Effects of phenylbutazone on bone activity and formation in horses

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Objective—To determine the effects of phenylbutazone (PBZ) on bone activity and bone formation in horses.

Animals—12 healthy 1- to 2-year-old horses.

Procedures—Biopsy was performed to obtain unicortical bone specimens from 1 tibia on day 0 and from the contralateral tibia on day 14. Fluorochromic markers were administered IV 2 days prior to and on days 0, 10, 15, and 25 after biopsy was performed. Six horses received PBZ (4.4 mg/kg of body weight, PO, q 12 h) and 6 horses were used as controls. All horses were euthanatized on day 30 and tissues from biopsy sites, with adjacent cortical bone, were collected. Osteonal density and activity, mineral apposition rate (MAR), and percentage of mineralized tissue were assessed. Serum samples from all horses were analyzed for bone-specific alkaline phosphatase activity and concentration of PBZ.

Results—MAR was significantly decreased in horses treated with PBZ. Regional acceleratory phenomenon was observed in cortical bone in both groups but was significantly decreased in horses treated with PBZ. Osteonal activity was similar at all time points in all horses. In control horses, percentage of mineralized tissue filling the cortical defects was significantly greater in defects present for 30 days, compared with defects present for 14 days. Differences in percentage of mineralized tissue were not detected in horses treated with PBZ.


Standard postoperative management of horses with fractures includes the use of nonsteroidal anti-inflammatory drugs (NSAID).14 Nonsteroidal anti-inflammatory drugs inhibit inflammatory responses by blocking prostaglandin (PG) synthesis via the cyclooxygenase pathway of arachidonic acid metabolism.10 Phenylbutazone (PBZ) is the most commonly used NSAID in horses with fractures for alleviation of trauma- and surgery-induced inflammation and pain, thus allowing the horse to bear weight on the affected limb and reducing the risk of breakdown in the contralateral limb.1-10 However, some PG are important factors in the early phase of bone healing.11-16 A local increase of PG concentration is a response of bone to trauma and PG may stimulate differentiation and proliferation of osteoprogenitor cells during early bone healing.17-18 Prostaglandin E2 in particular is thought to be an important factor in the early phase of bone healing through modulation of inflammation, blood flow, and influence on transmembrane ion transport.19 In horses, PBZ causes a considerable reduction in the production of prostaglandin E2 in inflammatory exudates.18

Although the effects of PBZ on bone healing have not been investigated, there is evidence that PBZ may decrease cellular metabolism of other mesenchymal cells.19-22 Recent research on the in vivo effects of PBZ on equine articular chondrocytes revealed a substantial decrease in proteoglycan synthesis in cartilage explants exposed to interleukin-1β.23 Proteoglycan synthesis in equine articular chondrocytes recovered within 2 weeks after treatment with PBZ was discontinued. Results of research in laboratory animals such as rats, rabbits, and dogs suggest that NSAID delay fracture healing.24-26 Several studies have recorded a drug- and dose-related effect of ketorolac, salicylic acid, and indomethacin on fracture healing in rats and rabbits.21-23 In rabbits, indomethacin decreased bone mineral content, mechanical bending strength, and blood flow at 2 weeks after osteotomy.22,25 Sudmann et al26 demonstrated that indomethacin decreased Haversian remodeling in rabbits.26 It has also been reported that other NSAID, such as diclofenac acid, ibuprofen, indomethacin, and aspirin, decrease ingrowth of bone into porous-coated implants in rabbits.27

It is our concern that the use of NSAID in the early postoperative period may adversely affect the biological response of bone to trauma. If inhibition of PG by PBZ adversely affects the rate of bone formation and results in a delay in bone healing, alternatives to the use of PBZ in the postoperative management of horses with fractures would be desired. We hypothesized that PBZ would decrease bone metabolic activity and rate of bone healing. The purpose of the study reported here was to evaluate the effects of PBZ on osteonal activity, mineral apposition rate (MAR), and healing of unicortical defects in the tibia of horses.

Materials and Methods

Horses—Twelve clinically normal horses (2 Thoroughbreds, 2 Quarter Horses, 3 Paints, 1 Standardbred, 1 Appaloosa, 1 Arabian, and 2 grades; 4 stallions, 3 geldings, and 5 mares) between 18 and 30 months of age were randomly assigned to the control group or the treated group.
Fluorochrome bone labeling technique—Osteonal density, osteonal activity, and MAR were assessed by use of fluorochrome bone labels. Oxytetracycline hydrochloride was dissolved in 1 L of sterile 0.9% sodium chloride solution and administered IV through an indwelling catheter. Calcein was dissolved in a 2% solution of sodium bicarbonate and administered IV through an indwelling catheter. Under UV light, oxytetracycline fluoresces yellow-orange and calcein emits bright green fluorescence. The labeling schedule for all horses was: calcein (20 mg/kg) was administered 2 days before the first biopsy specimen was obtained (day –2), oxytetracycline hydrochloride (25 mg/kg) was administered immediately after the first biopsy specimen was obtained (day 0) and again on day 10, and calcein (20 mg/kg) was administered after the second biopsy specimen was obtained (day 15) and on day 25 (Fig 1).

Bone biopsy procedure—One tibia from each horse was randomly chosen as the site for the first biopsy specimen (biopsy site 1) obtained on day 0; the second biopsy specimen (biopsy site 2) was obtained from the corresponding site in the contralateral tibia on day 14 (Fig 1). For the biopsy procedures, horses were sedated by administration of xylazine hydrochloride (0.4 mg/kg, IV); anesthesia was induced by administration of diazepam (0.2 mg/kg, IV) and ketamine hydrochloride (2.2 mg/kg, IV) and maintained with halothane vaporized in oxygen in a semiclosed circle system. After preparation for aseptic surgery, the skin, subcutaneous tissues, fascia, and periosteum at the proximomedial aspect of the left or right tibia, respectively, were incised. The periosteum was elevated and reflected, and full thickness cortical bone biopsy specimens were obtained, using a 12-mm bone trephine powered by a commercial cordless electric drill. Biopsy specimens were stored in 70% ethanol until processed. Fascia and subcutaneous tissues were closed with 2-0 absorbable suture in a simple continuous pattern. Skin was closed with 2-0 nonabsorbable suture in a simple interrupted pattern.

Sample collection—At the end of the study, horses were euthanized with an overdose of pentobarbital administered IV. The tibiae were collected from all horses and cleaned of soft tissues. The sections containing the unicortical defects (caused by the biopsy procedures) were excised, including a 2-cm margin of cortical bone, and fixed in 70% ethanol until processed.

Specimen preparation—For histomorphometric analysis, biopsy specimens were embedded in polymethylmethacrylate and cut into segments 200 µm thick with a calibrated saw with a diamond cutting wheel. Segments were then ground with an embedded tissue grinder to 30 µm thick. A computer-assisted image analysis system with camera lucida were used for microscopic examination of unstained specimen slides (Fig 2 and 3).

Bone-specific alkaline phosphatase assay—The effects of the biopsy procedure on serum bone-specific alkaline phosphatase activity and the effects of PBZ and biopsy procedure on bone-specific alkaline phosphatase activity were evaluated by use of a commercially available nonisotopic immunoassay kit validated for use in horses. Blood was collected from all horses before the first biopsy procedure was performed (day 0), on each day for 4 days after the procedure, and 12 days after the first biopsy procedure. For assessment of a possible effect of PBZ on bone-specific alkaline phosphatase activity beyond the last day of PBZ administration, blood was also collected 4 days after the second biopsy was performed (day 18) and 12 days after the second biopsy was performed (day 26). Blood samples were centrifuged; serum was collected and frozen at –70°C until analyzed. For analysis, immunoassay wells were filled with 125 µl of assay buffer followed by 20 µl of standard, control, or sample and incubated at room temperature (26°C) for 3 hours. After incubation, wells were emptied and washed 4 times with 250 µl of wash buffer. Wells were filled with 150 µl of working substrate solution and incubated for an additional 30 minutes at 26°C. After incubation, 100 µl of stop solution was added to each well and followed by optical reading at 405 nm.

Phenylbutazone analysis—Serum for analysis of PBZ concentration and analysis of bone-specific alkaline phosphatase activity was obtained and frozen at –70°C from all horses before...
PBZ administration and on days 4, 12, 18, and 26 of the study. For documentation of PBZ concentration, serum samples were analyzed by use of high performance liquid chromatography\(^2\) with a lower detection limit of 0.05 μg/ml.\(^2\)

**Osteonal density**—Biopsy specimens were evaluated under bright light illumination at 40X magnification, using a calibrated orthogonal grid. Osteons within the grid were counted and the procedure repeated 4 times. Mean number of osteons was determined and expressed as mean number of osteons/mm\(^2\).

**Osteonal activity**—Sections were evaluated under UV light at 200X magnification and 100 osteons were counted on each specimen slide. Of these osteons, the number containing fluorochrome label was determined and the percentage of fluorescing osteons was calculated. Osteonal activity was determined in the central portion of biopsy specimens and in the cortical bone adjacent to defects caused by the biopsy procedure. Results were expressed as percentage osteonal activity.

**Mineral apposition rate**—In all specimens, osteons were evaluated under UV light at 200X magnification and 100 osteons were counted on each specimen slide. Of these osteons, the number containing fluorochrome label was determined and the percentage of fluorescing osteons was calculated. Osteonal activity was determined in the central portion of biopsy specimens and in the cortical bone adjacent to defects caused by the biopsy procedure. Results were expressed as percentage osteonal activity.

**Statistical analyses**—Data were reported as mean ± SEM. Data were analyzed by use of commercially available software. Data for body weight were reported as median and range. Repeated-measures ANOVA followed by the Duncan post hoc test was used to compare serum bone-specific alkaline phosphatase activity, MAR, and osteonal activity in PBZ-treated horses with that of control horses. Osteonal density and percentage of mineralized tissue filling the biopsy defect were analyzed by use of Student t-test. Differences within groups and between groups were considered significant when \(P < 0.05\).
Results

Horses—Oral administration of PBZ was well tolerated by all horses. Only 1 horse had clinical signs compatible with PBZ toxicity (edema and oral ulceration) toward the end of the drug administration period. Clinical signs resolved after discontinuation of scheduled PBZ administration. All horses maintained or gained weight during the study period. In 1 control horse, a catastrophic fracture occurred through the first biopsy site while the horse was recovering from anesthesia; this horse was euthanized. Another control horse had mild lameness for 24 hours after the second biopsy was obtained; the lameness resolved without treatment.

Fluorochrome labeling—There was incorporation of all fluorochrome markers into cortical bone. Microscopic differentiation of all markers was possible, except the separation between the first and second label, which did not allow measurement of the interlabel distance between these 2 labels. Calcein was observed as a bright green band on the outermost perimeter of active osteons, followed by 2 pale orange seams of oxytetracycline label. The 2 innermost labels were bright green and represented calcein administered at days 15 and 25 of the study (Fig 4).

Phenylbutazone concentrations—Phenylbutazone was not detected in control horses at any time. Phenylbutazone was detected in all treated horses, with a mean serum concentration of 9.09 ± 3.6 µg/ml on day 4 and 9.80 ± 3.0 µg/ml on day 12. Phenylbutazone was detected in 3 horses on day 18 (mean, 4.82 ± 4.3 µg/ml) and in 1 horse on day 25 (2.02 µg/ml).

Serum bone-specific alkaline phosphatase activity—Bone-specific alkaline phosphatase activity was detected in all serum samples. In both groups, a decrease in bone-specific alkaline phosphatase activity was detected throughout the study period (Fig 5). In control horses, bone-specific alkaline phosphatase activity at the beginning of the study (day 0) was significantly (P < 0.001) higher (98.15 ± 22.17 U/L) than its activity 4 days after the second (day 18) biopsy (50.48 ± 10.67 U/L) and 12 days after the second (day 26) biopsy (46.74 ± 7.29 U/L) was performed. Although bone-specific alkaline phosphatase activity decreased with time, detectable differences were not found among PBZ-treated horses. Also, differences in bone-specific alkaline phosphatase activity were not detected between control horses and PBZ-treated horses at any time.

Osteonal density—Mean osteonal density was 50.1 ± 6.1 osteons/mm² for biopsy site 1 (day 0) in control horses and 38.3 ± 7.1 osteons/mm² in PBZ-treated horses before PBZ administration. For biopsy site 2 (day 14), mean osteonal density for control horses was 51.2 ± 6.1 osteons/mm² and 37.2 ± 2.7 osteons/mm² for PBZ-treated horses. Significant differences in osteonal density from biopsy site 1 or biopsy site 2 were not detected between control horses and PBZ-treated horses.

Osteonal activity—Basal (day 0) osteonal activity was not different in control horses (47.5 ± 5.8%), compared with PBZ-treated horses (31.4 ± 9.8%) before administration of PBZ. Osteonal activity in cortical bone adjacent to biopsy site 2 (day 14) was not different in horses after 2 weeks of PBZ administration (47.3 ± 4.5%), compared with control horses (37.6 ± 6.9%). Osteonal activity in cortical bone adjacent to biopsy site 1 after 30 days of healing time was not different in control horses (49.4 ± 6.8%), compared with PBZ-treated horses (56.0 ± 5.6%). Osteonal activity in cortical bone adjacent to biopsy site 2 in PBZ-treated horses (47 ± 2.7%) appeared to be lower than that in control horses (59.0 ± 5.1%), but no significant (P = 0.055) difference was found.

Mineral apposition rate—All 5 bone labels could be distinguished microscopically with fluorescent light. However, the short labeling interval of 2 days between label 1 and label 2 precluded measurement of interlabel distance in most horses, regardless of group; therefore, MAR between labels 1 and 2 was not included in statistical analyses.

In control horses, MAR between labels 2 and 3 in cortical bone adjacent to biopsy site 1 (1.67 ± 0.16 µm/d) was significantly (P < 0.001) greater than MAR between labels 2 and 3 (1.19 ± 0.08 µm/d) and 3 and 4 (1.25 ± 0.18 µm/d) in cortical bone adjacent to biopsy site 2, respectively. This represented a local increase in MAR in cortical bone adjacent to biopsy site 1 during the 10 days after the first biopsy was performed. In control horses, MAR was also significantly (P < 0.001) greater between labels 2 and 3 in cortical bone adjacent to biopsy site 1 (1.67 ± 0.16 µm/d) than MAR between labels 2 and 3 in biopsy site 2 (day 14; 1.19 ± 0.11 µm/d), and also represented the effects of the biopsy procedure on regional bone formation. Significantly (P < 0.001) greater MAR in control horses between labels 3 and 4 in cortical bone adjacent to biopsy site 1 (1.71 ± 0.18 µm/d) compared with MAR between labels 3 and 4 (1.25 ± 0.18 µm/d) and between labels 4 and 5 (1.28 ± 0.05 µm/d) in cortical bone adjacent to biopsy site 2 also represented the effects of the biopsy procedure on regional bone formation in cortical bone adjacent to biopsy site 1 (Table 1).

In PBZ-treated horses, MAR between labels 2 and 3 (1.30 ± 0.10 µm/d) and between labels 3 and 4 (1.63 ± 0.15 µm/d) in cortical bone adjacent to biopsy site 1 were significantly (P < 0.001) greater than MAR in cortical bone adjacent to biopsy site 2 between labels 2 and 3 (0.90 ± 0.03 µm/d) and labels 3 and 4 (1.13 ± 0.10 µm/d), respectively.

Comparison between the control and treated groups revealed a significant difference in MAR between groups. Mineral apposition rate between labels 2 and 3 in cortical bone adjacent to biopsy site 1 in PBZ-treated horses (1.30 ± 0.10 µm/d) was significantly (P < 0.001) less than MAR in control horses (1.67 ± 0.16 µm/d).

Mineralized tissue—In control horses, the percentage of mineralized tissue filling the unicortical defect from biopsy site 1 (28.42 ± 6.27%) was significantly (P = 0.04) greater than for the defect from biopsy site 2 (16.71 ± 4.32%). In PBZ-treated horses, per-
centage of mineralized tissue filling the first biopsy defect (17.72 ± 4.42%) was similar to that of the second biopsy defect (13.19 ± 4.01%). Comparison between groups did not reveal significant differences for the percentage of mineralized tissue in biopsy defect 1 and biopsy defect 2.

Discussion

Results of the study reported here revealed that PBZ significantly decreased MAR. Mineral apposition rate between labels 2 and 3 represented the rate of bone formation from day 0 until day 10 of the study. During this time, cortical bone adjacent to biopsy site 1 was under the influence of the biopsy in control horses and under the influence of the biopsy and PBZ in the treatment group, whereas cortical bone in the contralateral tibia was not under the regional influence of biopsy. This finding supports results of another report that indicate NSAID inhibit Haversian remodeling.

We also observed that cortical biopsies induced a local increase in MAR adjacent to the biopsy sites in both groups of horses because MAR was higher between day 0 and day 10 (labels 2 and 3) in cortical bone adjacent to biopsy site 1, compared with cortical bone adjacent to biopsy site 2, which most likely represented a regional acceleratory phenomenon (RAP).32,33 Also, within-group comparison in control horses revealed a significant increase in MAR between labels 2 and 3 in cortical bone adjacent to biopsy site 1, compared with MAR for these labels in biopsy site 2. This effect was not observed in PBZ-treated horses and suggests that PBZ may have reduced the RAP. Within both groups, comparison between labels 2 and 3 in cortical bone adjacent to the first and second biopsy defects revealed an increase in MAR within the first 10 days after the first biopsy was obtained. Phenylbutazone did not completely inhibit this increase in MAR, but PBZ clearly reduced the magnitude of the RAP.

To our knowledge, RAP has not been described in horses. Regional acceleratory phenomenon is characterized by an enhancement of healing processes in both soft and hard tissues. Regional acceleratory phenomenon promotes healing at a faster rate, compared with healing without the RAP.34 The RAP not only leads to an increase in local blood supply, but also acts in the recruitment of osteoblasts and other cellular mechanisms that are essential to bone healing.34 It has been proposed that PG play a role in the activation of the RAP.

Bone injury not only leads to an increase in bone metabolism at the site of injury, but also causes a systemic response in bone metabolism, termed systemic acceleratory phenomenon (SAP).35 The protocol of the study reported here did not allow for the evaluation of SAP; however, MAR in cortical bone adjacent to biopsy site 2 between any of the 5 labels analyzed were not different from each other. In both groups, MAR in bone adjacent to biopsy site 2 was less than MAR between the corresponding labels in cortical bone adjacent to biopsy site 1. This may suggest that the effect of RAP on osteoblastic activity in response to the second biopsy was decreased, compared with the effect of RAP observed after the first biopsy. A recent study in rats recorded that inflammatory-mediated osteopenia prevented the systemic stimulation of bone formation through SAP, but did not inhibit the RAP at the site of bone trauma.35 Whether systemic effects of local bone injury mimic the effects of inflammation-mediated osteopenia is not known. However, in both instances, inflammatory cells and their associated mediators of inflammation may exert similar effects on bone metabolism. It is not known whether changes in exercise may have caused a reduced RAP associated with the second biopsy. Because all horses in our study were confined to 3 × 5 m box stalls immediately after the first biopsy procedure, we feel that changes in exercise may have caused a reduced RAP associated with the second biopsy, which was performed after 14 days of stall confinement. However, stall confinement does not appear to explain the slight increase in MAR over time in PBZ-treated horses. It is conceivable that variability between horses and between groups may have influenced MAR.

Phenylbutazone also had an inhibitory effect on the mineralization of tissue filling the unicortical biopsy-induced defects. Control horses had nearly twice as much mineralized tissue in the first biopsy-induced defect, compared with the second biopsy-induced defect. This finding was in sharp contrast to PBZ-treated horses, in which there was a similar amount of mineralized tissue in both biopsy-induced defects. This data suggests that PBZ may have an inhibitory effect on bone metabolic activity and decreased the effects of RAP because woven bone formation has been stated to be a fundamental part of RAP. Further research is warranted to more fully investigate the effects of PBZ on healing of cortical defects.

Interestingly, there was an overall decrease in bone-specific alkaline phosphatase activity with time in all horses. To our knowledge, there is no information available regarding the effects of NSAID or stall confinement on bone-specific alkaline phosphatase activity.

The decrease in enzyme activity observed in the horses of this study may have been caused by the change in housing of the horses. The decrease in enzyme activity and exercise may have led to a reduction in mechanical stimulation of the appendicular skeleton and ultimately in decreased bone metabolic activity. Bone-specific alkaline phosphatase is a tetrmeric glycoprotein localized on the cell surface of osteoblasts and is used as an indicator of matrix production and mineralization.36,37 In humans, bone-specific alkaline phosphatase activity is used to diagnose and monitor metabolic bone diseases, such as osteoporosis. It is possible that failure to detect changes in alkaline phosphatase activity are related to the size of the defect created by the biopsy procedure, the short duration of the study, and decreased exercise.

Serum concentrations of PBZ varied widely among treated horses, but values were compatible with other reported data.37 Profound differences between horses are expected after oral administration of PBZ. Absorption of orally administered PBZ is variable and depends on a variety of factors such as diet, time of
administration relative to feeding time, and amount of protein in the diet. The highest serum concentration (25.3 µg/ml) was found in 1 horse after 4 days of PBZ administration; however, this horse did not have any clinical signs compatible with PBZ toxicosis.

Local regulation of bone formation and resorption is a complex process involving autocrine, paracrine, and systemic hormonal mechanisms.13,17,18-20 Clinical observations and experimental studies have documented that PG have profound effects on both bone formation and bone resorption.13,14,16-17,38-40 These paracrine substances are produced by bone cells as well as fibroblasts and other cells of the hematopoietic lineage in response to various stimuli.16,17,38-40 Prostaglandinds of the E series have been studied extensively. Prostaglandin E2 and, to a lesser degree, prostaglandin E1, have complex effects on osteoclasts and osteoblasts; their in vivo and in vitro effects are often contradictory. Prostaglandin E1 and E2 promote recruitment and differentiation of osteoprogenitor cells in the periosteum.13,14,16,17 These effects may be responsible for observed increases in periosteal and endosteal new bone formation after systemic administration of PG; however, the effects of PG on bone formation do not appear to be limited to the endosteal and periosteal envelope alone, but also extend to cancellous bone. Whether the effects of PG inhibit or increase osteoblast function may be concentration-dependent, because inhibition of osteoblastic activity is known to develop at concentrations higher than those necessary to induce an increase in osteoblast function.16,17

Results of the study reported here confirmed our hypothesis that horses receiving PBZ had a decrease in bone activity, compared with horses not receiving PBZ. The results of our study do not support the hypothesis that PBZ decreased serum bone-specific alkaline phosphatase activity. There is evidence that bone is able to recover from PBZ-induced inhibition of bone formation. Further research may be necessary to evaluate the specific effects of PBZ on bone metabolism. The small sample size of our study resulted in high variance between subjects and may have precluded finding more significant results. Also, the short study period limited our study to the effects of PBZ on bone metabolic activity and the early phases of healing following bone injury.

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