Concentrations of substance P and prostaglandin E_2 in synovial fluid of normal and abnormal joints of horses

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Objective—To correlate substance P content of synovial fluid with prostaglandin E_2 content, radiographic evidence of joint abnormality, and anatomic location of the joint for normal and osteoarthritic joints of horses.

Sample Population—Synovial fluid from 46 normal joints in 21 horses and 16 osteoarthritic joints in 10 horses.

Procedure—Normal and osteoarthritic joints were identified by clinical and radiographic examination, by response to nerve blocks, during scintigraphy or surgery, or by clinicopathologic evaluation. Substance P and prostaglandin E_2 contents of synovial fluid were determined by radioimmunoassay. Radiographs of joints were assigned a numeric score reflecting severity of lesions. Joints were assigned a numeric score reflecting anatomic location.

Results—Median concentrations of substance P and prostaglandin E_2 were significantly increased in osteoarthritic joints, compared with normal joints. A significant correlation was found between concentrations of substance P and prostaglandin E_2 in synovial fluid, but a correlation was not detected between substance P concentration in synovial fluid and anatomic location of the joint or between radiographic scores of osteoarthritic joints and concentrations of substance P or prostaglandin E_2.

Conclusions and Clinical Relevance—A correlation existed between concentrations of substance P and prostaglandin E_2 in synovial fluid obtained from normal and osteoarthritic joints. However, content of substance P in synovial fluid cannot be predicted by the radiographic appearance of the joint or its anatomic location. Substance P and prostaglandin E_2 may share an important and related role in the etiopathogenesis of osteoarthritis, lending credence to the importance of neurogenic inflammation in horses. *(Am J Vet Res* 2000;61: 714–718)

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Osteoarthritis is a disease of synovial joints characterized by variable degrees of pain, synovitis, and degeneration and erosion of articular cartilage.[1] The etiopathogenesis of osteoarthritis is not completely understood. Many investigators have focused on cytokines as principal mediators of this disease process.[2-7] Others have found evidence for involvement of the nervous system and its products, particularly substance P and other mammalian neurokinins,[8-13] in the early phases of joint inflammation.

Substance P is a neuropeptide composed of 11 amino acids.[14] It is widely distributed within the CNS and peripheral nervous system. In the latter, it resides in small-diameter primary afferent neurons, where it is implicated in the reception, propagation, and transmission of nociceptive impulses to the CNS.[14,15] Evidence implies that release of substance P in response to stimulation of primary afferent neurons initiates a local inflammatory response.[14,15] Substance P can induce vasodilation[16] and activate macrophages, B lymphocytes, polymorphonuclear cells, platelets, and mast cells.[13] Infusion of substance P into joints enhances the production of prostaglandins and collagenases by synoviocytes[17] and also increases the severity of joint inflammation.[8,18] Conversely, use of capsaicin in rats to inhibit substance P has been documented to suppress joint inflammation.[18]

It is likely that substance P plays a primary role in the etiopathogenesis of osteoarthritis in horses. Equine synovial membranes are innervated by substance P-containing nerve terminals,[19-21] and the amount of substance P in abnormal synovial fluid of horses is increased substantially.[19,22] In the study reported here, we measured the content of substance P in synovial fluid obtained from horses with normal articulations and those with osteoarthritis and correlated the values with content of prostaglandin E_2, an eicosanoid that also has an intimate role in articular inflammatory and nociceptive pathways.[23,24] In turn, Substance P concentrations were correlated with radiographic evidence of joint abnormality and anatomic location of the joint.

Materials and Methods

Animals—The research protocol was approved by an institutional animal care and use committee. Control horses consisted of 21 adult horses of various sex, breed, and age; samples of synovial fluid were obtained from 46 diarthrodial joints in these horses. For 10 affected horses, samples of synovial fluid were obtained from 16 diarthrodial joints with osteoarthritis. Criteria for selection of horses with osteoarthritis included horses that had clinical lameness for > 1 month, involvement of ≥ 1 joint as ascertained by clinical and radiographic examination, responded to nerve blocks, or had osteoarthritis diagnosed during scintigraphy,

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surgery, or clinicopathologic evaluation. Horses whose joints recently had been injected with therapeutic agents were excluded from the study. Control horses did not have a history of lameness associated with the joints from which samples were obtained and did not have clinical or radiographic signs of joint abnormality. Synovial fluid samples were obtained aseptically with hypodermic needles and syringes, using mild tranquilization with xylazine hydrochloride (0.22 to 0.55 mg/kg of body weight, IV) when necessary. Joints were assigned a score (scale of 1 to 7) that reflected their anatomic location within the limb (**Appendix 1**).

Substance P radioimmunoassay—The generation and characterization of anti-substance P has been described.[25-27] A highly specific and sensitive radioimmunoassay for substance P was developed by several of the investigators (MSAK, RKA). Bolton-Hunter reagent[a] conjugated to substance P was iodinated, using the chloramine-T method.[26] Iodinated substance P was purified and used in the radioimmunoassay procedures. Anti-substance P was used at a final dilution of 1:120,000. At that dilution, sensitivity of the assay was 0.5 pg/ml. Prior to analysis, samples of synovial fluid were centrifuged at 10,000 × g for 30 minutes to remove any particulate material. Filtration through a sephadex G-25 column[b] was subsequently completed to separate peptides from larger molecular weight substances. Synovial fluid samples then were frozen at –80 C; samples were thawed at room temperature immediately prior to assay. Duplicate aliquots of 100 µl of synovial fluid were assayed for substance P.

Synovial fluid is viscous and proteinaceous; therefore, the authors performed preliminary studies to determine the best procedure to use to measure the content of substance P in synovial fluid. Recovery of exogenous substance P (8 ng/ml of synovial fluid) added to synovial fluids obtained from normal joints was monitored, using various extraction procedures for the neuropeptide. Among the methods tried were extraction of substance P with 2N acetic acid, methanol, 1N hydrochloric acid, or boiling phosphate-buffered saline solution as well as synovial fluid assayed without extraction. Extraction efficiency of these methods were as follows: 2N acetic acid, 52%; methanol, 61.4%; 1N hydrochloric acid, 65%; and boiling phosphate-buffered saline solution, 58%. Recovery for synovial fluid assayed without extraction was 83%. Thus, substance P recovery was optimal in unextracted synovial fluid.

Effect of synovial fluid on displacement characteristics of the substance P antibody-antigen reaction was evaluated. Analysis of results indicated that synovial fluid did not alter displacement characteristics of substance P radioimmunoassay standard curves. Substance P radioimmunoassay standard curves were run with substance P for concentrations ranging from 0.2 to 500.0 pg. Because substance P concentrations in synovial fluid could not be predicted, 25-, 75-, and 150-µl samples of synovial fluid were assayed in duplicate, ensuring a dose-response authentication of the assay as well as increased probability of at least 2 samples of synovial fluid having values within the usable slope of the standard curve.

A literature search was conducted[c] for the years 1965 through 1998, and reports of substance P content in normal and abnormal synovial fluid obtained from horses were recorded for comparative purposes.

Prostaglandin E_2 radioimmunoassay—Prostaglandin E_2 content was measured in synovial fluid, using a commercial radioimmunoassay kit[d] with ^{125}I tracer. Limit of detection for the assay was 5.3 pg of prostaglandin E_2/ml. A literature search was conducted[c] for the years 1965 through 1998, and reports of prostaglandin E_2 content in normal and abnormal synovial fluid obtained from horses were recorded for comparative purposes.

Radiographic analysis—Using standard radiographic techniques, routine multiple views were obtained of joints from which synovial fluid was obtained. Radiographs were evaluated by an observer (AT) unaware of the history or physical examination findings for each horse. Scores were assigned, using a scale of 0 to 5, for each of the following 10 criteria: periarticular soft-tissue swelling; soft-tissue mineralization; change in position of fat pad; increase in joint space; decrease in joint space; evidence of enthesiophytes; evidence of osteophytes; sclerosis of subchondral bone; erosive lesions; and evidence of avulsion or osteochondral chip fractures (**Appendix 2**). Scores for each of the 10 criteria were summed for each horse, and that value subsequently was correlated with data on content of substance P and prostaglandin E_2.

Statistical analysis—Unless indicated otherwise, all data were reported as median and range. Mann-Whitney analyses for nonparametric data were used to test for differences in concentrations of substance P and prostaglandin E_2 in synovial fluid obtained from normal and abnormal joints. Spearman-Rank correlation tests were used to determine the significance of correlations between substance P and prostaglandin E_2 concentrations in synovial fluid obtained from normal and abnormal joints, concentrations of substance P and prostaglandin E_2 in synovial fluid from abnormal joints and radiographic scores, and concentrations of substance P in synovial fluid from abnormal joints and anatomic location of the joints. For all analyses, a value of $P < 0.05$ was considered significant.

Results

Analysis of synovial fluid—Synovial fluid was obtained from 4 distal interphalangeal, 12 metacarpophalangeal and metatarsophalangeal, 26 tarsal, 16 carpal, and 4 femorotibial joints. Analyses indicated a significant ($P < 0.001$) increase in median synovial fluid content of substance P (22.9 pg/ml; range, 9.5 to 69.6 pg/ml) and prostaglandin E_2 (361.6 pg/ml; range, 7.4 to 2,205.9 pg/ml) in 16 osteoarthritic joints, compared with 46 normal joints (8.0 pg/ml; [range, 2.5 to 19.3] and 5.7 [range, 0.1 to 30.8] pg/ml, respectively). Synovial fluid prostaglandin E_2 values were increased (656.8 pg/ml; range, 19.2 to 1151.6 pg/ml) in all 7 osteoarthritic joints that had osteochondrosis dissecans lesions. When data for synovial fluid from normal and abnormal joints were combined (n = 62), a signif-

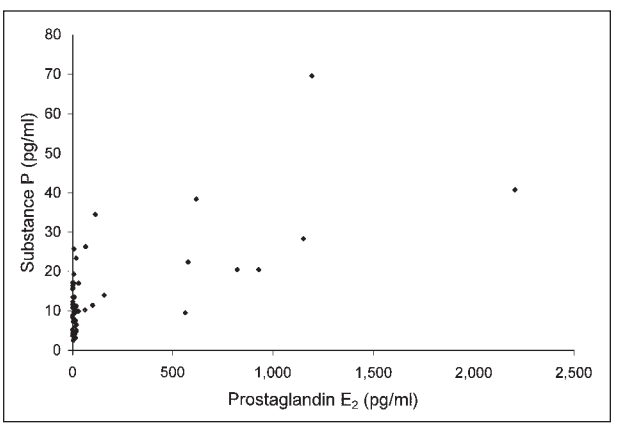

Figure 1—Graphic representation of the significant correlation (r = 0.68; P = 0.01; n = 62) between concentrations of substance P and prostaglandin E_2 in synovial fluid obtained from normal and osteoarthritic joints of horses.

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icant correlation (r = 0.68; P = 0.01) was found between concentrations of substance P and prostaglandin E_2 (**Fig 1**). Mean substance P concentrations in osteoarthritic joints were not significantly correlated with anatomic location of the joints.

Radiographic findings—Radiographic scores assigned for each of the 10 criteria in osteoarthritic joints ranged from 0 to 4. Maximum total score for a single joint was 16, and minimum total score was 2. Radiographic scores of the osteoarthritic joints were not significantly correlated with substance P or prostaglandin E_2 concentrations in synovial fluid. With the sample size available, the power of our test was only 80% for detecting a significant ($P < 0.05$) correlation larger than 0.65.

Discussion

The significant increase in synovial fluid concentrations of substance P and prostaglandin E_2 from osteoarthritic joints in the study reported here is consistent with other observations in horses,[19,22,24,28-31] which helps corroborate involvement of both biomolecules in the inflammatory process. Median concentration of substance P in synovial fluid from osteoarthritic joints in the horses in our study (22.9 pg/ml; range, 9.5 to 69.6 pg/ml) corresponded moderately well with data for osteoarthritic joints in horses in a study conducted by Caron et al[19] (mean ± SEM; 81.8 ± 10.1 pg/ml). Similarly, concentration of substance P in synovial fluid from normal joints in our study (8.0 pg/ml; range 2.5 to 19.3 pg/ml) correlated with those for normal joints of horses reported elsewhere (mean ± SEM, 50.2 ± 3.9 pg/ml[19]; mean ± SD, 50.6 ± 7.4 pg/ml and 75.2 ± 11.2 pg/ml[20]). Median concentration of prostaglandin E_2 in synovial fluid from osteoarthritic joints in the horses in our study (361.6 pg/ml; range, 7.4 to 2,205.9 pg/ml) also corresponded moderately well with data for osteoarthritic joints in horses in other studies (mean ± SD, < 12.5 to 940.0 pg/ml[24]; 144.9 ± 22.1 pg/ml[28] and mean ± SD, 53.9 ± 28.3 pg/ml[30]). Similarly, the concentration of prostaglandin E_2 in synovial fluid from normal joints in our study (5.7 pg/ml; range 0.1 to 30.8 pg/ml) corresponded with data for normal joints of horses reported elsewhere (< 12.5 pg/ml[24]; mean ± SEM, 26.5 ± 3.3[28] and 36.5 ± 12.2 pg/ml[30]). However, substance P synovial fluid concentrations reported here for osteoarthritic joints (22.9 pg/ml; range, 9.5 to 69.6 pg/ml) were substantially lower than those reported by Hardy et al (mean ± SEM, 720 ± 280 pg/ml[22]), who used an isolated equine limb in which the inflammatory response was induced by intra-articular administration of interleukin 1β.[22] Similarly, prostaglandin E_2 values (361.6 pg/ml; range, 7.4 to 2,205.9 pg/ml) were lower than those recorded in 2 other studies in which the inflammatory responses were initiated by intra-articular infusions of lipopolysaccharide (mean ± SEM, 3,600.0 ± 370.0 pg/ml[29]) or carrageenan (mean ± SEM, 79,190 ± 33,830 pg/ml[31]). Chemical induction of joint inflammation may prompt substantially greater release of substance P and prostaglandin E_2 than naturally developing disease. Alternatively, differing durations of inflammation may account for the discrepancies. All 3 studies[22,29,31] in which inflammation was chemically induced had samples of synovial fluid collected shortly after initiating the inflammatory process (2 to 9 hours) when prostaglandin E_2 and substance P activity was likely to be peaking.[32] By comparison, the synovial fluids retrieved from joints undergoing naturally developing inflammatory arthritis[19,24,28,30] reflected a chronic condition. An association between duration of disease and synovial fluid content of prostaglandin E_2 has been reported.[24] In that study, investigators recorded high concentrations of prostaglandin E_2 more frequently in horses during the early phase of osteoarthritis (3 of 4 horses) than in horses with chronic osteoarthritis (5 of 15 horses).

Other factors that can cause variability of synovial fluid content of substance P or prostaglandin E_2 within and between studies include differences in biomolecule extraction and assay techniques,[13] sample size of a study, severity of pathologic changes in affected joints, and anatomic location of joints from which samples are obtained. The techniques reported here for extraction and radioimmunoassay of substance P differed from those used by others to evaluate synovial fluid of horses. Sample size for determining content of substance P and prostaglandin E_2 in normal joints and osteoarthritic joints in this study substantially exceeded those reported elsewhere in which investigators used 4 to 17 joints[24,28-31] and 6 joints.[19,22]

A correlation was not identified between substance P or prostaglandin E_2 concentration and radiographic score, the principal indicator of severity of pathologic changes in joints used in this study. This could have reflected an inadequate sample size. With the sample size available (ie, 16 samples), we could only be sure (80% power) of detecting with 95% confidence a correlation larger than 0.65. Alternatively, a substantive association may not exist, because substance P and prostaglandin E_2 are principally indicators of active synovial inflammation, whereas radiographs frequently bear little association to current soft-tissue inflammatory activity within the joint capsule. Evaluation of a synovial biopsy specimen or soft-tissue scintigraphic activity, which more closely reflect ongoing synovial inflammation, may offer a better correlation in future studies.

In this study, a correlation was not detected between anatomic location of joints and synovial fluid content of substance P for normal or osteoarthritic joints. Investigations in horses and other species have documented that the density of nerve endings in the synovial membranes that secrete substance P is greater in the more distal joints of the appendicular skeleton than in the more proximal joints.[10,21] It has been implied that this accounts for the greater frequency of osteoarthritis in the more distal articulations.[10,21]

Data generated in humans are not helpful in clarifying the association between substance P content, prostaglandin E_2 content, and severity of joint disease. Marshall et al[14] documented significantly greater synovial fluid content of substance P in samples obtained from patients who had sustained a traumatic knee injury, compared with those who were afflicted with one of several other arthritic conditions. Conversely, investigators in another study[33] failed to detect any correlation between synovial fluid content of substance P and various degrees of joint disorders in the temporomandibular joint. If such

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an association can be clarified, it is possible that synovial fluid content of substance P or prostaglandin E_2 might become useful indicators of existing inflammatory status or for predicting long-term outcome.[34]

Unlike May et al,[24] who reported that only 1 of 8 synovial fluid samples from joints with osteochondrosis dissecans had increased prostaglandin E_2 (> 12.5 pg/ml) concentrations, all 7 joints afflicted with osteochondrosis dissecans in the study reported here had increased synovial fluid prostaglandin E_2 values (656.8 pg/ml; range, 19.2 to 1,151.6 pg/ml). In addition to those factors previously referred to, this discrepancy may reflect the more clinically active nature of the horses' disease in this study.

Insufficient data prevented further conclusions about the correlation between cause of the osteoarthritis and prostaglandin E_2 or substance P concentrations. However, a significant positive correlation (r = 0.68; P = 0.01) was detected between synovial fluid concentrations of prostaglandin E_2 and substance P. Although correlations between synovial fluid concentrations of substance P and other cytokines have been reported,[35] a correlation between substance P and prostaglandin E_2 concentrations had only been implied to exist [22,36,37] or had been documented in vitro.[17] The correlation may reflect the recognized ability of substance P to induce prostaglandin E_2 production by synoviocytes.[17] In view of the early peaking of prostaglandin E_2 and substance P concentrations in equine synovial fluid in other studies,[22,29,31] it is likely that both biomolecules retain a role during the acute-phase inflammatory process. The early involvement of substance P also can be inferred from its ability to promptly release interleukin 1 and tumor necrosis factor-α,[38] both of which are considered to act in the early part of the acute-phase inflammatory response.[39] In the late part of the acute-phase response, interleukin 1 and tumor necrosis factor-α affect many systemic changes, including increased production of acute-phase proteins such as prostaglandin E_2[39] and, possibly, substance P, often resulting in irreversible cartilage deterioration.[7]

Our data lend credence to the hypothesis that substance P plays a role in the etiopathogenesis of clinical joint disease, setting the stage for consideration of novel analgesic and therapeutic strategies. Pharmacologic approaches for reducing the neurogenic component of inflammation include interference with the production or release of neuropeptides from afferent terminals, enhancing degradation of neuropeptides, or blocking neuropeptide receptors.[40] The beneficial effects of capsaicin, an extract of chili peppers that depletes the substance P content of small-diameter primary afferent nerve fibers, and reserpine, which depletes sympathetic neurotransmitters, have been reported anecdotally and documented scientifically.[41] Therapeutic use of opioids, which have a modulating effect on the release of substance P from peripheral afferent fibers through their interaction with δ and μ receptors, has been reported.[42] Novel agents that block enzymes required in the synthesis of substance P also are being investigated.[40]

[a]Pierce Chemical Co, Rockford, Ill.

[b]Sigma Chemical Co, St Louis, Mo.

[c]Medline, National Library of Medicine, Bethesda, Md, 1998.

[d]PerSeptive Biosystems, Framingham, Mass.

Appendix 1

Joint score, assigned on the basis of location

Forelimb	Hind limb	Score
Shoulder	Hip	1
Humeroradial	Femorotibial	2
Radiocarpal	Tarsocrural	3
Intercarpal	Intertarsal or tarsometatarsal	4
Metacarpophalangeal	Metatarsophalangeal	5
Proximal interphalangeal	Proximal interphalangeal	6
Distal interphalangeal	Distal interphalangeal	7

Appendix 2

Radiographic scoring system for each of 10 criteria

Periarticular soft-tissue swelling
0 = None. 1 = Loss of tissue planes. 2 = Slight or confined to capsule. 3 = Mild capsular distention. 4 = Severe distention, early involvement of other tissues. 5 = Massive swelling involving all periarticular tissues.

Soft-tissue mineralization
0 = None. 1 = Suspicious. 2 = Capsular streaking. 3 = Patchy capsular or periarticular. 4 = Merging reaction with osteophytes and enthesiophytes. 5 = Massive involvement of periarticular tissues.

Position of fat pad
0 = Clearly defined. 1 = Visible but reduced in size. 2 = Not identified with certainty. 3 = Not visible. 4 = Moderate soft-tissue swelling obscuring fat pad. 5 = Massive soft-tissue swelling obscuring fat pad.

Increase in joint space
0 = Normal. 1 = Suspicious. 2 = Recognized immediately, joint capsule not distended. 3 =Obvious, with joint capsule distention. 4 = Widely separated joint. 5 = Accompanied by subluxation or ligament rupture.

Decrease in joint space
0 = Normal. 1 = Slight or uneven orientation of space. 2 = Narrowed but space still seen between end of bones. 3 = End of bones touching in some places. 4 = Accompanied by subchondral sclerosis. 5 = Ankylosis or trabecular bridging.

Evidence of osteophytes
0 = None seen. 1 = Suggestion of mineralization of cartilage lip. 2 = Joint margin(s) moderately raised. 3 = Easily recognized lip of joint margin. 4 = Prominent lipping around joint margin. 5 = Irregular prominent lipping with fragmentation or mineralization in adjacent tissues.

Evidence of enthesiophytes
0 = None seen. 1 = Faint haziness at capsule attachments or ligament insertions. 2 = Lines or ridges of mineralization at capsule attachments or ligament insertions. 3 = Easily recognized bone spur at attachment site. 4 = Prominent periosteal new bone at attachment site. 5 = Prominent periosteal reaction merging with extensive soft-tissue mineralization.

Sclerosis of subchondral bone
0 = None seen. 1 = Suspicious of denser or slightly more extensive subchondral plate. 2 = Patchy or localized sclerotic zones. 3 = Confined sclerotic reaction involving most of subchondral plate. 4 = Sclerosis extending unevenly into epiphysis. 5 = Extensive sclerosis involving all of subchondral plate and extending throughout much of epiphysis.

Erosive changes and lesions in joint
0 = None seen. 1 = Dimpling of subchondral margin, slight irregularity, or not sclerotic. 2 = Shallow erosion of subchondral bone without sclerosis. 3 = Shallow erosion of subchondral bone with restricted zone of sclerosis. 4 = Prominent irregular erosion or cystic lysis of subchondral bone and underlying epiphysis with or without sclerosis. 5 = Massive erosive or lytic lesion extending into epiphysis with sclerosis and reactive periostitis.

Evidence of osteochondral fragment
0 = None seen. 1 = Subtle nondisplaced osteochondral fragment. 2 = Small well-defined nondisplaced or separated osteochondral fragment. 3 = Large fragment (> 10% of total joint width). 4 = Osteochondral fragment accompanied by moderate proliferative response. 5 = Multiple osteochondral fragments or fragmentation with advanced proliferative response.

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