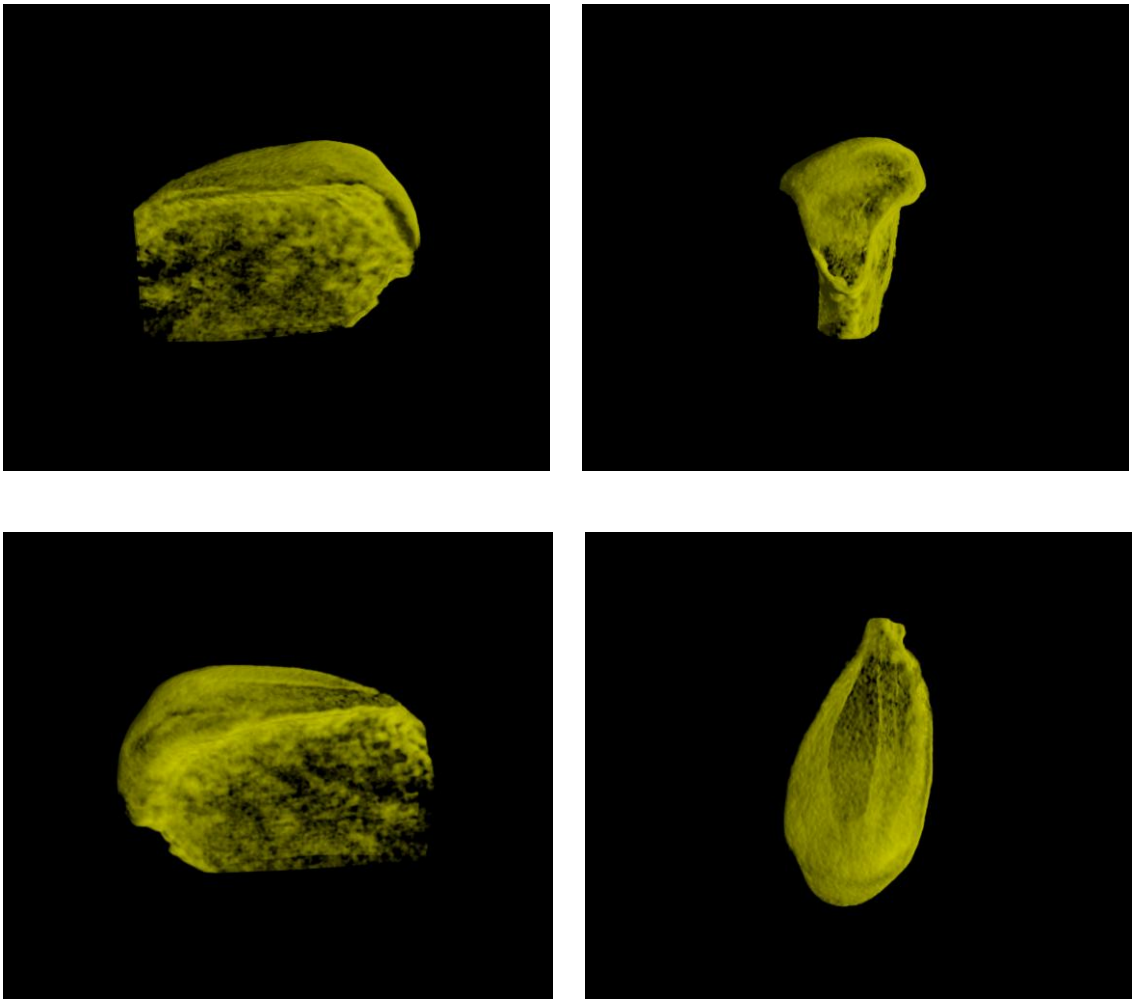


Figure 9 – 3 Dimensional cartilage imaging at day 0.
From Upper left a. Lateral view b. posterior view c. Medial view d. Superior view

Functional appliance

The shape of the condyles which had functional appliance therapy varied significantly from control condyles. Changes began to be evident by day 7 with increasing morphological changes evident by day 21. The remodelling of cartilage was evident in both class 3 and class 2 functional appliance animals in the axial cross sections and 3 dimensional analysis. Typically in the class 3 functional appliance animals, the morphological characterstics included a broader shaped condyle from the superior aspect, with remodeling evident posteriorly as seen from the lateral or medial aspect. This

posterior tubercle was characteristic of nearly all class 3 animals and is thought to be associated with the point of attachment of the retrodiskal tissue. The cartilage was also found to be greater at and immediately inferior to the lateral and medial ridges of the condyles, most likely where the capsular attachment exists. This finding of an increase in lateral thickness of cartilage was consistent in most functional appliance condyles irrespective of class. Anteriorly, an increase in the width of the condylar head was also found to occur in class 3 condyles in comparison to the control condyles, corresponding to the area of the lateral pterygoid attachment.

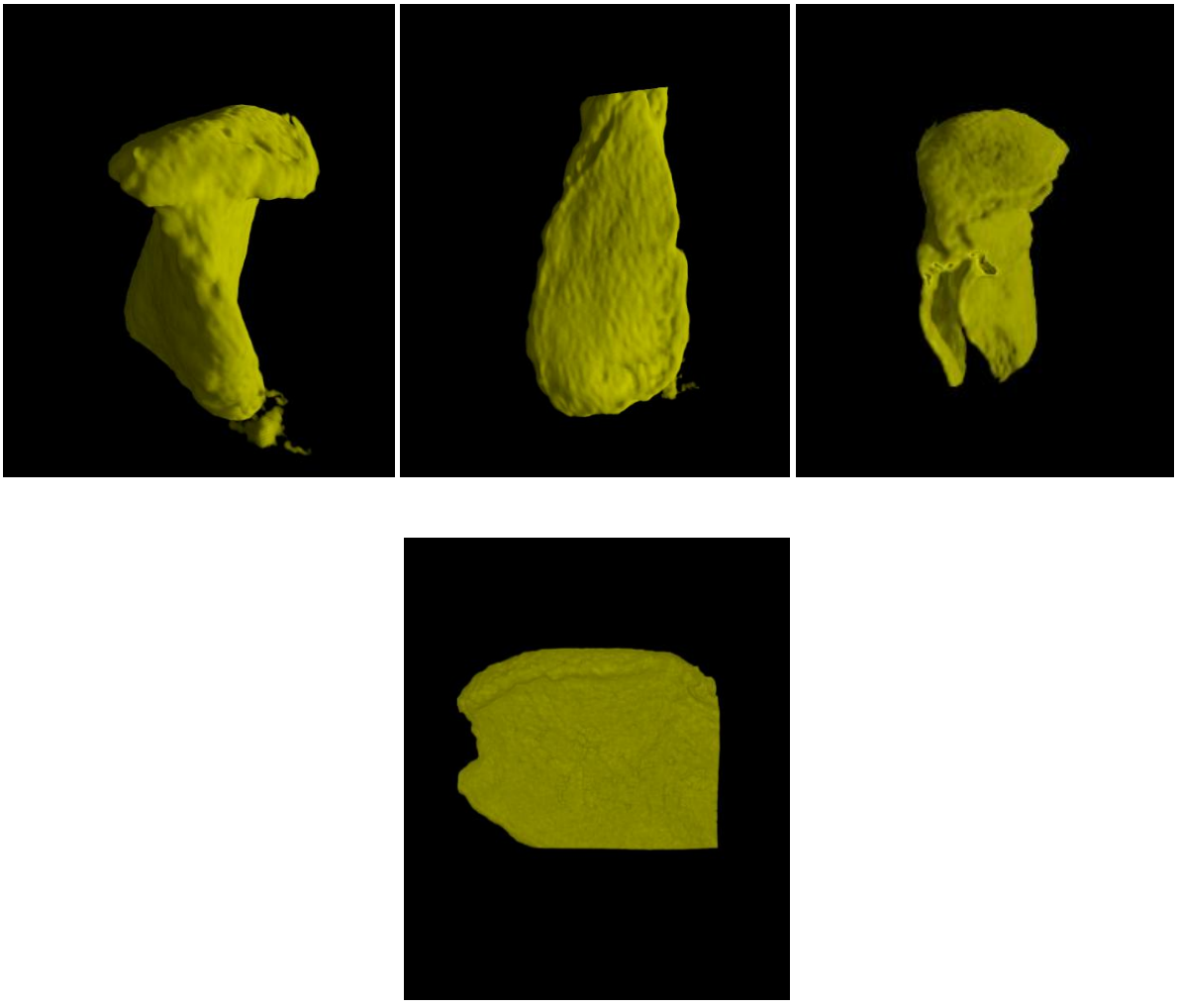


Figure 10 - Condyle at day 21 after functional appliance (Class 3) and supplementation
From left 1.) posterior view, 2.) superior view 3.) anterior veiw 4.) medial view

Class 2 condyles displayed slightly different morphological characteristics to control and class 3 condyles. Routinely, evidence of significant remodelling was seen in 3- dimensional and axial slices on the mediosuperior surface of the condyles. This feature occurred in some but not all of the class 3 animals. The period after appliance removal displayed increased remodelling of the cartilage, particularly in those animals which were class II and had been receiving supplement therapy.

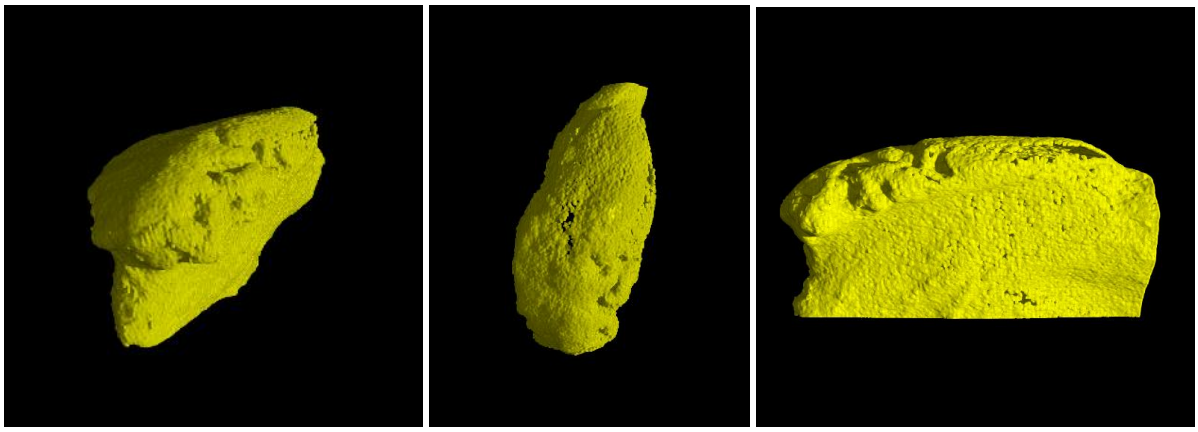


Figure 11 - Condyle after Functional appliance (class II) and supplement at day 28.
This undulating surface on the mediosuperior surface was evident on mostly class II functional appliance condyles by day 21 and persisted into day 28.

The induced cartilage changes in the control and the appliance groups appeared to be increased in the groups receiving supplements. An increase in cartilage remodelling was particularly noticeable in the day 28 samples; which had been receiving supplementation and had the funcitional appliances removed. This evidence was seen as increased thickness of cartilage in the axial sections with undulating topography on the superior condylar surface, as well as cartilage in fill in these remodelling regions.

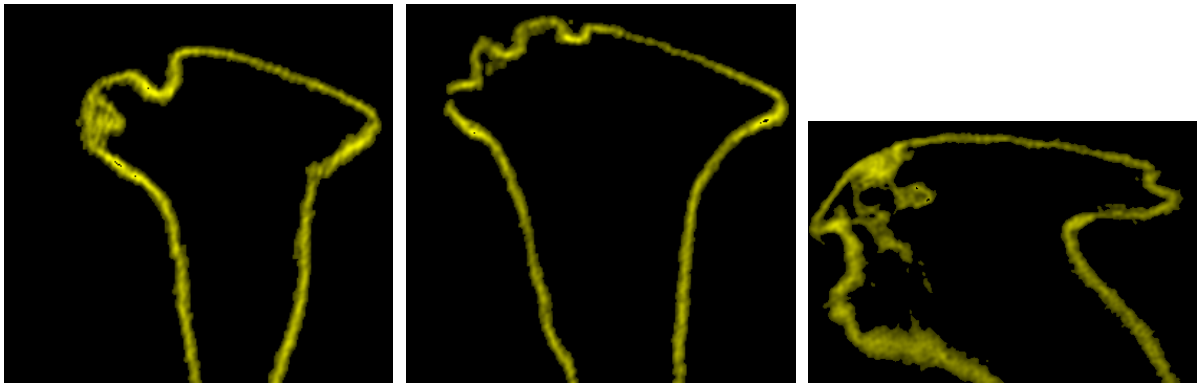


Figure 12 – 2 Dimensional cartilage imaging of condyle at day 28, after both functional appliance and supplement therapy. Cartilage segmentation in the.) Anteroposterior dimension b.) Anteroposterior dimension c.) Mediolateral dimension

Discussion

Condylar cartilage is considered a secondary cartilage and while belonging to the articular cartilage group it differs in its biological features due to its ability to undergo adaptive changes in response to external stimuli, both mechanical [6,11,12,56] and biochemical [20,4,21,23,25]. Functional appliance therapy in orthodontics is utilized in the treatment of Class II mandibular retrusion during the pubertal growth spurt to enhance mandibular growth whilst restraining maxillary growth to improve the skeletal and dental relationships of patients[10,57]. More recent evidence has indicated condylar remodeling may still occur with functional appliance therapy past the pubertal growth spurt [16,58,59]. Biochemical stimulation of condylar cartilage has been attempted with positive results using growth hormone [4], insulin-like growth factor-1 and fibroblast growth factor-2 [23,25] and anabolic steroids, [21] however long term prescription of these substances in healthy adolescents may be questionable. Glucosamine and chondroitin sulphate have historically been prescribed for the treatment of osteoarthritis due to their disease and symptom modifying capabilities as well as impressive long-term use safety profile [33,60].These neutraceuticals act synergistically [61] to stimulate collagen synthesis by the chondrocytes [35], produce an anticatabolic [36] and chondroprotective effect [32,35,37,38] and exhibit mild anti-

inflammatory properties [39-41]. Studies suggest these compounds function as "biological response modifiers" in response to mechanical stress in cartilage tissue [35].

Limited literature exists on the therapeutic use of G +CS for condylar cartilage, with only two studies reporting their use for the treatment of temporomandibular disorders and resultant decreases in TMJ tenderness, decrease in TMJ sounds and decrease in the number of over-the counter analgesic medications taken by subjects with temporomandibular joint pain [47,48]. Similarly, no evidence exists in the literature for the 3-dimensional analysis of condylar cartilage remodeling under functional appliance therapy nor glucosamine and chondroitin sulphate supplementation. The purpose of this investigation was to analyse quantitatively and qualitatively the effect of these dual therapies concomitantly utilizing micro-computed tomography for 3-dimensional visualization.

Study design

Animal selection and housing

Historically, the rat model has posed a favorable design for functional appliance investigation due to logistics and advantageous cost structure in comparison to large animal work. Female Sprague-Dawley rats of five weeks of age were selected for functional appliance treatment due to the large amount of literature existing in this particular model [6,7,16,50,62] as well as the time point coinciding to their peak pubertal growth rate [63]. Two animals died during the experimental procedure of unknown causes. Housing of animals individually in the supplement receiving groups was considered important to monitor their daily consumption of drug and food. Those not receiving the drugs were housed four to a cage. Whilst individual housing of animals would have been ideal to maintain consistency between the groups, the monetary costs involved in this study design were too great for

this investigation. There was no statistical significance in the sacrifice weights between groups, 1, 3 and 4 which may indicate the effect of housing may not have affected growth of the animals significantly. However, due to the significant anatomic variations in condylar sizes, the use of the box volumes of the condyles as covariates in the statistical analysis were effective in compensating for these size differences.

Drug delivery

The use of homogenized Nutrigel paste with G and CS supplements was deemed successful due to the ability to monitor and ensure drug administration without the inherent stress of restraint and gastric lavage. This finding was consistent with the literature [51], where Nutrigel with the same dose regimes of G and CS were administered for an experimental duration of 52 days, in 8-9 week old female Sprage-Dawley rats. Nutrigel was required to mask the G and CS supplementation as experience in a concurrent study found less homogenized mixes resulted in Nutrigel consumption around the larger supplement particles. Similarly the use of a paste like sustenance was beneficial in the maintenance and long term success of appliance design as heavy grinding associated with rat pellets may result in appliances breakage. Whilst daily monitoring during feeding identified normal behavioural and health characteristics of the animals, the animals in all groups failed to gain significant weight, possibly due to a lack of the high caloric and fat content of rat pellets in their diet. This lack of weight gain may contribute negatively to the results of this study, with perhaps smaller growth changes occurring within this investigation. Towards the end of the experimental period a few individual animals, not limited to the supplement group, displayed some gastrointestinal effects. Whilst initially thought to be a side effect of the supplementation, the lack of specificity for this group alone indicates that perhaps this was due to a reduction in the fiber content in the rat's diet in comparison to normal rat pellets. This finding along with the minimal gain in weight in the animals,

were not discussed in the Beren *et al* study. A follow up study of G and CS supplementation in rats utilizing Nutrigel as the base found small amounts of ground rat pellets on top of the Nutrigel and supplement mix resulted in weight gain and normal excretory function in the animals. Therefore the author advises that a repeated investigation may be required, with additional ground rat chow added to the food delivery in order to maintain normal gastrointestinal function and correct weight gain.

Appliance design

While studies have shown consistent morphological changes to occur in non-human primates subjected to functional appliance treatment [2,8,13], there have been some discrepancies reported in the literature in similar experiments within rats [4,64,65]. This discrepancy may be due to the difficulty in functional appliance design for the rat model, in order to achieve a reproducible and consistent mandibular advancement in the entire experimental population [66]. The morphology of the rat's dental arches along with the specific temporomandibular and symphyseal joints within these animals, provides them with large freedoms of movement in lateral and rotational movements of the lower jaw [67]. In addition, the rest position of the rat's mandible lies approximately 6mm behind the incisal edge to edge position and the position at which the molars come into occlusion is even further posteriorly [67]. This ability for large lateral excursions and their ability to retrude the mandible to a position of minimum discomfort and maximum rest creates difficulty in appliance design for mandibular hyperpropulsion of the mandible in the rat [66]. An appliance design suitable for hyperpropulsion should take into consideration an adequate vertical dimension in order to prohibit the animal from achieving a retrusion of the mandible backwards and downwards and if bonded as an anterior bite plane to the lower incisors should extend sufficiently anteriorly to inhibit functional retrusion [66]. In this investigation, some animals maintained a functional protrusion yielding different

regional remodeling changes in the condylar cartilage in comparison to those animals which functionally retruded. A follow- up study utilizing the same appliance design however with a more forward angulation of the pediatric strip crown forms created a more consistent functional protrusion. This appliance design was found to be effective, robust and enduring throughout the experimental period without negatively impacting on their ability to consume food.

Image analysis

Gadolinium chloride ($GdCl_3$) staining of cartilage prior to scanning with the MicroCT has been proven effective in enhancing the contrast of the cartilage[54]. The staining procedure within this study improved the visualization of the condylar cartilage and the ability to segment the cartilage from the bone digitally. The reorientation of all scanned samples into the specified condylar orientation using RotateScanLine software permitted accurate and comparable linear measurements which constituted the dimensions required to obtain the box volumes. Reorientation also enabled accurate digital hemi section of the condyles into anatomically corrected anterior and posterior halves. The segmentation tool on VGStudioMax was effective in calculating volumetric measurements of cartilage alone and total volume of the condyle. The volume of cartilage segmented from the remaining condyle as a result of the enhanced contrast from the staining, most likely incorporated only the articular, resting and proliferative layers of cartilage. Selection of grayscale values to incorporate more of the cartilage lead to measurement errors due to technical difficulties in eliminating the inclusion of bone in these measurements. Ease of segmenting cartilage from the bone was at its highest when cartilage thickness was greater for example in young animals, or those subjected to functional appliance therapy. However percentage error in cartilage measurements increased as cartilage became thinnest and was harder to distinguish and separate from the bone.

Qualitative analysis

The overlap of certain morphological characteristics discussed here in relation to class 3 and class 2 animals may be due to the animals ability to functionally alter their mandibular position. While assessments were made of the animals functional adaptations, these were only specific time points in the experiment and cannot be extrapolated to be a fixed adaptation. Animals were seen to be class II in a habitual resting position however postured forward to eat and chew. The morphological characteristics discussed were generalisations of each class type and overlapping features were found in a few samples.

Quantitative analysis

Total and Posterior Condylar Volume

Growth of the condyle has been found to display changes in response to hard and soft diet types in growing rats [68-70]. Documentation of the effects of a soft diet versus a hard diet in growing male Sprague-Dawley rats showed smaller condyles in length and width due to the reduced functional loading of the condyle[68]. Topographic changes in cartilage thickness were also noted with thinner anterior cartilage and thicker posterior cartilage in the soft diet groups[68]. In addition, the gradient of cartilage maturation and endochondral ossification differed in the regional areas of the condyle, with maturation slower in the superior part of the condyle and faster in the posterior part in animals fed a soft diet. These regional differences suggested the rate of differentiation and maturation of mesenchymal cells into chondrocytes may be influenced by the loading level of the cartilage[68,69]. The acceleration of differentiation and mesenchymal maturation of chondrocytes in soft diet groups compared to hard diet groups resulted in a thinner layer of cartilage, with an increase in erosion and endochondral ossification as a result of the accelerated maturation [68,69]. The decreases in condylar length and width and therefore total volume in the appliance free (control) groups within this study

were consistent with the findings in the literature. The normal growth pattern of the condyle over the 28 day period was a general reduction in volume as would be expected.

Functional appliance therapy in this study appeared to sustain the volume of the condyle rather than permit its natural decrease, perhaps due to functional stimulus and loading. When separated for functional retrusion (class 2) and functional protrusion (class 3) the effect could be seen clearly. The total condylar volumes of those animals in class 3 relation were found to sustain similar volumes for the time period. However in contrast, those animals in class 2 relation displayed a general increase in total volume which may be attributed to an increase in functional loading of the condyle. An enhancement of the transition from chondrogenesis to osteogenesis in the mandibular condyle due to functional appliance therapy has been documented in the literature in female Sprague-Dawley rats [17,16], with peak osteogenesis occurring at day 21 after appliance placement[17,50]. The maintenance of condylar volume in the functional appliance groups in comparison to no appliance animals may be interpreted as an increased stimulation of osteogenesis, with peak changes also coinciding to day 21.

Similar changes to total condylar volume were found to occur in relation to posterior condylar volume. General growth trends without functional appliances indicated decreasing posterior volume. The effect of functional appliance therapy again appeared to sustain this volume, however with a marginal increase in the class 2 group over time.

Limited literature exists on G and CS and their effects on bone. However in an investigation on treatment of osteoarthritis, recent evidence showed the stimulus for the initiation and progression

of cartilage degeneration may be originating from the altered bone metabolism in the subchondral bone[71]. Receptor activated nuclear factor-kB ligand (RANKL) is a factor essential for bone osteoclast differentiation and bone loss, while its decoy ostegoprotegerin (OPG) can block the binding of RANKL to its receptor RANK, thereby preventing osteoclastogenesis and as a result inhibit, bone resorption[72]. Chondroitin sulphate supplementation was investigated on human osteoarthritic subchondral osteoblasts, with results indicating the CS supplementation with or without vitamin D3 stimulation down regulated bone remodeling factor, RANKL and upregulated OPG [73], thereby inhibiting bone resorption. Therefore, by increasing the OPG: RANKL ratio CS may exert positive effects on the subchondral bone in osteoarthritic patients[73]. While results prove promising on the reduction of bone resorption due to CS supplementation in adult osteoblasts, no literature exists on the effects of these supplements on bone growth as experienced in the process of endochondral ossification. In this investigation, the effects of supplement therapy on total and posterior condylar volume were found to be insignificant.

Total and Posterior Cartilage Volume

A number of animal studies suggest that the proliferation and growth of the mandibular condylar cartilage (MCC) is altered after a change in the postural position of the mandible[1,4,6,74]. It has been proposed the growth of the MCC is adaptive, therefore the growth rate or direction of growth is transiently altered in response to a change in the MCC's normal functional setting (e.g. the growth of the midface or change in occlusion) [4,74] and then returns to baseline levels of growth once equilibrium has occurred. Mandibular condylar cartilage has been shown to exhibit characteristics of both growth plates and articular cartilage; however exhibiting a unique characteristic of biological adaptation to its biophysical environment. The ability to modify and enhance condylar cartilage remodeling with functional appliance therapy in the animal model has been well documented within

the literature [1,4,6,74]. Similarly attempts for a biochemical enhancement of cartilage growth have also been attempted [4,21,23-25].

Glucosamine and chondroitin sulphate supplements are commonly consumed neutraceuticals in the management of osteoarthritis due to their anti-inflammatory [39-41], chondroprotective [36,60] and potentially chondrogenic abilities [32,35]. Extensive research into these supplements exists due to their favorable safety profiles for long term use in patients suffering with degenerative cartilage disease. Investigations into the effects of these substances on cartilage volume as measured in 3 dimensions have never been published; neither have their effects on growing and healthy cartilage. Studies have shown chondrocytes subjected to G and CS supplementation under mechanical stresses will increase proteoglycan synthesis and that these substances may in fact act as "biological response modifiers" [32,35]. Tissues from younger animals however, were found to be less responsive to stresses under G and CS supplementation, than cartilaginous tissues from older animals[35].

In this investigation, normal total and posterior cartilage growth exhibited increases in volume by day 7 with decreases in volume continuing thereon. The presence of supplement therapy prior to the commencement of the functional appliance therapy had significant effects on the cartilage. Currently no literature exists on the effects of supplement therapy on epiphyseal growth plates or condylar cartilage growth. This finding may warrant future investigation into this field. Normal growth trends persisted in animals receiving supplements only, however at slightly greater cartilage volumes for the duration of the experimental period. Increases in cartilage volume as a result of functional appliance therapy in Sprague-Dawley rats of this age, with gradual decreases in cartilage as endochondral ossification ensues were consistent with the literature[14,18,49]. Within this study, functional appliance therapy alone increased the total and posterior cartilage volumes with peak

changes occurring by day 7 following with decreases in volume of cartilage thereafter while the posterior total condylar volume increased marginally by day 21 perhaps illustrating the enhanced transition from chondrogenesis to osteogenesis[49]. Interestingly, the posterior cartilage volume response differed in respect to class 2 and class 3 animals, with increased volumes found by day 21 in class 3 in comparison to class 2 animals. Experiments on functional protrusion have classically shown enhanced cartilage proliferation on the posterior aspects of the condyles[2,6,8] due to the stretching of the retrodiskal elastic band between the condyle and the glenoid fossa[8]. A functional retrusion enforced with a distally applied extra oral force to lower mandibular molars in *Macaca mulatta* monkeys showed with radiographical and histological examination; resorption at roof of glenoid fossa and anterior surface of the postglenoid tubercle, with extensive resorption at posterior aspect of condyle while anteriorly the condyle enlarge and compensatory bone deposition at posterior of insertion of lateral pterygoid muscle[13]. Relapse was found to occur with the relocation of the joint in the anterior direction with extensive remodelling was seen to take place during this period. Within this study, similar changes in condylar remodeling were found to occur with functional protrusion and functional retrusion, however with mildly differences in the retrusion probably due to the anatomical differences between the species used. In contrast however, functional retrusion in this investigation resulted in marginally increased volumes of cartilage and total condylar volume in contrast to the resorption noted from 2 dimensional data in the study mentioned above. While undulating surfaces in class 2 animals were certainly noted in axial slices through the condyles, it is however suggested these may be features of remodeling as volumetrically these samples maintained if not mildly increased their total and cartilage volumes. It is proposed in this study the extensive remodeling seen on the mediosuperior aspects of the condyles 2 and 3 dimensionally would most probably represent the areas of compression during function in the class 2 animals. The increase in remodeling after the appliance removal at day 28 concurs with the literature[13].

The effect of supplementation with functional appliance therapy appeared to be an enhancement of the normal biological response to the functional appliance therapy. This cartilage response to mechanical stresses under supplementation is seen in the animals which had functional appliance therapy, particularly in the increased response of class 2 animals. In addition, the new stimulus after the removal of the functional appliances in both class 2 and class 3 animals receiving supplementation, resulted in cartilage volume increases which may indicate the supplements do in fact act as "biological response modifiers"[35].

Cartilage remodeling has been documented to occur in adult rats as a result of functional appliance therapy[16], whilst adult chondrocytes under supplementation have been found show greater response to mechanical stresses. Therefore, further study into the concomitant supplementation and functional appliance therapy on post pubertal or early adult rats may be an area of future investigation.

The results of this study suggest the growth of the condyle is not entirely genetically determined but has the capacity for adaptation and stimulation, not only mechanically but biochemically.

Conclusions

The purpose of this study was to investigate the effects of glucosamine and chondroitin sulphate supplementation on condylar cartilage during functional appliance therapy. The following findings are reported;

1.) Normal growth followed a decreasing trend in condylar volume.
2.) The effect of functional appliance therapy was to sustain the volume of the condyle over the duration of functional appliance therapy.

3.) Functional appliance therapy alone resulted in increases in cartilage volume over untreated animals. The effect of functional appliance therapy was to elicit changes in cartilage volume with peak increases occurring at day 7 followed by decreases as endochondral ossification ensued.
4.) Supplement therapy increases the volume of cartilage with or without functional appliance therapy.
5.) Supplement therapy appears to enhance the biological response to functional appliance therapy.
6.) Those animals which functionally retruded may have greater total volume and cartilage volume during functional appliance therapy due to increased loading of the condyle.
7.) Supplementation appeared to increase the response of cartilage between day 21 to day 28 as a new stimulus was initiated after the removal of appliances. This may indicate an increase in the stability of the outcomes of functional appliance therapy.

Whilst results have proved promising, further research in this field is warranted.

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