

Histologic and cytologic changes in normal equine joints after injection with 2.5% injectable polyacrylamide hydrogel reveal low-level macrophage-driven foreign body response

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OBJECTIVE

The data presented in this paper are derived from an in vivo study performed to characterize the nature of the synovial integration process of a 2.5% synthetic cross-linked injectable polyacrylamide hydrogel (2.5 iPAAG) injected IA in horses.

ANIMALS

10 healthy horses not suffering from OA or signs of joint disease were administered 50 or 100 mg 2.5 iPAAG in a total of 13 metacarpophalangeal or middle carpal joints.

METHODS

Injected joints were examined at 0, 14, 42, and/or 90 days postinjection. Parameters investigated included clinical examination, synoviocentesis, gross pathology, histology, and scanning electron microscopy.

RESULTS

All horses remained clinically normal, with no adverse events recorded throughout the study period. Gross postmortem did not reveal any significant findings. Arthrocentesis cytology parameters remained within clinically normal levels throughout the study. Synovial histology demonstrated that cellular infiltration of macrophages, villus hyperplasia, and vascularization were significantly higher in 2.5 iPAAG-injected joints compared to controls. Scanning electron microscopy confirmed that the 2.5 iPAAG demonstrated an extensive tissue integration as a 3-D scaffolding structure with intact cross-linked strands.

CLINICAL RELEVANCE

Results confirm that an IA injection of 2.5 iPAAG induces a typical foreign body response that is predominately macrophage driven with no evidence of fibrosis or mineralization. Integration of the gel is evident by 14 days, with no free gel remaining in the joint cavity at this time.

Keywords: equine, 2.5% iPAAG, histology, synovitis, macrophage

Osteoarthritis (OA) is a leading cause of lameness and morbidity and presents significant treatment challenges in animals and humans.¹ Although the pathophysiology of OA is still incompletely understood, it is now well recognized that the OA disease process typically starts with disease of the synovial membrane itself and is driven primarily by macrophages.² These synovial tissue macrophages appear to exist in a hybrid state of

activation that overall displays regulatory responses that are crucial for joint homeostasis but are unable to control inflammation when regulatory functions become impaired or overwhelmed.

A 2.5% synthetic cross-linked injectable polyacrylamide hydrogel (2.5 iPAAG) has been used for years in the bulking of soft tissues such as skin and the bladder neck.³ Intra-articular injection of 2.5 iPAAG is now used to treat OA in horses⁴⁻⁶ and humans,⁷ with significant effects on joint pain, joint effusion, lameness, and capsular stiffness. The 2.5 iPAAG is biocompatible, nonabsorbable, nonresorbable, and nondegradable, and since the material is chemically inert the

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mode of action is considered purely mechanical.^{8,9} Nevertheless, histologic studies of mice, rats, rabbits, pigs, goats, horses, and humans have shown that 2.5 iPAAG administration supports cellular growth and integration and possesses a permanent and stable augmentation effect due to constant water exchange with its host tissue.^{3,10} Data from clinical studies support that the clinical benefits remain long-term, up to 2 years in horses⁴ and up to 3 years in humans.⁷

This observational in vivo study was performed to characterize the nature of cellular changes in the synovium in response to 2.5 iPAAG injected IA into normal equine joints.

Methods

The study was conducted under animal ethics committee approval, and informed owner consent was obtained. A total of 10 Thoroughbred horses were included in the study, ranging in age from 3 to 5 years (median, 3.8 years), with 5 geldings and 5 mares. These animals were normal, healthy animals not suffering from OA or signs of joint disease (as determined by clinical lameness examination, including joint health and mobility and flexion tests) and chosen from available horses being retired from racing (for reasons unrelated to lameness such as age, poor racing performance, or financial constraints of the owners), horses rested from racing at a nearby training facility, or resident horses at the study facility.

Horses were kept in small 100 X 100-m grass paddocks consistent with local conditions, except for the

first 48 hours after treatment when they were restricted in a 4 X 4-m stall. All 10 horses were administered either 2 mL (50 mg) or 4 mL (100 mg) 2.5 iPAAG (ArthramidVet; Contura Vet) injected into a specific joint(s): the metacarpo-phalangeal (MCP) or middle carpal joint (MCJ), chosen at random in sequence as the horses were enrolled by the treating veterinarian. Doses were predetermined, as the purpose of the study was to demonstrate target animal safety at 1 or 2 times the recommended label dose in a single joint, or up to or 3 times the label dose in the target animal for regulatory purposes. Therefore, selected horses had multiple joints injected with varying doses but not all joints were sampled for synoviocentesis or histology.

Timing of sample collection was predetermined to address specific questions about what was happening at a cellular level in the joint tissues between 14 and 42 days when tissue integration and a clinical benefit are known to occur and to assess any longer-term effects of up to 90 days postinjection. Overall, synovial fluid was aspirated from a total of 10 horses/13 joints (**Table 1**). Synovial fluid was aspirated prior to injection of 2.5 iPAAG (at day 0) in 7 of the 10 horses, and these joints acted as controls.

Five horses had synovial fluid aspirated at day 14 postinjection from the same treated joint (3 MCP and 2 MCJ). Four horses had synovial fluid aspirated at day 42 postinjection, all from MCP joints. One horse had fluid aspirated at day 90 postinjection only but from a total of 4 treated joints (2 MCP and 2 MCJ).

On each of days 14 and 42, 2 horses (a total of 4) owned by the study sponsor were humanely

Table 1—Individual laboratory results from 10 horses/13 joints pre- and post-treatment with either 50 or 100 mg of 2.5% synthetic cross-linked injectable polyacrylamide hydrogel (iPAAG).

Horse	Articulation	Dose of 2.5% iPAAG	Timing	TNC ^a	RBCs	TP	Mononuclear cells	Lymphocytes	Neutrophils
H1	Right fetlock (MCP)	100 mg	Day 0	0.1	0.05	8.00	17%	83%	0%
H1	Right fetlock (MCP)		Day 14	1.7	0.05	6.00	99%	1%	0%
H2	Left carpus (MCJ)	50 mg	Day 0	0.1	0.05	22.00	67%	33%	0%
H2	Left carpus (MCJ)		Day 14	0.6	0.05	18.00	62%	38%	0%
H3	Left carpus (MCJ)	50 mg	Day 0	0.1	0.05	24.00	75%	25%	0%
H3	Left carpus (MCJ)		Day 14	0.9	0.05	20.00	61%	39%	0%
H4	Left fetlock (MCP)	50 mg	Day 14	1.2	0.05	8.00	68%	32%	0%
H5	Right fetlock (MCP)	50 mg	Day 14	0.2	0.05	7.00	26%	74%	0%
H6	Right fetlock (MCP)	50 mg	Day 0	0.1	0.05	24.00	29%	71%	0%
H6	Right fetlock (MCP)		Day 42	1.1	0.05	30.00	68%	32%	0%
H7	Left fetlock (MCP)	50 mg	Day 0	0.5	0.41	—	17%	11%	72%
H7	Left fetlock (MCP)		Day 42	1.4	0.05	10.00	43%	57%	0%
H8	Left fetlock (MCP)	100 mg	Day 0	0.1	0.05	27.00	67%	33%	0%
H8	Left fetlock (MCP)		Day 42	1.3	0.05	29.00	31%	69%	0%
H9	Right fetlock (MCP)	100 mg	Day 0	1.30	1.05	—	22%	41%	36%
H9	Right fetlock (MCP)		Day 42	2.60	0.05	14.00	28%	72%	0%
H10	Left fetlock (MCP)	50 mg	Day 90	0.1	0.05	6.00	99%	1%	0%
	Right fetlock (MCP)	50 mg	Day 90	0.4	0.05	6.00	95%	5%	0%
	Left carpus (MCJ)	50 mg	Day 90	0.3	0.05	17.00	99%	1%	0%
	Right carpus (MCJ)	50 mg	Day 90	0.3	0.05	15.00	99%	1%	0%

All results always remained within normal laboratory limits throughout the study. Horses 7 and 9 had blood contamination at the time of taking the pretreatment sample, meaning that no TP levels could be accurately determined at that time point. RBC (normal reference range, < 0.05 X 10¹²/L) and neutrophil levels were also markedly higher in pretreatment samples and as a result were excluded in the analysis.

MCJ = Middle carpal joint. MCP = Metacarpo-phalangeal. TNC = Total nucleated cell count (normal reference range, 0 X 10⁹ to 0.5 X 10⁹/L). TP = Total protein (normal reference range, 0 to 30 g/L).

^aStatistically significant difference in TNC (T-statistic, -7.67; pre = 0.33 X 10⁹/L; post = 1.37 X 10⁹/L; difference = 1.04 X 10⁹/L; 95% CI, P = .0003).

euthanized (sedation with xylazine, 1.5 mg/kg, IV, followed by a pentobarbital, 100 mg/kg, IV, bolus) and the fetlock or carpal joints were removed for gross and histologic examination of the joint capsule. Untreated joints on the contralateral limbs were used as controls.

The numbered slides used in the final analysis (**Table 2**) were those that had distinct cellular and architectural detail evident and that enabled the pathologists to precisely characterize the nature of the synovial reaction to the gel. All horses and limbs were observed daily for 2 days (48 hours) following treatment for swelling, effusion, and lameness and at weekly intervals throughout the study by an experienced equine veterinarian blinded to which joint had been treated.

Statistical analysis

Statistical analysis was conducted in Genstat 23 (VSN International) to determine any significant treatment effects on synovial fluid cell parameters and synovial membrane histology findings in response to administration of 2.5 iPAAG in normal equine joints. Synovial fluid cell parameters were analyzed by *t* test on paired pre- and post-treatment values within joint. Synovial membrane histology scores were analyzed by *t* test. No analysis of the difference between horses, time to sampling (14, 42, or 90 days), joints (MCP or MCJ), or interobserver findings was performed. Statistical significance was set to *P* < .05, at the 95% CI.

Arthrocentesis

Synovial fluid samples were obtained by an experienced veterinarian using a 1-inch, 20G needle attached to a 5-mL syringe and using aseptic technique following surgical preparation of the skin.¹¹ Horses were sedated

with 0.4 mg/kg xylazine hydrochloride and 0.01 mg/kg butorphanol tartrate IV. Synovial fluid (2 to 3 mL) was obtained, and all samples were transferred into a sterile EDTA test tube and transported in a chiller box (at 4 °C) on the same day as collection from the test site to an independent laboratory for testing. Sample color was observed and recorded and all samples routinely tested for total nucleated cell count (TNC), RBCs, total protein (TP), mononuclear cells, lymphocytes, and neutrophils. Results were recorded (Table 1).

Postmortem examination

Four horses were euthanized, and both the injected joints (2 MCP joints, 1 at 14 and 1 at 42 days; and 2 MCJ, 1 at 14 and 1 at 42 days) and the contralateral noninjected joint were opened and examined. Any subjective findings were recorded.

Samples of the joint capsule were taken from the following sites: metacarpo-phalangeal, dorsal and palmar aspects, and carpus, dorsal, and palmar parts of both the proximal and distal aspects of the middle-carpal joints.

Biopsy samples were either fixed in neutral-buffered 10% formalin for 24 hours and trimmed for standard histologic processing or collected into 0.9% saline and stored in a chiller box (at 4 °C) and transported overnight for scanning electron microscopy.

Synovial histology

Tissue samples were embedded in paraffin wax for histologic examination. Histologic sections were cut at 5 μm, stained with H&E stain, and examined for pathologic changes using light microscopy.

A histologic scoring system was devised and results from 19 slides examined from the 4 treated joints summarized (Table 2). Changes in the joint capsule were

Table 2—Histologic findings.

Site	Slide No.	Dose	Time postinjection	Subjective scores for cell hyperplasia, ^a villus hyperplasia, and the presence of 2.5 iPAAG in synovial membrane layers				Subintimal 1 macrophage infiltration ^b	Subintima vascularity ^c
				1 Intima	2 Subintima	3 Deeper CT/fat/capsule	4 External tendon/ligament		
Column No.				1	2	3	4	5	6
				Intima	Subintima	Deeper CT/fat/capsule	External tendon/ligament	Subintima	Subintima
RFF	6	100 mg	14 d	0	1	1	1	1	3
RFC (prox)	13	50 mg	42 d	0	1	1	1	1	2
RFC (distal)	15	50 mg	42 d	0	1	1	1	1	1
LFC (prox)	17	50 mg	42 d	0	1	1	1	1	1
LFC (distal)	19	100 mg	42 d	2	2	2	1	1	2
RFF	22	100 mg	42 d	2	1	2	1	2	2
LFF	23	50 mg	42 d	0	1	1	1	1	2
RFC (prox)	26	50 mg	42 d	3	1	1	1	2	1
RFC (distal)	27	50 mg	42 d	2	1	1	1	2	1
LFC (prox)	30	50 mg	42 d	2	1	1	1	3	1
LFC (distal)	31	100 mg	42 d	3	1	1	1	3	1
RFF	33	50 mg	42 d	NA	1	2	1	2	3
LFF	36	50 mg	42 d	0	1	1	1	2	3
RFC (prox)	38	50 mg	14 d	3	2	1	1	3	2
RFC (distal)	41	50 mg	14 d	3	3	2	1	2	0
RFF	43	50 mg	14 d	2	1	1	1	2	2
LFF	45	50 mg	14 d	1	1	1	1	1	2
LFC (prox)	47	50 mg	14 d	3	3	1	1	3	0
LFC (distal)	48	50 mg	14 d	1	1	1	1	2	2

Subjective scoring from a single observer for synovial cell hyperplasia, villus hyperplasia, presence of 2.5 iPAAG gel, stromal adipocytes, macrophage infiltration, and vascularity in synovial membrane layers (absent = 0, mild = 1, moderate = 2, marked = 3). Site coding is as follows: L = Left/R = Right; F = Fore/H = Hind; F = Fetlock; C = Carpus. Results of *t* test analysis are shown for each variable. Significant differences were shown (*P* < .001) for synovial hyperplasia,^a cellular infiltration,^b and vascularity^c between 2.5 iPAAG-treated joints and controls (set at fixed effect).

CT = Connective tissue. Prox = Proximal.

assessed and scored against noninjected controls by a single experienced observer using a subjective 4-point grading system they had developed (absent = 0, mild = 1, moderate = 2, and marked = 3) for synovial cell hyperplasia, villus hyperplasia, presence of 2.5 iPAAG in synovial cells, stroma and joint capsule, and the presence of stromal adipocytes. The same slides were also sent to a second board-certified veterinary anatomic pathologist for additional interpretation and comment.

Scanning electron microscope

A sample of fresh 2.5 iPAAG gel was prepared in the same way as tissue samples from injected joints. Samples were mounted in a brass cryo holder, then transferred to a Gatan Alto 2500 cryo preparation chamber/cryo stage (Gatan Inc). The chamber was kept at a constant temperature of -130°C . The sample was then sublimed (-98°C , 1 to 2 minutes) to remove a small amount of water from the surface of the tissue. The sample was then coated with approximately 3 nm of platinum to make it conductive for viewing. Samples were viewed in a JEOL JSM-6700F field emission scanning electron microscope (SEM; JEOL Ltd).

Results

The data in this *in vivo* study demonstrated that an IA injection of the 2.5 iPAAG induces a simple macrophage-driven phagocytic response to foreign material.¹²

Clinical parameters

Following injection with or without 2.5 iPAAG, all horses and joints remained clinically normal, with no adverse events recorded throughout the study period. No lameness, joint effusion, or reaction to flexion was reported at any time point.

Arthrocentesis cytology

Individual horse results were tabulated to enable a comparison between pre- and postinjection values (Table 1). In a total of 7 horses, preinjection samples from the same joint acted as controls. Horses 4, 5, and 10 had only postinjection samples taken. Horses 7 and 9 had blood contamination at the time of taking the preinjection sample, meaning that TP levels could not be accurately determined at that time point. Red blood cells and neutrophil levels were also markedly higher in preinjection samples as a result, and these were excluded in the final analysis.

Although case numbers were limited, all cytology parameters stayed within normal laboratory reference ranges throughout the study period. Furthermore, injection with 2.5 iPAAG did not cause any statistically significant changes in percentage of mononuclear cells, lymphocytes, TP content, or RBCs. A statistically significant increase was however observed between pre- and postinjection samples for TNC (mean pre, $0.33 \times 10^9/\text{L}$ vs post, $1.37 \times 10^9/\text{L}$; $P = .003$, 95% CI).

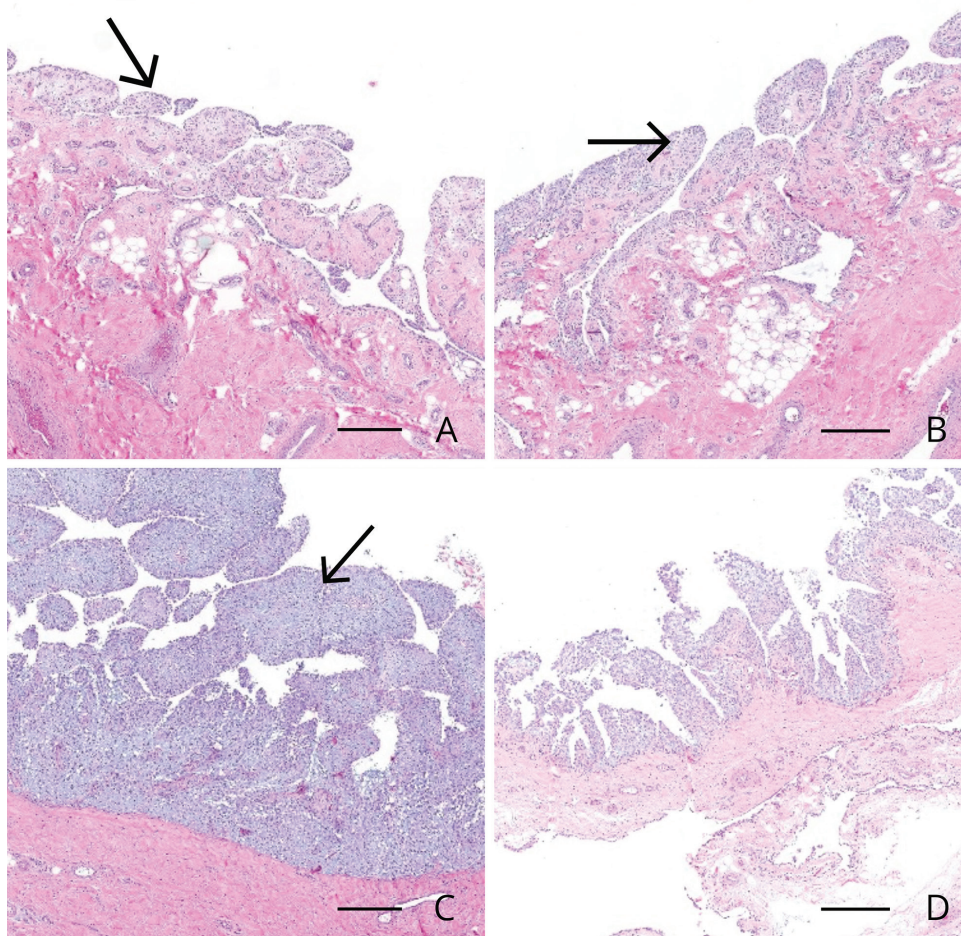


Figure 1—Amount and distribution of the gel. Photomicrographs show sections from equine fetlock joints 14 (A) and 42 (B) days after injection with 50 mg of 2.5% synthetic cross-linked injectable polyacrylamide hydrogel (2.5 iPAAG) showing small amounts of gel (blue) in the superficial subintima (arrows). The gel has a relatively intense fibrillar/beaded appearance, and there are further deposits of pale-staining finely fibrillar basophilic material in superficial and/or deeper layers of the synovium. Photomicrographs show sections harvested from the middle carpal joint (MCJ) 14 (C) and 42 (D) days after treatment with 50 mg 2.5 iPAAG showing intermediate amounts of gel in the subintima and thickened villous projections. H&E stain; bar = 100 μm .

In general, elevations in TNC were most apparent at day 14, with mostly medium-sized mononuclear cells (macrophages), some of which contained small, punctuated vacuoles (activated) and with fewer small, well-differentiated lymphocytes scattered throughout a background of pale-pink homogeneous glycosaminoglycan. These findings were interpreted as a mild nonsuppurative inflammation. These differences were less apparent but still present at day 42, with levels by that time point being more allied with preinjection levels. By 90 days (horse 10, 4 joints), nucleated cells were nearly all macrophages with only occasional rare small lymphocytes, and 20% of the macrophages were foamy cells (with vacuolated cytoplasm). Neutrophil levels remained consistent between pre- and postinjection in all samples at all time points (except in those cases with blood contamination).

Gross postmortem pathology

Gross postmortem did not show any significant findings. Two injected MCP joints (14 and 42 days) and 2 injected MCJ (14 and 42 days) were examined, with the same joint on the contralateral limb acting as a control. Upon opening the joint cavity, there was no evidence of 2.5 iPAAG gel coating the cartilage surface or the joint capsule or deposited in the joint space. Free 2.5 iPAAG could not be discerned in the apparently normal joint fluid, and smooth, glistening yellow synovium and

connective tissues appeared similar between injected and noninjected joints. There was some evidence of OA (minor cartilage defects/articular surface wear lines) present in both the MCP joints of 1 horse (injected and noninjected joints, data not shown).

Histology

Histology results from 2 independent observers are collated and described below. No analysis for interobserver variation took place. Synovial membrane histology scores were analyzed by *t* test at 95% CI (Table 2) with controls set at fixed effect. Overall scores for histologic assessment of the synovium for synovial hyperplasia (mean, 1.3; SE, 0.18; $P < .001$), cellular infiltration (mean, 1.8; SE, 0.18; $P < .001$), and vascularity (mean, 1.6; SE, 0.21; $P < .001$) were significantly higher in the 2.5 iPAAG-injected group compared to controls. No differences between the MCP and MCJ were assessed.

In injected joints, basophilic blue gel was not noted overlying the synovial membrane (ie, in the joint space) but was present in the superficial subintima in mostly small amounts in most sections (**Figure 1**). In the superficial subintima, the gel had a relatively intense fibrillar/beaded appearance and there were further deposits of pale-staining, finely fibrillar basophilic material in superficial and/or deeper layers of the synovium and external connective tissues, particularly in perivascular locations.

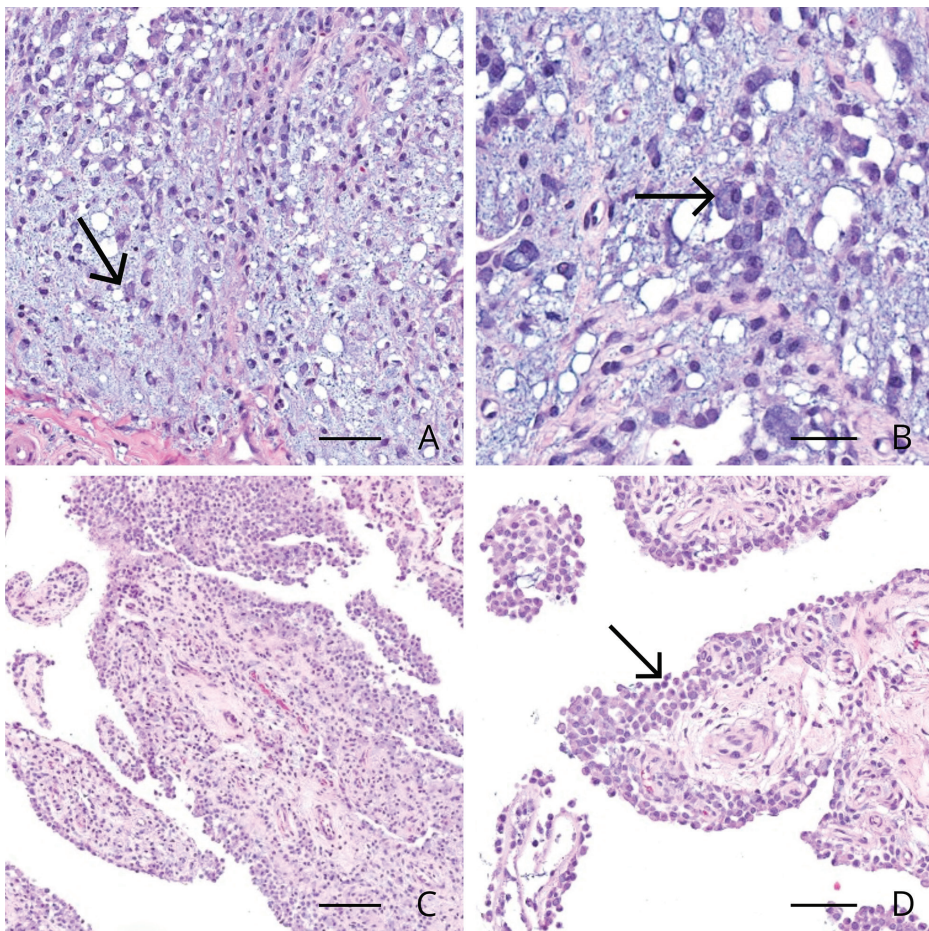


Figure 2—Inflammatory reaction in response to the gel. H&E stain. An inflammatory response is noted, consisting of variable but, in some sections, large numbers of macrophages and surface synoviocytes infiltrating the gel material. A—Fourteen days after treatment, depicting significant amounts of (blue) gel material with numerous macrophages infiltrating it in an attempt to phagocytose the material; bar = 50 μ m. B—A higher-magnification view (X40) 42 days after treatment showing the cytoplasm of macrophages (arrows) filled with basophilic gel material, and the gel material between them is granular with vacuoles (holes); bar = 20 μ m. In addition to the macrophage infiltration, in many sections (C) harvested 42 days after treatment the numbers of synoviocyte layers were increased (hyperplasia) and those cells swollen (hypertrophy); bar = 100 μ m. A higher-magnification view (D; X20) showing again the synoviocyte hyperplasia and hypertrophy (same horse) and the unique demonstration of a reactive synovial lining layer (arrows) with no evidence of neutrophil infiltration, fibrin deposition, cell death, or other inflammatory cell types; bar = 50 μ m.

An inflammatory response was noted, consisting of variable but, in some sections, significant numbers of macrophages and surface synoviocytes (type A cells, which are also macrophages) infiltrating the gel material (**Figure 2**). Nodular aggregates of macrophages were not noted (ie, there were no organized granulomas). There were occasional binucleate histiocytic (macrophage) cells with no evidence of neutrophilic inflammation, fibrin deposition, cell death, or other inflammatory cell types

but rather a simple phagocytic response to foreign material. There was no evidence of mineralization.

In contrast to the control joints, villous hyperplasia (**Figure 3**) was seen in many of the injected joints (to various degrees), with long, thick, and/or branching villous projections with mild to moderate hypertrophy 4 to 6 cells thick. Hypervascularity was also noted in most sections and in some involved all layers, including external tendon/ligament tissue. Fibroblasts were more prominent around blood vessels in inflamed synovium,

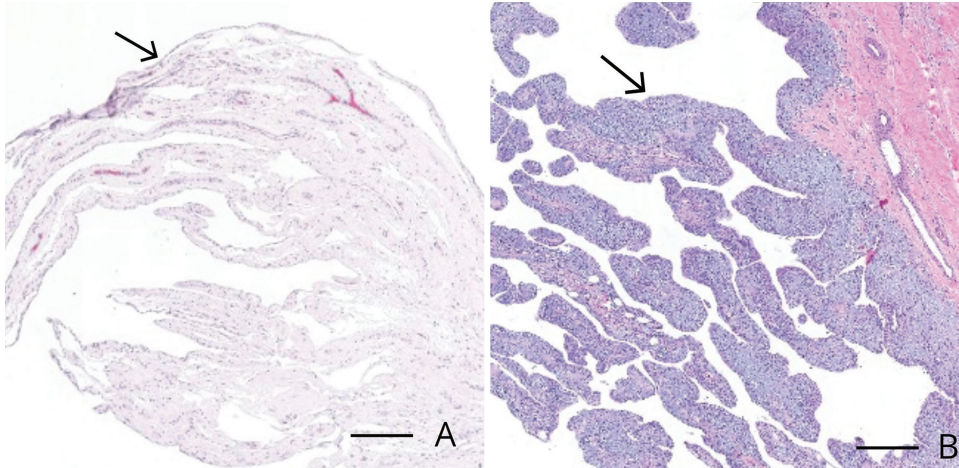


Figure 3—Villous hyperplasia is further demonstrated in these images comparing an untreated MCJ with a single layer of synoviocytes (A) with an MCJ 42 days after treatment (B) and depicting long, thick, and branching villous projections (core of subintima) lined on each side by synoviocytes and in which significant amounts of subintimal (blue) gel are noted. H&E stain; bar = 100 μ m.

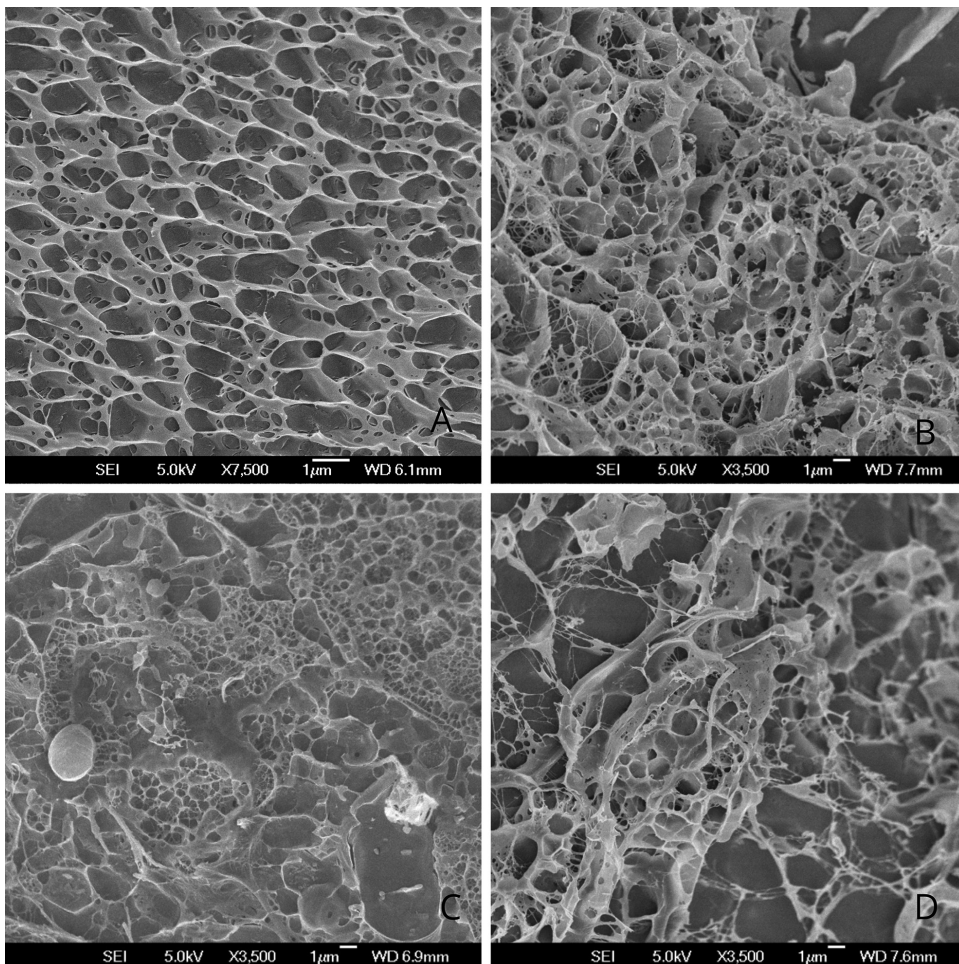


Figure 4—Scanning electron microscope images of the fresh gel (A) X 7,500 μ m showing typical uniform 3-D cross-linking structure. B, C, and D—Images X 3,500 μ m of joint tissue 42 days after injection with 50 mg 2.5 iPAAG showing integration of the gel with the surrounding host tissue. Cells and thin strands of connective tissue (collagen) fibers can be seen inside the gel, and the gel structure appears unaltered in appearance.

but overall differences between injected and noninjected joints were considered to be subtle. In control joints, the number of intimal blood vessels were variable and relatively numerous in some areas, indicating this could have been preexisting (presumably) subclinical disease.

Scanning electron microscope

Scanning electron microscope (Figure 4) confirmed that fresh 2.5 iPAAG had a physical and uniform 3-D scaffolding structure with intact cross-linked strands. In injected joints, tissue integration with the surrounding host tissue (synovial membrane) was extensive, as evidenced by gel traversed by thin strands of connective tissue (collagen) fibers. The gel structure appeared unchanged by this process, at least to 42 days in this study.

Discussion

This observational study investigating the synovial reaction, localization, and retention of 2.5 iPAAG in normal equine joints demonstrates that the 2.5 iPAAG causes a minimal transient cellular response in the synovial fluid before localizing in the subintima of the synovial lining, where it induces a reactive sublining layer that is predominately monocytic in nature.

Our findings are consistent with those of previously published studies of horses,¹⁰ with the notable exception that no free gel was discernible at gross postmortem in the joint cavity or adhering to the inner joint capsule as described at 4 months in an induced OA goat model by Tnibar et al.⁸ This was the first time some of the changes in synovial fluid cellular parameters have been characterized with the 2.5 iPAAG gel. This was also the first time that the uniformity of the 2.5 iPAAG molecule has been clearly demonstrated in situ with SEM imaging (Figure 4), an observation that in part may explain why the 2.5 iPAAG has such a unique biocompatibility and safety profile across multiple published studies of both animals⁹ and humans.⁷

The dose of 2.5 iPAAG recommended on the product label is 1 to 4 mL/joint (2 mL for the equine fetlock joint). The product has a nonpharmacological mode of action, so these recommended dose rates are based on what has been shown to be safe and effective in several independent studies,^{4-6,9} and up to 4 mL may be used at the discretion of the veterinarian. The data presented in this study were used for a margin of safety submission in the test country (at 1 and 2 times the recommended dose in a single joint and up to 3 times the dose in the target animal); therefore, up to a 4-mL (100-mg) dose (2 times the recommended dose) was used in an individual MCP or MCJ. No adverse events were reported. Interestingly, in human orthopedics a single injection of 6 mL of 2.5 iPAAG is now being used to alleviate symptoms in participants with moderate to severe knee OA.⁷ This may suggest that veterinary practitioners might also consider administering higher doses in some patients depending on the severity and duration of disease.

Christensen¹⁰ reported that tissue integration of the gel was evident as early as 7 to 14 days after injection in the horse, and as a result 14 days was the

first time point used in the current study. Injection with 2.5 iPAAG did not cause any statistically significant changes in the percentage of mononuclear cells, lymphocytes, TP content, or RBCs in synovial fluid. However, a statistically significant increase was observed for TNC following injection. For all parameters, however, it should be noted that case numbers were limited, and findings should be viewed in terms of clinical relevance. All parameters assessed in the synoviocentesis sampling remained within normal laboratory reference ranges throughout the study (between baseline day 0 and day 14, 42, or 90 postinjection). This minimal, transient cellular reaction in response to the gel, which is predominately macrophage driven, further supports the low level of irritation, antigenicity, and high degree of safety seen with administration of 2.5 iPAAG.

In consideration of these findings, the authors now recommend that patients may benefit from a period of reduced exercise (walking, swimming, treadmill, or light cantering exercise) while this transient inflammatory response and the integration process is taking place, at least for the first 10 to 14 days following treatment. It may also be important for veterinarians to understand this phenomenon when managing any post-treatment joint flare. On the basis of available published studies, the complication rate for IA injection of 2.5 iPAAG is estimated at 0.04%.⁹ In the authors' own combined experiences this is similar with only occasional cases (< 1:2,000 or 0.05%) exhibiting a mild transient joint edema that responds well to conservative therapy.

Gross postmortem examination showed no observable amounts of gel material overlying the synovial surface, and no free gel was observed in the joint space at any time point. Three of the authors performed the examinations concurrently, and findings could have differed if examinations had been performed at other time points or in diseased joints. Christensen et al.¹⁰ reported macroscopic findings from 13 horse joints (7 horses aged 5 to 13 years; median, 10 years) presented with veterinarian-diagnosed OA that had been injected with 2.5 iPAAG between 7 days and 2 years previously. They reported that free 2.5 iPAAG inside the cavity could not be discerned from joint fluid on naked-eye inspection but also described the PAAG appearing "as a thick, smooth, glistening, yellow substance or in the coffin joint as small, clear deposits along the inner part of the longitudinal tendon facing the cavity." Tnibar et al.⁸ in an induced OA model in goats at 4 months reported gel was seen "in various amounts adhering to the inner side of the joint capsule in all the treated OA goats." Diseased (OA) joints will often show signs of gross inflammation, such as synovitis and villous hyperplasia,¹³ which may affect the gross appearance and possibly even the uptake of 2.5 iPAAG in situ. However, 2.5 iPAAG is transparent and histologic findings in horse, goat, and rabbit joints all show similar histologic findings to those in our study; the gel is clearly demonstrated to be fully integrated into the subintima of the joint capsule between 7 and 14 days after injection.^{8,10} In rabbits, free gel was still discernible in the cavity at 10 days but not at 30 days.¹⁰ Therefore, in the authors' opinion, it is important not to interpret these statements as free gel residing in the joint cavity, but rather

that the transparent gel is integrated within a thin layer of transparent synoviocytes with induced synovial hypertrophy and hyperplasia.

The gel acts as a bioscaffold, enabling cell migration and vessel integration.^{9,10} In our study, the gel was seen as a blue granular (basophilic) material with a local tissue response consisting predominately of macrophages and surface synoviocytes that appear to be attempting, unsuccessfully, to phagocytose the gel material. This finding contrasts with an earlier study¹⁴ of a 4% PAAG that showed synovial macrophages were able to phagocytose that gel. This property presumably therefore relates to variations in chemistry and manufacturing that imparts differences in the molecular size, stability, and stiffness of different PAAG products.¹⁵ This may also explain the superior and long-term efficacy seen with 2.5 iPAAG in contrast to simple visco-supplement treatments like hyaluronic acid that are metabolized quickly after injection,¹³ triamcinolone acetonide, and hyaluronic acid combinations,^{6,9} as well as 4% PAAG that shows only a reduction in lameness grade in the single efficacy study published so far.¹⁶ In comparison, efficacy studies with 2.5 iPAAG have used complete resolution of lameness as their successful outcome measure.^{4-6,9} A direct comparison between 2.5 iPAAG and 4% PAAG could be a focus of future studies.

The synovial hypertrophy and villus hyperplasia observed in this study was described as a low-level phagocytic response to foreign material that was inflammation by definition. This type of local tissue response to a foreign body is typical in approved medical devices in humans¹² and importantly does not imply a high level of irritation or antigenicity. In our study, the inflammation showed no evidence of neutrophilic inflammation, fibrin deposition, mineralization, cell death, or other inflammatory cell types. Our findings support that the 2.5 iPAAG does not appear to act as a nidus for infection, nor does it cause fibrosis. Less is known about the performance or safety of other PAAG products, and the widespread and largely indiscriminate use of these products in some countries has caused serious long-term complications, mainly infection and granulomatous reactions.^{9,15} It is therefore important for veterinarians to consider this when selecting a particular PAAG product for clinical use.

Our findings clearly demonstrate that by 14 days 2.5 iPAAG is fully integrated into the subintima with the formation of a *de novo* intimal cell layer and does not remain in the joint space. It is not known whether integration of gel into the subintima affects joint pharmacokinetics, and specific comparisons between 2.5% iPAAG injected and noninjected joints should be a focus of future studies.

It has been suggested that the notable macrophage-driven cellular reaction in the synovial intima may act to improve the nature of the synovial fluid.^{10,14} Other studies, as well as the authors' own experiences, have recognized the limitations in the detection of inflammatory cytokines in synovial fluid, even in samples from patients experiencing marked inflammation.^{2,4,16,17} However, synovial fluid quality could be assessed with a simple viscometer, rheological analysis, tribology, or similar objective measures.¹⁸ Recent

advances in transcriptomic RNA analysis may also provide new opportunities in the future.¹⁹ We used nonclinical joints, and we did not attempt to evaluate the effects of the 2.5 iPAAG on any measures of synovial fluid quality or markers of inflammation, but this should be a focus for future studies.

Scanning electron microscopy was undertaken to gain a better understanding of the gel's physical response to integration. This enabled us to observe the 3-D structure of the gel. It is in the manufacturing process that the manufacturer utilizes its unique and proprietary (IL-X cross-linking) technology and where polymerization is progressing under tight temperature control to provide a minimum of variation in formed chain length and distribution of cross-links. It is this stable and homogenous gel structure that is accredited for the 2.5 iPAAG's stability, biocompatibility, and safety in comparison to other hydrogels.^{9,15} This uniformity is clearly demonstrated in the SEM images (Figure 4) and further illustrates the gel acts as a bioscaffold, undergoing extensive tissue integration. There was no evidence of any untoward effect on the gel structure itself (ie, the gel structure appeared stable and remained unchanged in appearance, at least by 42 days).

The 2.5 iPAAG does however induce significant macrophage-driven villous hypertrophy and hyperplasia as compared to noninjected joints and significant hypervascularity, although the differences between injected and noninjected joints for this variable were considered subtle. Notwithstanding, these findings may be inconsistent with what is normally considered desirable for joint health.¹³ All of the control joints were set at fixed effect for statistical analysis, which may have influenced the outcome of the analysis, but subjective observations from 2 independent and experienced equine pathologists also supported that there were significant histologic changes induced by the 2.5 iPAAG. However, multiple clinical trials have now demonstrated superior long-term (up to 2 years) clinical efficacy with complete resolution of lameness in 65.3% to 83.3% of horses treated with 2.5 iPAAG across a range of equine disciplines.^{4-6,9} Previous studies have suggested either a biomechanical mechanism of action through an increase in joint capsule elastance⁸ or some means of improvement in synovial membrane function.⁸⁻¹⁰ What is increasingly recognized, however, is that macrophages play a central role in both the synovial inflammation leading to OA and the restoration of the joint to homeostasis.^{2,20}

Our study had several limitations: the population of horses used in this study all had a history of being used previously for athletic pursuits (including racing) and so findings could indicate preexisting subclinical disease; a limited number of experimental animals were included, none with clinical evidence of OA; and subjective clinical measures were used, including reaction to flexion and effusion, different types of joints, variation in 2.5 iPAAG doses, and different time periods for follow-up. Some of our findings also highlight the importance in future studies of knowing the preexisting level of pathology when trying to estimate the effect of the gel, and even though our experimental design was aimed at minimizing variability, synovial histologic parameters could vary with joint and site within the joint, and this could also

have contributed to a degree of variability among samples. Also, no attempt was made in these normal joints to stain for nerve endings or perform joint capsule elastance studies of equine samples to compare with previous studies performed of goats with OA.⁶

In conclusion, injection of 2.5 iPAAG induces a low-level foreign body response, which is predominately macrophage driven, and with no evidence of fibrosis or mineralization. By 14 days, the gel is fully integrated into the subintima, with no free gel remaining in the joint cavity. These findings support previous studies that have found 2.5 iPAAG is safe, and our findings can be used to guide future directions for research and evidence-based treatment decisions with the likes of 2.5 iPAAG.

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