Squamous cell carcinoma is the most common ocular neoplasm and the second most common tumor overall in horses. Common sites of predilection for SCC in horses include the squamous epithelium of the head, eye, ocular adnexa, and genitalia. The etiology of the disease is incompletely understood, but suggested predisposing factors include exposure to solar radiation and certain viruses, absence of pigmentation, chronic physical irritation, and hormonal or immunologic influences. Draft breeds (eg, Belgians and Clydesdales), Paint Horses, and Appaloosas have a higher prevalence of SCC than other breeds. Although SCC rarely metastasizes to remote sites, the tumor is locally invasive. As a result of its local effects, ocular SCC tends to cause destruction of the globe and blindness. Treatment options are numerous and include surgical excision, cryotherapy, radiofrequency hyperthermia, immunotherapy, radiation therapy, laser surgery, intralereal chemotherapy, or a combination of those modalities. Treatment options for ocular and adnexal SCC vary depending on location of tumor, depth and size of tumor, the horse's use, presence or absence of metastatic disease, equipment availability, and financial constraints. Unfortunately, recurrence has been reported as a complication of all presently available treatment methods. Cyclooxygenases are a family of enzymes responsible for conversion of arachidonic acid to prostaglandins. Multiple isoforms of COX exist, with COX-1 and COX-2 being the most biologically active. Although these isoforms have similar enzymatic activity, they are expressed by separate genes and differ in physiologic function. Traditionally, COX-1 has been described as being expressed constitutively in healthy tissues and as being active in numerous homeostatic functions, including gastrointestinal tract cytoprotection, maintenance of platelet function, and maintenance of renal blood flow. In contrast, COX-2 has been described as an inducible enzyme that is overexpressed in inflammatory and neoplastic conditions via signals such as cytokines, hormones, and tumor promoters. However, the simplified paradigm of COX-1 being constitutive and associated with physiologic homeostasis and COX-2 being inducible and associated with pathologic conditions has not been upheld by research. It is now known that COX-2 often plays a critical physiologic role and is constitutively expressed in many tissues, including the kidneys, pancreas, stomach, colon, uterus, and eyes.
High levels of COX-2 expression have been detected in human and veterinary neoplasms, including colorectal cancer, breast cancer, hepatocellular carcinoma, and SCC of the head and neck in humans and epithelial neoplasias such as nasal epithelial SCC, nasal carcinoma, renal cell carcinoma, transitional cell carcinoma, and nasal adenocarcinoma in dogs. Correlations have been made in humans with SCC involving the head and neck between overexpression of COX-2 in neoplastic tissues and poor prognostic factors (eg, enhanced tumor vascularization and increased likelihood of metastasis). Mechanisms responsible for such changes are being investigated. Correlations have been found between COX-2 overexpression, prostaglandin E\(_2\) concentration, vascular endothelial growth factor (a potent angiogenic factor) concentration, and angiogenesis in SCC of the head and neck in humans, suggesting that there are inductive relationships among these factors.

To the authors’ knowledge, investigation of the role of COX in naturally occurring equine ocular and periorcular SCC has not been reported. The objective of the present study was to determine whether COX is overexpressed in SCC-affected ophthalmic tissues in horses.

**Materials and Methods**

**Signalment**—Age, breed, and sex of affected and control horses were recorded and evaluated by use of descriptive statistics. Results were compared with findings reported in the literature to determine whether significant differences existed between our population of horses and those in earlier reports.

**Tissue samples**—Squamous cell carcinoma—affected tissues were acquired from the cornea (n = 5 horses), eyelid (5), and third eyelid (5) of client-owned horses. Owners signed a consent form prior to horses undergoing general anesthesia and surgery at the University of Missouri-Columbia Veterinary Medical Teaching Hospital. Tissues were collected as part of routine surgical tumor resection typically recommended for treatment, and tumor sections were submitted to the University of Missouri-Columbia Veterinary Medical Diagnostic Laboratory for histologic examination. Site-matched control tissues were obtained from 5 horses with normal results of complete ophthalmic examinations prior to humane euthanasia for reasons unrelated to the present study. Those horses were eutanatized via IV injection of sodium pentobarbital. Tissues from control horses were obtained within 10 minutes of euthanasia with an 8-mm punch biopsy (for corneal tissues) or by sharp dissection with Metzenbaum scissors (for eyelid and third-eyelid tissues) and immediately fixed in 10% neutral-buffered formalin for 48 hours prior to sectioning.

All specimens underwent H&E staining and were evaluated by a veterinary pathologist (JRT) to confirm the presence of SCC in affected tissues and absence of neoplastic tissue in control tissues.

**Immunohistochemistry**—Standard streptavidin biotin techniques were used for detection of COX-1 and COX-2 proteins. Five-micron-thick paraffin sections were cut and placed on positively charged glass slides. Sections were microwaved for 1 to 2 minutes on high power or until the paraffin melted. Sections were deparaffinized, and heat-induced epitope retrieval was performed at 90° to 100°C in buffer solution for 30 minutes with 20 minutes of cooling at ambient temperature. Slides were rinsed several times with distilled water. Endogenous peroxidase activity was quenched by exposure to 3% hydrogen peroxide for 5 minutes, and slides were rinsed in distilled water and placed in Tris-buffered saline (0.9% NaCl) solution with Tween. Tissues were blocked with avidin-biotin blocking solution for 15 minutes and rinsed with the Tris and Tween buffer solution. A protein block was applied for 10 minutes. Primary antibodies were applied, and slides were incubated for approximately 12 hours at 4°C.

For COX-1 detection, goat polyclonal anti-human COX-1 was used. For COX-2 detection, rabbit polyclonal anti-C terminal peptide of murine COX-2 was used. All slides were developed by use of diaminobenzidine with a diaminobenzidine enhancer. Kupffer cells in the liver were stained as the positive control for COX-1, and lung tissue from swine with gram-negative bacterial pneumonia was stained for use as a positive control for COX-2. For negative controls, histologic sections were incubated in nonimmune serum.

**Microscopy**—Cyclooxygenase-1 and COX-2 staining were evaluated by use of light microscopy. With slides under 400X magnification, the 3 most intense areas of COX staining per slide were identified and photographed with a digital camera. The corresponding locations of tissues stained for COX and stained with nonimmune serum (negative immunohistochemical control) were also identified and photographed. Quantification of staining was performed with a commercially available software program that uses a color-sensitive selection method to determine the area of staining expressed in pixel units. In each photomicrograph, total area of possible staining and total area of stain uptake were determined. The total area of possible staining was determined by outlining the area of tissue and quantifying the number of pixels included in the outlined area. Stain uptake was determined by pixel-color sensitive selection. The percentage of stain uptake per potential area of staining was calculated. Control tissue sections were imaged in an identical manner.

**Statistical analysis**—Immunohistochemical data were analyzed by use of commercially available statistical software. Normally distributed data were analyzed with a paired t test. Data that were not normally distributed according to the Kolmogorov-Smirnov test were analyzed by use of the Mann-Whitney rank sum test. Values of P ≤ 0.05 were considered significant.

**Results**

**Signalment**—Signalment data were evaluated with descriptive statistics (Appendix). The mean ages of affected and control horses were 12 and 14 years, respectively. Geldings (73%) were overrepresented among affected horses, consistent with the hospital population sex distribution. The Appaloosa breed was most frequently affected (33%), a finding that was consistent with previous reports of ocular-adenexal SCC.
Histopathologic findings—Histologic evaluation of SCC-affected tissue sections revealed classic features of the tumor in the given locations. All tumors had features consistent with invasive carcinoma, including dyskeratosis, poor differentiation, and local penetration of basement membranes with invasion of surrounding tissues.

In the corneas, there was significantly ($P = 0.008$) more immunoreactivity for COX-1 (Figure 1) and COX-2 (Figure 2) in corneal SCC than in control tissues. Immunoreactivity for COX-1 and COX-2 was observed in tissues from eyelids with SCC and those of healthy controls, but no significant difference in immunoreactivity for COX-1 ($P = 0.151$) or COX-2 ($P = 0.099$) in eyelid SCC, compared with control tissues, was detected. Immunoreactivity for COX-1 and COX-2 was observed in third-eyelid tissues with SCC and those of healthy controls, but no significant difference in immunoreactivity for COX-1 ($P = 0.310$) or COX-2 ($P = 0.440$) in third-eyelid SCC, compared with control tissues, was detected.

Discussion

Results reveal that COX-1 and COX-2 immunoreactivity are higher in equine corneal tissue with SCC than in healthy corneal tissue. Immunoreactivity for COX-1 and COX-2 in eyelid SCC was also higher than in healthy tissue, although the differences were not significant. Evaluation of a larger population size may enable better determination of whether COX is consistently expressed at a higher level in affected tissues at that site. Similarly, because healthy tissues from horses with SCC were not examined, this study was not designed to evaluate overexpression of COX in anatomic sites other than the ocular and periocular tissues analyzed. The immunohistochemical techniques and image analysis methods used have been well established.\(^1\)\(^-\)\(^3\) Additionally, care was taken to handle all tissues identically with regard to fixation and processing.

Selective COX-2 inhibitors are a group of nonsteroidal anti-inflammatory drugs that inhibit COX-2 function while only minimally affecting the critical physiologic functions of COX-1 such as gastrointestinal epithelial cytoprotection, maintenance of renal blood flow, and maintenance of platelet function.\(^1\) Increased expression of COX-2 has been described in various neoplasms in humans and other animals. Specifically, overexpression of COX-2 in some neoplasms has been associated with a high degree of tumor ag-
gression and other poor prognostic indicators. A satisfactory explanation for the association between increased COX-2 expression and an unfavorable prognosis is presently lacking. However, correlations have been published linking COX-2 overexpression in neoplastic tissue to both stimulated angiogenesis and mitosis and inhibition of apoptosis, both of which enhance oncogenesis. It has been proposed that COX inhibition is useful in management of COX-associated neoplasia because of antiangiogenesis, antimitotic, and proapoptotic effects of certain selective COX-2 inhibitors. Moreover, both selective COX-2 inhibitors and nonselective COX inhibitors induce tumor regression in vivo and in vitro. More specifically, COX-2 inhibition has an antiangiogenic effect through antagonization of endothelial cell proliferation (which is induced by COX-2) and induction of defective vascular assembly. Thus, inhibition of COX-2 may lead to disruption of the blood supply to the neoplasm, resulting in cell death and tumor regression.

Results of stain uptake in the present study make it tempting to speculate that COX-2 is expressed in greater quantities than COX-1 in equine corneal SCC, as has been published in reports of carcinomas in other species. However, results of the present study, in which immunohistochemical techniques alone were used, cannot confirm a relationship between COX-1 and COX-2 in equine cornea for several reasons. Biopsy specimens varied in size, different antibodies were used for the 2 different COX isoforms with differing molecular weights, and antibodies against nonequine COX isoforms were used in equine tissues. Corroborative western blot techniques with loading of lanes with equal amounts of protein would represent 1 technique in which the bands at the appropriate molecular weight could be used to quantify the proteins of interest as a percent of total protein. Importantly, COX-2 is overexpressed in experimental models of conjunctivitis. Horses with ocular and periocular SCC have different degrees of concurrent conjunctivitis, depending on the location and size of the primary tumor. If COX-2 is found to be overexpressed, compared with COX-1, in future studies of equine ocular SCC, consideration should be given to whether or not the difference is the result of the primary tumor or secondary ocular inflammation.

More effective treatments for ocular and periocular SCC in horses is needed because this neoplasm is
common and presently recommended treatments are often associated with recurrence of disease. Moreover, suboptimal or inadequate treatment of SCC often leads to blindness in affected horses, resulting in an adverse impact on the horse’s function. To the authors’ knowledge, use of a nonsteroidal anti-inflammatory drug (piroxicam) for treatment of mucocutaneous SCC in a horse has been described in a single case report.¹ The present study was undertaken to determine the levels of expression of COX-1 and COX-2 in equine ocular and periorcular SCC. To the authors’ knowledge, similar studies have not been reported previously. Our findings revealed that equine corneal SCC tissue had high immunoreactivity for COX proteins. There was no microscopic or gross evidence of an explanation for the differences in COX expression other than location in the present study.

Immunohistochemical analysis was used successfully to detect differences in COX expression and is a potentially useful technique for characterization of this tumor type in these sites. Results can be used as a foundation for future investigations of the role of COX in the oncogenesis and progression of ocular and periorcular SCC in horses. Studies in which expression of COX-2 in ocular and periorcular SCC is investigated by use of specific and quantifiable biomarkers for the isoenzyme (such as prostaglandin E₂) are warranted, as are clinical studies to determine the efficacy of use of COX-2 selective inhibitors in horses with ocular SCC.

References


Appendix

Summary of signalment data for 20 horses evaluated in a study of SCC of ocular adnexal tissues.

<table>
<thead>
<tr>
<th>Breed</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Tissue site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tennessee Walking Horse</td>
<td>16</td>
<td>Mare</td>
<td>SCC-eyelid</td>
</tr>
<tr>
<td>American Paint Horse</td>
<td>10</td>
<td>Mare</td>
<td>SCC-eyelid</td>
</tr>
<tr>
<td>Foxtrotter</td>
<td>3</td>
<td>Gelding</td>
<td>SCC-eyelid</td>
</tr>
<tr>
<td>Appaloosa</td>
<td>9</td>
<td>Gelding</td>
<td>SCC-eyelid</td>
</tr>
<tr>
<td>Foxtrotter</td>
<td>11</td>
<td>Foal</td>
<td>SCC-eyelid</td>
</tr>
<tr>
<td>American Paint Horse (overo coat pattern)</td>
<td>4</td>
<td>gelding</td>
<td>SCC-third eyelid</td>
</tr>
<tr>
<td>American Saddlebred</td>
<td>3</td>
<td>Mare</td>
<td>SCC-third eyelid</td>
</tr>
<tr>
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<td>16</td>
<td>Gelding</td>
<td>SCC-eyelid</td>
</tr>
<tr>
<td>American Paint Horse</td>
<td>5</td>
<td>gelding</td>
<td>SCC-third eyelid</td>
</tr>
<tr>
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<td>SCC-third eyelid</td>
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<tr>
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<td>SCC-eyelid</td>
</tr>
<tr>
<td>Quarter Horse</td>
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<td>SCC-eyelid</td>
</tr>
<tr>
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<td>SCC-eyelid</td>
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<tr>
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<td>SCC-eyelid</td>
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<tr>
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</tr>
<tr>
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<tr>
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<tr>
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<td>gelding</td>
<td>Control</td>
</tr>
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