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Evaluation of Chitosan-Glycerol Phosphate in Experimental Osteochondral Joint Defects in Horses

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Abstract

The aim of the present study was to investigate the long-term effects of chitosan-glycerol phosphate (chitosan-GP) gel with the articular environment following implantation into experimental osteochondral defects in horses. The animals were submitted to experimental arthroscopy of both tibiotarsal joints for creation of osteochondral defects on the medial aspect of the lateral trochlear ridge. One hindlimb was randomly selected for treatment with chitosan-GP gel, while the contralateral hindlimb served as untreated control. Horses were followed at different time points over 24 months, by clinical, radiographic, ultrasonographic, and synovial fluid analysis. Enlargement of all operated joints occurred in both hindlimbs during the first 30 days. No radiographic signs of osteoarthritis appeared, in treated or untreated joints. Ultrasonography revealed similar defect filling tissue, and synovial fluid with similar characteristics in either treated or untreated control joints, with osteochondral defect areas progressively decreasing. Synovial fluid analysis have shown no differences in the total protein, prostaglandin E_2, chondroitin sulfate, and hyaluronic acid concentrations, and white blood cell count, when comparing treated to untreated control joints. In conclusion, Chitosan-GP is biocompatible with the equine articular environment, and did not cause relevant clinical effects, inflammatory response or toxic effects in the horse joints.

Keywords: Horse; Joint; Osteochondral defects; Chitosan-GP; Prostaglandin E2; Chondroitin sulfate; Hyaluronic acid; Synovial fluid; Ultrasonography; Radiography

Introduction

Chitosan is a hydrophilic polysaccharide obtained by alkaline deacetylation of chitin. Chitin, in turn, is an insoluble linear polysaccharide composed by repeating units of β(1,4)-D-N-acetylglucosamine, abundant in the exoskeleton of arthropods such crustaceans, mollusks, and insects. It is also present at the cell wall of certain fungi [1]. Chitin preparations, obtained as a byproduct of shrimp and crab industry, are commercially available.

Partial deacetylation (75% or above) of chitin renders the polymer fully soluble in dilute acids, such as formic and acetic acids, below pH 6 [2], and this polymer is named "chitosan". Chitosan may be used as a gel for direct application (depending on its concentration, molecular weight, pH, and deacetylation degree [3,4]), or production of derivatized biomaterials.

The applications of chitosan-based products have markedly increased over the last 30 years, ranging from pharmaceutical excipient and drug delivery systems [5] to complex tissue engineering and use as scaffold for cell migration [6].

Among the limitations in the clinical use of chitosan is the need for sterilization, given exposure to high temperatures may affect the product solubility, appearance and chemical structure, while gamma radiation may interfere with the product's biological properties due to fissure formation and dose-dependent loss of viscosity [4].

Many chemical derivatizations have been used to modify chitosan mechanical properties and stability, as well as to promote new biological activities [7,8]. Chitosan-glycerol phosphate (GP) is one of these derivatized products. The addition to chitosan of polyols, such as sodium glycerol phosphate, creates a hydrogel that forms a viscous liquid at room temperature or below, and converts to a semisolid gel at body temperature [9-11]. Chenite et al. [9] described a procedure to obtain a neutral chitosan-GP preparation (pH 7.15), which resists to autoclaving (pH 6.8-7.2), and is biodegradable. So, this is a promising biomaterial to treat osteochondral defects.

A wide array of matrices or hydrogels have been used as biomaterials in joints, including polysaccharides (agarose, alginate, hyaluronic acid and chitosan), proteins (collagen and fibrin), and other polymers (polyethylene glycol, poly-lactic acid) [12]. The ideal scaffold should mimic the naturally occurring environment in the articular cartilage extracellular matrix, allowing chondrocyte proliferation and synthesis of cartilage ECM, which is composed by a dense network of collagen fibers [13], proteoglycans, and other proteins [14]. Chitosan-based hydrogels are promising due to their structural analogy to glycosaminoglycans.

Cell implant, using both fully differentiated chondrocytes and progenitor cells (mesenchymal stem cells) have been used to produce hyaline-like cartilage [15,16], and the biomaterials to be used as cell-delivery vehicles must meet stringent requirements in that the material

should be non-toxic, non-immunogenic, and must either integrate with the repair tissue or degrade without generating toxic by-products or leaving gaps, debris or fissures in the tissue [17].

The biocompatibility of chitosan with chondrocytes has been studied in vitro [18-20], and many authors have shown that it can be used, alone or in combination with other polymers, as a scaffold for chondrocyte proliferation [21-24]. Furthermore, chitosan and chitosan-derived polymers have been shown to be biocompatible with the articular environment in rats [25], rabbits [26,27], and sheeps [28]. It seems that chitosan-based gels remain in place for at least 1 week, giving time for migration of chondrocytes and synthesis of extracelular matrix components [29].

However, studies in horses are lacking. This is of particular interest due to the high incidence of osteochondral lesions in athletic horses (e.g., intra-articular fractures, dissecting lesions, or refractory osteoarthritis), and because the regeneration in high load joints, submitted to strong shear forces, is certainly different from the processes that occur in smaller animals, such as rats and rabbits.

The reports on the successful use of chitosan, both as drug releasing agent and as cell scaffold, have motivated the investigation of its applicability in osteochondral defects of horses. Chitosan-GP was chosen because of its thermo- and auto-gelling properties, which permit that it should be loaded into an osteochondral defect in living joints as a viscous liquid, and upon application, forms a gel and adheres to bone and cartilage.

The aim of the present study was to evaluate the long-term biocompatibility of chitosan-GP in the horse articular environment, following implant into experimentally created osteochondral defects. Physical exams, radiographic and ultrasound images, as well as synovial fluid analyses are here described. The analyses of tissue samples obtained at the beginning of the experiments and on the 180th day, by chondral biopsy, have already appeared [30].

Materials and Methods

Animals

The experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee (protocol number: 1245/2007). Six healthy Mangalarga male horses, aged 3 years and weighing 300-315 kg, owned by the University, were used in this study. Laboratory, radiographic and ultrassonographic exams were performed before inclusion in the experimental group, and all were considered normal. Horses had no history of tibiotarsal lesions and no clinical signs of hindlimb lameness on dynamic examination or on hock flexion test. The animals were housed in single 12 m^2 boxes (3 x 4 m) and fed pellets (1% of the animal body weight), coast cross hay and water ad libitum.

Chitosan-glycerol phosphate (GP) gel preparation

Chitosan-GP gel was prepared as proposed by Chenite et al. [9]. In brief, chitosan (200 mg, Cat #9012-76-4, 419419, Sigma-Aldrich, MO, USA) of 310-375 kDa (based on the viscosity range of 800-2000 mPaS), deacetylation grade >75% (200 mg), was solubilised in 0.1 M hydrochloric acid (9 mL). To this solution, 560 mg of sodium glycerol phosphate (Cat #55076-41-1, Sigma-Aldrich, MO, USA) dissolved in 1 mL of water were added, slowly and under continuous agitation. A pale gel resulted, which was transferred to glass tubes, sterilized in autoclave (120˚C, 20 min), and stored for no more than 24 h at room temperature.

Application of chitosan-GP gel

Tibiotarsal joint arthroscopy was performed with horses in dorsal recumbency under general inhalation anesthesia. One joint was randomly selected as the experimental "treated-joint". An osteochondral defect (10 mm diameter and 5 mm deep) was created on the medial aspect of the lateral trochlea of the talus, in right or left leg, using a bone burr attached to a power shaving system. This osteochondral defect was filled up with chitosan-GP gel (0.5 mL, containing 10.25 mg of chitosan-GP). The gel was delivered using an insulin syringe (1 mL). For gel delivery, fluid infusion was replaced by CO_2, using a laparoscopic insuflattor calibrated to maintain 30 mmHg until the end of the procedure, allowing the gel retention in the defect (Figure 1). The chitosan-GP gellification and adhesion to the defect was confirmed 1-2 min after application. Afterwards, the gas was drained and the skin was sutured with a Sultan pattern using no 0 suture, and compressive bandaged.

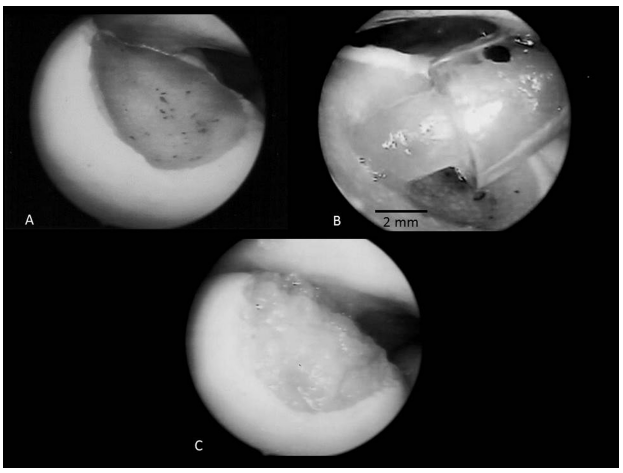

Figure 1: Arthroscopic image of the experimental procedure: (A) osteochondral defect on the medial aspect of the lateral trochlear ridge of the talus; (B) filling of the osteochondral defect with chitosan-GP; (C) osteochondral defect filled up with chitosan-GP.

One of the experimental horses died due to anesthetic complications, after the complete arthroscopic procedure and chitosan-GP application. This animal was used to check if the implant remained in place. The joint was submitted to 100 complex movements of flexion and extension, and was afterwards opened. It was found that the implant remained firmly attached to the osteochondral defect, presented smooth surface, and was hard to remove from the defect, indicating its molding and firm adhesion to the surrounding tissues.

Amikacin 15 mg/kg BW, IV, q48 h for 3 d, and phenylbutazone 4.4 mg/kg BW, IV, q24 h for 3 d, were administered 2 hours preoperatively and continued postoperatively. The surgical wound was cleaned daily until suture removal was done on the 10th postoperative day. The horses were rested for 180 days.

In the contralateral joint, an identical ostrochondral defect was created, but this one received no implant, and served as "untreated-control".

On the 180th day, arthroscopy was repeated, and a chondral biopsy was performed [30].

Physical, radiographic and ultrasonographic exams

Physical exams were performed daily, by blind evaluators, from day 0 (preoperative) to day 14, and weekly from days 21 to 180. The animals were again examined at the 720th day (2 years postoperative). The tibiotarsal joints were evaluated for effusion, enlargement, presence of heat, response to palpation, and response to passive flexion tests.

Radiographic images of the tibiotarsal joints were obtained on days 0 (preoperative), 1 (immediately postoperative), 7, 14, 21, 30, 60, 90, 120, 150, 180 and 720 using a Poskom PXP 20 HF X-ray machine. Radiographic images were analysed by two blind specialists. The following views were obtained from both experimental and control joints: lateromedial, dorsoplantar, dorsolateral-plantaromedial, dorsomedial-plantarolateral, and flexed lateromedial. However, only the dorsomedial-plantarolateral images were used to assess the experimental osteochondral defects. These radiographic images were scanned and analysed by "ImageJ" software (NIH, USA). The following parameters were observed: area of the osteochondral defect (cm^2), appearance of the defect margins (well-defined or poorly defined, regular or irregular), characteristics of the material inside the defect (homogeneous or heterogeneous, radiolucent or radiopaque), and presence of sclerotic lines (presence or absence). Osteoarthritis diagnosis was based on the criteria proposed by Kirker-Head et al. [31].

Ultrasonography was performed on days 0 (preoperative), 1 (immediately postoperative), 7, 14, 21, 60, 120, 180, and 720, using a linear 7.5 MHz probe and ALOKA 900 ultrasound machine, again by two blind investigators. The ultrasonographic parameters considered were: osteochondral defect area (cm^2), area of the medial recess of the tibiotarsal joint (cm^2), and echogenicity of the material filling the osteochondral defect bed (anechoic, hypoechoic or hyperechoic; homogeneous or heterogeneous).

Synovial fluid analysis

Synovial fluid samples were collected before surgery (day 0, preoperative), and on days 7, 14, 21, 30, 60, 90, 120, 150 and 180 postoperative. The samples were aliquoted: one of them (100 µL) was immediately used for white blood cell (WBC) counts, while others (0.5 mL each) were stored at -80°C and used to quantify total proteins, urea, prostaglandin E_2 (PGE_2), and glycosaminoglycans. These analyses were performed at the end of the experiment (after the 180th day).

As above mencioned, WBC counts, both total and differencial, were performed in synovial fluid fresh samples. Total WBC were counted in Neubauer chamber (Hirschmann, Eberstadt, Germany), while differential counts were performed in smears stained with May-Grümwald-Giemsa dye.

Total protein was measured by the biuret method using an automated biochemical analyzer (Randox RX Daytona Chemistry Analyzer – Crumlin, County Antrim, UK). PGE_2 was quantified by ELISA, using the PGE_2 EIA kit (Monoclonal, Cayman Chemical Co., Ann Arbor, MI, USA) [32].

Hyaluronic acid (HA) and chondroitin sulfate (CS) concentrations were determined as previously described [33]. In brief, synovial fluid samples (100 µL) were submitted to proteolysis, debris was removed by centrifugation, and the glycosaminoglycans were precipitated by addition of methanol (3 volumes). After standing at -20°C for 24 h, the precipitate formed was collected by centrifugation (3,000 x g, 15 min), vaccum dried, and resuspended in water (50 µL). Aliquots (5 µL) were submitted to agarose gel electrophoresis in 0.05 M 1,3-diaminopropane buffer, pH 9, and stained with Toluidine Blue in 50% ethanol:1% acetic acid (for CS), and then in 0.05 M sodium acetate buffer, pH 5 (for HA), as previously described. The compounds were quantified by densitometry of the agarose gel slabs.

To correct for the possible dilution of the synovial fluid samples, urea concentrations were measured by urease-glutamate dehydrogenase, using an automated biochemical analyser. Total protein, PGE2, HA and CS concentrations are given as urea ratios.

Statistical methods

Data were evaluated for normality using the Kolmogorov-Smirnov test. Analysis of variance was employed for comparison between groups and time points (baseline and postoperative days). Means were compared using the Tukey-Kramer test. The Kruskal-Wallis and the Mann–Whitney non-parametric tests were used for intra-group and inter-group comparisons respectively. The level of significance was set as 5% ($P<0.05$).

Results

Physical, radiographic and ultrasonographic exams

Physical exams revealed enlargement of all operated joints (~15%), both experimental and controls, up to the 30th day postoperative ($P<0.05$) (Figure 2).

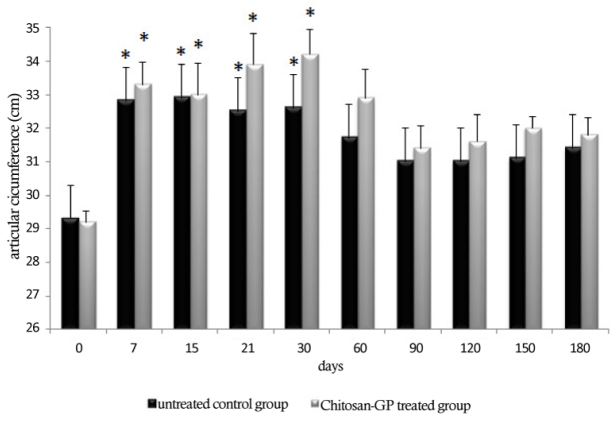

Figure 2: Articular circunference (mean ± standard error) of treated and untreated control tibiotarsal joints. *Differences statistically significant as compared to each group baseline ($P<0.05$).

This enlargement, which is due mainly to soft tissue swelling, slowly decreased afterwards. On the 180th day, joints were still slightly increased (~8%), but in the 720th day all were normal in size. All of the other parameters: effusion, presence of heat, response to palpation, and response to passive flexion tests, were absent at the 30th day and remained so up to the end of the experiment.

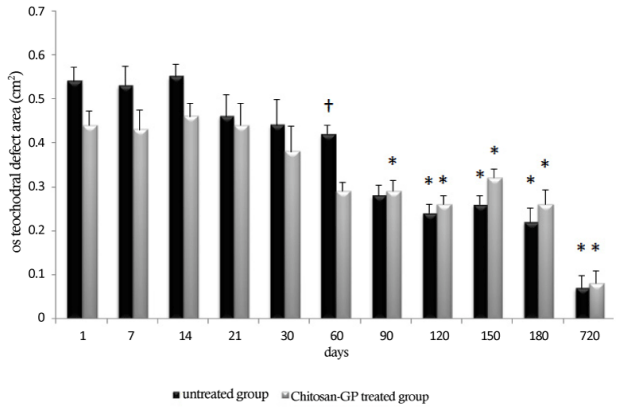

Figure 3: Osteochondral defect area (mean ± standard error) measured in postoperative radiographs of treated and untreated control tibiotarsal joints. †Differences statistically significant as compared to untreated control group (P<0.05); *Differences statistically significant as compared to each group baseline (P<0.05).

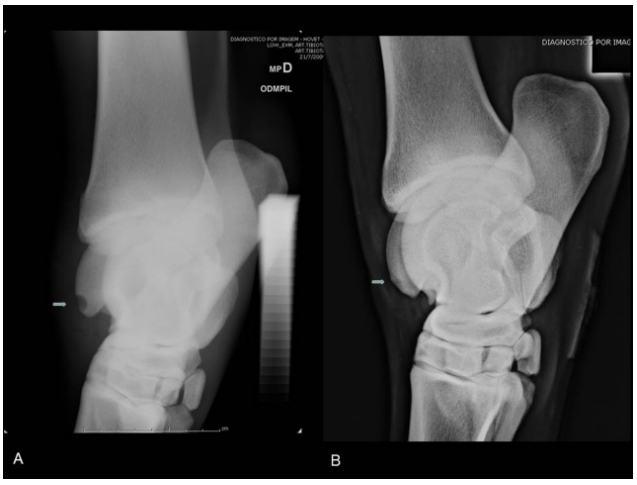

Figure 4: Radiographic appearance of the osteochondral defects (arrow) created on the medial aspect of the lateral trochlear ridge of the talus and treated with chitosan-GP gel on postoperative days 7 (A) and 720 (B).

Radiographic images obtained immediately postoperative revealed that chitosan-GP appears as a homogeneous and radiolucent material, clearly contrasting to the surrounding bone. Significant decrease (P<0.05) in the size of the osteochondral defect was observed from the 90th day on for treated, and from the 120th day on for controls joints. Comparing treated to control joints, significant differences in osteochondral defect area were observed exclusively on day 60 (P<0.05) (Figure 3).

Most chitosan-GP treated osteochondral defects had regular, well-defined margins on radiographic images up to day 21, while irregular, ill-defined margin predominated between days 30 and 180. In controls, the osteochondral defects presented well-defined margins up to day 60, and margin contour remained regular up to day 90, progressing to irregular ill-defined margins between days 90 and 180. At 720 days (24 months), most osteochondral defects still presented irregular ill-defined margins. A sclerotic rim surrounded treated as well as untreated osteochondral defects between days 14 and 180 days, but was no longer visible at the 720th day (24 months postoperative).

In chitosan-GP treated joints, the homogeneous radiolucent appearance of the osteochondral defect remained so up to the 21st day, and was slowly replaced by radiopache materials (days 30 to 180). Similar results were observed for untreated defects, except that radiopacity appeared later (days 90 to 180). Nevertheless, even at 24 months, radiopacity was still abnormal, and the osteochondral defects, filled with homogenous material of relatively low radiotransparency were observed in all joints (Figure 4).

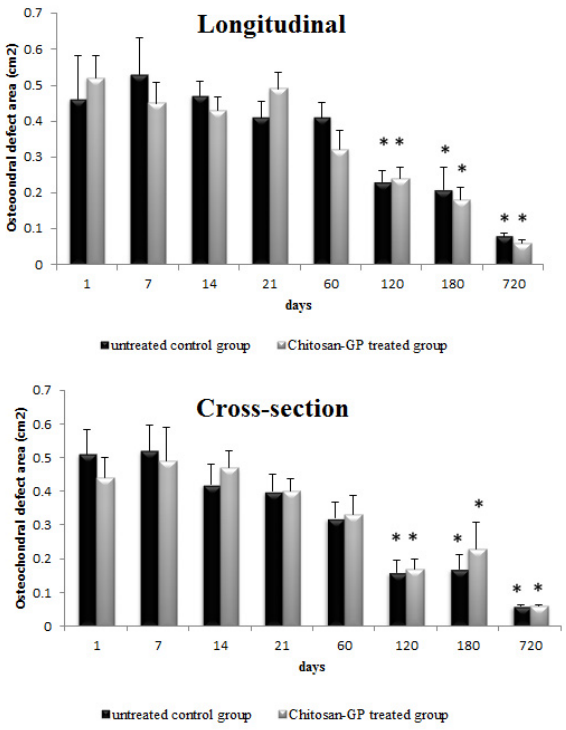

Figure 5: Osteochondral defect area (mean ± standard error) based on postoperative ultrasonographic measurements (cm^2; longitudinal and cross-sectional) performed in treated and untreated control tibiotarsal joints.

Ultrasonographic assessment revealed that osteochondral defect area decreased progressively along the experimental period in treated and untreated control joints alike. Longitudinal and cross-sectional defect area had significantly decreased in treated and untreated control joints at postoperative day 120, with no significant differences between groups. Incomplete defect repair was observed at 180 days. At the end of the 24-month (720 days) experimental period, defects were barely visible in both groups (0.06 cm^2 in both, longitudinal and transversal sections) (Figure 5). In one treated joint, the defect was no longer visible on postoperative day 720.

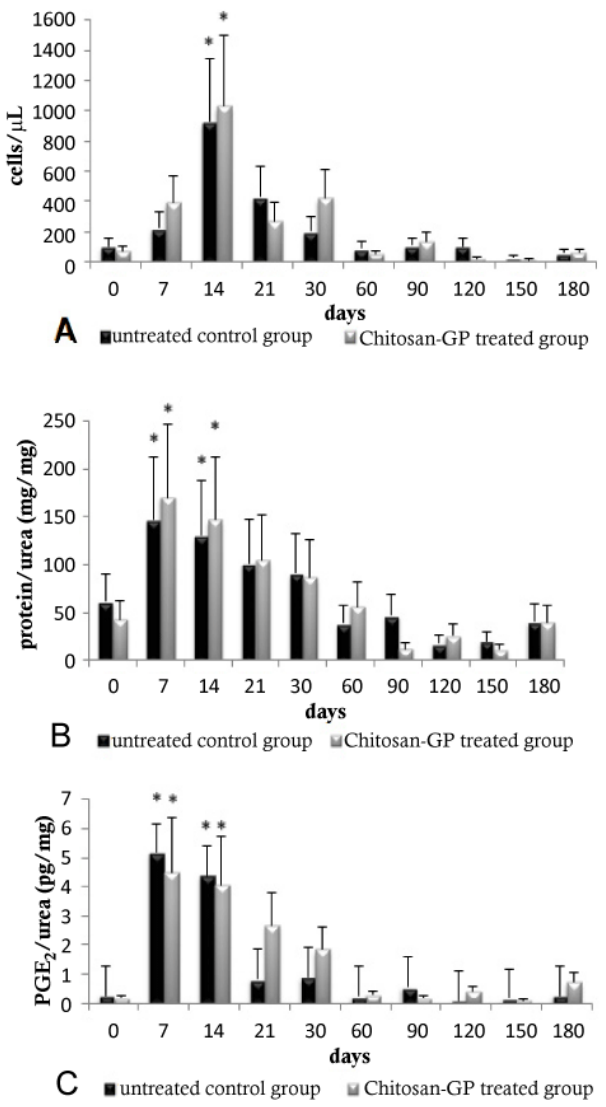

Figure 6: Nucleated cell counts (WBC, cell/µL), and concentrations (expressed as average ± standard error) of total protein, prostaglandin E2 (PGE_2) from untreated control and treated groups synovial fluid. All concentrations were expressed as urea ratios, to correct for possible fluid volume variations. *Differences statistically significant as compared to each group baseline (P<0.05).

Treated and untreated control osteochondral defects were filled with heterogeneous material up to postoperative day 21 and homogeneous material thereafter. Filling tissue echogenicity increased progressively from hypoechoic (up to day 180) to hyperechoic (day 720) in both groups.

Enlargement of the medial recess of the tibiotarsal joint up to day 60 postoperative, returning to preoperative normal values thereafter in all studied joints. Amorphous substance predominated in the medial recess of treated and untreated control tibiotarsal joints throughout the experimental period. Hyperecogenicity was noted on postoperative day 7, while anechoic material predominated at the other time points considered. On day 720, the medial recess of all joints studied contained anechoic material. Hyperechoic foci were more frequently observed in the treated group between days 1 and 60.

Synovial fluid analysis

Concerning the synovial fluid analyses, the WBC counts were increased in treated and untreated control joints at postoperative day 14 (P<0.05), with a predominance of neutrophils with discrete phagocytic activity. There was an increase in total protein concentrations on days 7 and 14 (P<0.05) in both groups when compared to their respective baselines. There was also an increase in PGE2 concentrations at days 7 and 14 (P<0.05), with a decrease after day 21, both in treated and untreated-control joints. From day 60 to 180, the PGE_2 concentrations were on the baseline in both groups (Figure 6).

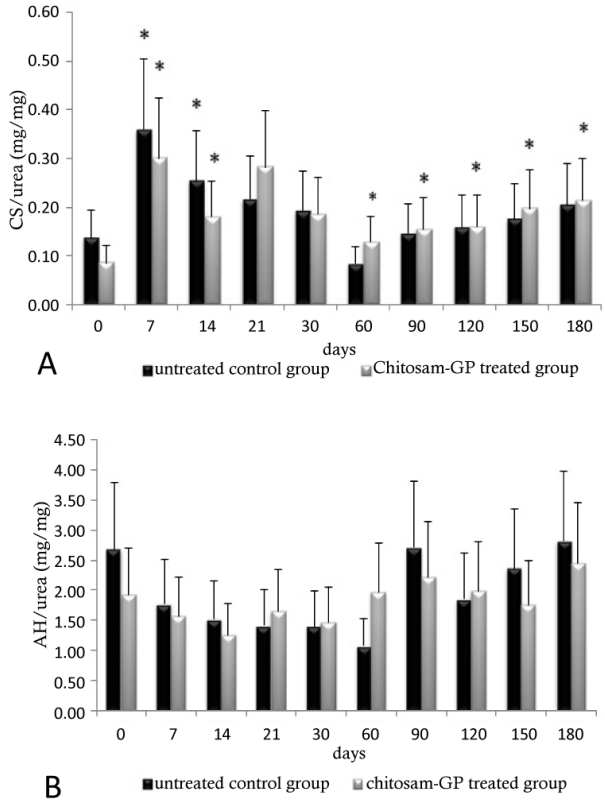

Figure 7: Concentrations (expressed as average ± standard error) of chondroitin sulphate (CS) and hyaluronic acid (HA) from untreated control and treated groups synovial fluid. All concentrations were expressed as urea ratios, to correct for possible fluid volume variations. *Differences statistically significant as compared to each group baseline (P<0.05).

The CS concentrations were increased at postoperative day 7 and 14 in both groups when compared to the baselines, and also at postoperative day 21 in treated joints. From day 60 to 180 an increase in CS concentration was observed when compared to the baseline values, statistically significant only in the treated group (P<0.05). No

significant difference was observed in the hyaluronic acid levels during the experiment (Figure 7).

Comparing chitosan-GP-treated to untreated-control synovial fluid, no significant differences were observed for WBC counts, total protein, PGE_2, CS and HA levels.

Discussion

The aim of the present study was to investigate the biocompatibility of chitosan-GP in horse osteochondral defects. Our results indicate that chitosan-GP forms a molding gel at body temperature that remains "in place", and is slowly substituted by tissues with radiographic and ultrasonographic "nearly-normal" characteristics, eliciting minimal inflammatory reaction. Clinical exames, X-ray and ultrasound images, obtained during a long-term follow up of chitosan-GP-treated joints in comparison to untreated-controls, as well as the synovial fluid analysis, support our conclusions. Biochemical and histological analysis of the healing tissues have already appeared [30].

There is little information on postoperative duration of clinical signs following creation of experimental osteochondral defects in horses [34]. While Hurtig et al. [35] reported postoperative pain, Sams and Nixon [36] observed minimal pain and joint effusion following experimental surgery. In a study by Litzke et al. [37], operated horses treated with anti-inflammatory drugs for 6 days following surgery showed no signs of lameness at the walk or trot, although chondral rather than osteochondral defects were evaluated. Frisbie et al. [38] also documented mild to moderate joint effusion and lameness up to the 10th week following the creation of osteochondral defects in horses.

Postoperative radiographic images in the present study suggested bone repair in treated and untreated control joints alike. Kirker-Head et al. [31] reported periarticular soft tissue swelling that decreased over time was the most prominent finding suggestive of osteoarthritis using the criteria proposed by.

At the end of the 24-month follow-up period, no degenerative changes were observed in operated joints. Radiographic findings suggest similar healing pattern in treated and untreated control experimental osteochondral defects.

Radiography enabled good assessment of the progression of the osteochondral healing process. Up to day 90, heterogeneous filling tissue and peripheral sclerosis were observed. From day 90 to day 180, the tissue filling the defect became progressively homogeneous and defect margins irregular and ill-defined, with more pronounced peripheral sclerosis. Irregular ill-defined margins and homogeneous filling tissue were observed 24 months. The sclerotic rim was no longer visible at this stage.

Differences in radiotransparency between the material filling the defect bed and the surrounding bone at 24 months suggest incomplete healing. In the study by Litzke et al. [37], radiotransparency persisted for 2 years after the creation of experimental partial-thickness articular cartilage defects in some horses. Therefore, full-thickness osteochondral defects, such as the ones in this trial, would likely take longer to heal.

Medial recess area was used to assess the degree of joint effusion and characterize the material contained within the joint. Anechoic material (i.e. no particles suspended) predominated in untreated control joints, whereas hyperechoic foci in the treated joints. Greater amounts of amorphous substance corresponding to enlarged synovial villi and fibrin were observed in treated joints in this study. Synovitis and joint effusion were therefore present in all cases, but these inflammatory signs subsided along the experimental period.

Longitudinal and cross-sectional defect areas were similar in both groups. The predominance of heterogeneous hypoechoic material in the defect bed in treated and untreated control joints on postoperative day 1 confirmed complete removal of the articular cartilage during the experimental surgery. At the end of the 24-month follow-up period the predominance of heterogeneous material in the osteochondral defect bed indicates incomplete healing. Incomplete healing and calcification at 24 months suggests the need for a longer healing period, supporting the results of radiographic assessment.

Articular defects in this study involved the removal of both, articular cartilage and subchondral bone (full-thickness or osteochondral defects [39]). Osteochondral defect repair tissue is of inferior quality compared to normal hyaline cartilage, therefore the selected experimental model may be considered appropriate for assessment and comparison of treatment efficacy. Surface area may also influence defect repair. According to Hurtig et al. [35], defects larger than 15 mm^2 tend to progress well in the first 5 months, but undergo degeneration over time. In contrast, femorotibial defects less than 3 mm in diameter tend to heal with minimal residual deformity [40]. In this study, circular defects measuring 0.78 cm^2 (78 mm^2) were created, thereby prolonging the healing period [30].

Experimental osteochondral defects creation promotes a mild, transient, inflammatory response in horses, since increased WBC counts, PGE_2 and total protein concentrations in synovial fluid were observed within 7-14 day in comparison to baseline values. Also, the synovial fluid CS concentration increased 7 -14 days after surgery, both in treated and untreated control joints. This time course suggests that the inflammatory mediators may stimulate cartilage catabolism, leading to CS increase in synovial fluid. It´s importante to note that these changes are not related to the chitosan-GP presence.

The WBC counts in treated and untreated control joints are lower than found by Nixon et al. [41] from synovial fluid of joints with defects filled with polylactic acid and fibrin in horses at the 7th postoperative day (2050 cells/μL). It was also lower than the results obtained by Wilke et al. [42] from synovial fluid of joints with defects filled with fibrinogen and mesenchymal stem cells in horses, on days 4 and 7 postoperatively (2320 cells/μL). Fortier et al. [43] observed increased WBC counts at 4 and 7 days after surgery in synovial fluid of joints with defects filled with or without IGF-I in horses. Regarding studies using chitosan-GP, Hoemann et al. [10] observed no significant WBC counts changes in defects receiving chitosan-GP when compared to the control group in sheep.

The synovial fluid CS concentration increased between 90 and 180 days, statistically significant on treated joints. This should reflects the cartilage high turnover rates that occurs during processes of repair, for this period no sign of joint inflammation was observed, since the synovial fluid showed normal concentrations of PGE2 and total protein, as well as WBC counts.

Our findings suggest that Chitosan-GP is compatible with the intra-articular environment in horses and did not cause relevant inflammatory or toxic effects in the horse joints. Future studies may show whether the GP Chitosan can be used as a scaffold for tissue engineering.

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