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Brian D. Nielsen
Major Professor's Signature

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IMPACT OF NUTRITIONAL SODIUM ZEOLITE A SUPPLEMENTATION IN THE EQUINE AND BOVINE

By

Kari Krick Turner

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ABSTRACT

IMPACT OF NUTRITIONAL SODIUM ZEOLITE SUPPLEMENTATION IN THE EQUINE AND BOVINE

By

Kari Krick Turner

This group of experiments focused on determining the effects of dietary sodium zeolite A (SZA) supplementation on animal mineral concentrations (plasma, milk or tissue) and joint and bone physiology. Sodium zeolite A is broken down into silicon and aluminum in the digestive tract and contains approximately 650 mg Si/kg SZA and 130,000 mg Al/kg SZA. Eight two-year-old Standardbreds were used to evaluate SZA's effect on osteochondrotic lesions in the metacarpo-, metatarsophalangeal and tarsocrural. To determine alterations in plasma and milk mineral concentrations following SZA supplementation, 20 lactating Holsteins were utilized. Twenty Holstein bull calves were used to determine the effects of SZA supplementation on plasma and tissue mineral concentrations, mineral retention and absorption, qualities of the fused third and fourth metacarpal bones, synovial fluid hyaluronic acid (HA) concentration, and cartilage glycosaminoglycan (GAG) content. Bone metabolism markers were analyzed in both cows and calves. There were no effects of SZA on osteochondrotic lesions in Standardbreds. Feed intake and milk production in the cows were decreased by SZA ($P<0.003$). Plasma phosphorus concentrations were decreased in the SZA-treated cows and plasma calcium concentrations were increased ($P<0.0002$). Milk aluminum concentration was increased, and phosphorus concentration was decreased in the SZA-treated cows ($P<0.03$). Plasma magnesium concentration was decreased by SZA

supplementation in the calves, and plasma phosphorus concentration tended to be decreased ($P<0.1$). Aluminum retention was increased in the calves ($P=0.001$), but plasma aluminum concentrations did not change. Aluminum content was substantially increased in all analyzed tissues except bone ($P<0.05$). There was little effect of SZA on bone or joint physiology, with an increase in deoxypyridinoline in the calves as the only difference ($P<0.05$). The results from the series of experiments described here suggest that SZA has an impact on mineral metabolism. The use of SZA as a dietary supplement to animals should be reconsidered. The substantial accumulation of tissue aluminum and therefore potential adverse consequences would preclude any benefits, regardless of whether or not the animal is intended for human consumption. The possibility of excess aluminum in human diets is a situation which should be avoided.

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And with that, I'm outta here. The world is Gator bait, and it's time I took a Chomp.

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CHAPTER 1

INTRODUCTION

Supplemental silicon, primarily in the form of sodium zeolite A (SZA), has been gaining popularity in the equine industry as a supplement for bone health. The interest arises predominantly from a study conducted at Texas A&M University (Nielsen et al., 1993). Researchers found SZA to be associated with both decreased skeletal injuries and increased training distance until injury. Moreover, plasma silicon concentrations were correlated with training distance before an injury occurred. However, the mechanism by which SZA was associated with the decreased injuries is unknown.

Trainers feeding silicon to their horses have reported a decrease in osteochondrosis in the joints of their horses. Although anecdotal, the underlying results of osteochondrosis – disturbed endochondral ossification and glycosaminoglycan loss – may be remedied by supplementation of silicon. Chicks supplemented with silicon had increased cartilage glycosaminoglycan concentrations (Carlisle, 1976), hastened bone mineralization (Carlisle, 1982) and improved endochondral bone growth (Carlisle, 1980) compared to controls on a silicon-deficient diet. The incidence of osteochondrosis in the horse population, specifically in Standardbreds, is quite high. Although surgery is a viable option, the inherent risks and costs of surgery mean it would be beneficial to have a non-invasive method that reduces osteochondrosis in horses.

Sodium zeolite A is being used in the feed industry as an anti-caking supplement, with allowable levels up to 2% SZA. However, there are many questions surrounding the feeding of SZA. Studies using SZA have found conflicting results in feed intake,

digestibility, and mineral metabolism, both within and between species. For example, feeding 2% SZA to horses has not been reported to affect feed intake (Frey et al., 1992; Nielsen et al., 1993; Lang et al., 2001b,c), but cows fed the same level reduced their feed intake (Johnson et al., 1988; Thilsing-Hansen et al., 2002). Alternatively, less than 2% SZA to cows increased feed intake (Roussel et al., 1992). Research needs to address this issue. Additionally, most studies have overlooked the effects of aluminum. As SZA is an aluminosilicate that breaks down in the digestive tract to aluminum and orthosilicic acid (bioavailable silicon), aluminum cannot be ignored as is can cause toxicities in animals and humans (Goodman, 1986; Nayak, 2002). The ramifications of feeding a supplement high in aluminum should be investigated, particularly in food producing animals. Additionally, beneficial effects have been seen when supplementing silicon. By identifying tissues that accumulate silicon following supplementation, future research can be planned accordingly.

The primary objectives of this project were to:

1. determine if supplementation of SZA decreases osteochondrotic lesion size in Standardbreds.
2. determine if supplementation of SZA to lactating dairy cows would affect plasma mineral metabolism as well as bone metabolism.
3. attempt to elucidate a mechanism by which SZA was associated with decreased skeletal injuries in racing horses by evaluating bone mechanical properties, turnover, and architecture.

4. evaluate the tissue mineral composition following SZA supplementation to determine the extent of tissue mineral accumulation in young calves.

CHAPTER 2

REVIEW OF LITERATURE

Silicon

Silicon (Si) is the second most abundant mineral found in the earth's crust (Carlisle, 1984). As silicon is not found free in nature, it exists mainly as silicates and the oxide, silica. Silicon is relatively inert, but can be reactive to halogens and dilute alkali. Most acids do not affect it. Silicon has three stable isotopes – ^{28}Si, ^{29}Si, and ^{30}Si, and two radioisotopes that can be used as tracers – ^{31}Si and ^{32}Si (Carlisle, 1984). Silicon is found throughout the environment, particularly in glass and sand. Forages and grains also contain silicon.

Silicon is found throughout the mammalian body in various tissues. Early data on the silicon content of mammalian tissues have reported variable results. This could be due to the use of glass in experiments that would result in leaching of silicon from glass Lang et al., 2001c).

Silicon is present in the blood as free soluble monosilicic acid, and is found in similar concentrations (~ 100 μg/dl) in monkey, rat, and human whole blood (Carlisle, 1984). Silicon appears to be freely diffusible throughout tissue fluids as other examined body fluids had similar silicon concentrations as the blood serum (Carlisle, 1984). Blood silicon levels remain fairly constant except in instances in which additional silicon is added to the diet (Carlisle, 1984). Supplemental silicon increases plasma silicon concentrations in horses (Frey et al., 1992; Nielsen et al., 1993; Lang et al., 2001b,c; Mazzella et al., 2005).

Various tissues throughout the body, particularly the connective tissues such as the aorta, trachea, tendon, bone, and skin are unusually rich in silicon (Carlisle, 1972). Carlisle (1972) suggested that the high silicon content of connective tissue is mainly from its presence as an integral component of the glycosaminoglycans and their protein complexes that contribute to the structural framework of the tissues. Furthermore, these tissues show a decline in silicon concentration as the animal ages. While the heart, liver, and muscle silicon concentrations remained unchanged over several years, concentrations in the aorta and skin decreased significantly (Carlisle, 1974). Moreover, a decrease in total glycosaminoglycan synthesis seems to occurs in human skin (Vuillermoz et al., 2005), lending some support to the hypothesis that the majority of silicon in connective tissue is associated with glycosaminoglycans.

Rat kidney, liver, bone, skin, spleen and lung were found to accumulate the greatest amounts of ^{31}-labeled silicic acid one hour after intracardiac injection of the labeled element

Few studies have investigated tissue silicon accumulation following silicon administration. One such study found that silicon was increased in several rat tissues post-intracardiac injection of ^{31}-labeled silicic acid (Adler et al., 1986). Initially, the skin, bone, and muscle contained the largest amounts of silicon, with 85 % of the total ^{31}Si in those three organs. However, as time progressed, the ^{31}Si concentration of the three organs decreased as the concentrations in the other organs did not change or increased. After four hours, 56% of the total ^{31}Si was found in the bone, muscle and skin. The decreases in those tissues and the subsequent increases in the other tissues could be a result of equilibrium. The same study also reported that the ^{31}Si was found almost

entirely in a nonprotein bound form in the plasma (Adler et al., 1986). Additionally, the blood-brain barrier excluded ^{31}Si from the brain as it was present only in negligible concentrations. This appears to be the only study that investigated the effects of supplemental silicon on tissue uptake. Silicon supplemented through dietary means, and not via injection into circulatory system, might have different effects. Also, a livestock species such as the horse or cow, might have dissimilar tissue responses to supplemental silicon as the tissues might have different concentrations than the rat due to the livestock diet of grains, forage, and oftentimes, sand. The effects of long-term supplementation of silicon are unknown as well.

Reported silicon concentrations in cow milk have been conflicting. Concentrations have been reported to be 0.81 ppm in normal cows (Parantainen et al., 1987), 0.39 ppm in mastitic cows (Parantainen et al., 1987), or absent entirely (Anderson, 1989). Differences may come from the use of glass labware, processing procedures, or simply cow differences due to diet or environment. Supplemental silicon, in the form of sodium silicate (Na_2SiO_3) and at a dosage rate of 1 gm/d, did not consistently or significantly increase the silicon content of milk in Holstein, Ayrshire, and Guernsey cows (Archibald and Fenner, 1957). However, the form of silicon may not be as bioavailable as other forms, such as sodium zeolite A, and the low dosage rate may not be sufficient to cause an increase in milk concentration. Finally, the study was conducted in only six cows, two per breed, which may explain the variability in results.

Unlike cows, an increase in milk silicon concentration has been reported in mares (Lang et al., 2001b). Within 24 hr of foaling, mares were supplemented with sodium zeolite A at a dosage rate of 2% dietary intake, for 45 days. Milk silicon concentration

was higher ($P < 0.01$) on d 45 than d 0 in treated mares, and on d 45, milk silicon concentration was higher ($P < 0.001$) in treated mares compared to control mares (Lang et al., 2001b). Additionally, plasma silicon concentration in treated mares' foals was higher ($P < 0.05$) on d 45 than d 0 and plasma silicon concentration on d 45 was higher ($P < 0.01$) in treated mares' foals compared to control mares' foals. This study suggests that supplemental silicon can increase silicon concentrations in milk, and that the milk silicon can be consumed and absorbed. Therefore, studies need to be conducted with a bioavailable source of silicon, and at a higher dose than in a previous study, to determine if milk silicon concentration can be increased in supplemented cows.

Silicon essentiality

Silicon has been suggested to be an essential element for several biological processes including bone formation and mineralization, glycosaminoglycan synthesis and composition, collagen formation, and brain function. As a result of the research implicating silicon as an essential element, the American Institute of Nutrition added silicon to the purified rodent diet (Reeves et al., 1993). Numerous studies have been conducted in rats and chicks, but no studies on silicon essentiality in other animals have been found. Additionally, the studies were conducted primarily by one research group (E.M. Carlisle) using extreme conditions. Essentiality was determined by the finding of adverse effects when silicon was deficient in the diet, as compared to when adequate to high levels of silicon were supplied in the diet. To obtain the deficient diets, extreme measures such as washing ingredients like casein to remove silicon (Carlisle, 1980a), were taken so that the highly purified diets induced responses to the low intakes.

Furthermore, the crystalline amino acid diets used in studies by Carlisle prior to 1980 were admittedly inadequate diets that produced less than optimum growth even in the control animals (Carlisle, 1981). Silicon deficiencies have not been reported under normal management circumstances, and requirements for humans and animals have not been established (Nielsen, 1991). However, the requirements for silicon are probably fairly low as diets used to induce adverse effects typically must be below 2 mg Si/kg diet (Nielsen and Poellet, 2004). Rats fed 4.5 mg Si/kg diet did not differ from rats fed 35 mg Si/kg diet, and both prevented the deprivation signs exhibited by rats fed 1 mg Si/kg diet (Seaborn and Nielsen, 1993). Additionally, essential elements are normally found in adequate quantities in milk, so that young animals may receive nutrition to enhance proper growth. Silicon has been found to be absent (Anderson, 1989) or present in low quantities (0.81 mg Si/kg milk) (Parantainen et al., 1987) in cow milk. Horse milk also contained low quantities of silicon (Lang et al., 2001b). There is some support to the suggestion that silicon is an essential element; however, a more appropriate term might be "probably essential", as suggested by Georgievskii (1982).

Bone formation and mineralization. Some evidence exists that suggests silicon may be an essential element for bone formation and mineralization. Silicon deprivation in chicks caused depressed long bone skeletal development, and also caused the skulls to be abnormally shaped and smaller (Carlisle, 1972). Dietary silicon deficiencies in rats caused depressed growth and skull abnormalities (Schwarz and Milne, 1972). . However, the diets used in the previous studies were inadequate to maintain optimum growth in the control group as well. Subsequent studies using a more appropriate diet

seem to support the earlier postulates that silicon is essential for bone formation. A study conducted in chicks revealed a difference between chicks consuming a silicon-supplemented diet and those consuming a silicon-deficient diet (Carlisle, 1980a). The matrix of the deficient chicks' skulls lacked the normal striated trabecular pattern. The deficient chicks also showed a nodular pattern of bone arrangement, indicative of a primitive type of bone. Additionally, collagen content was reduced in the deficient chicks. Silicon deficient chicks also had gross abnormalities in skull architecture, with fewer trabeculae and less calcification (Carlisle, 1981).

A study conducted in vitro showed that silicon was present in active growing areas of bones in young rats and mice (Carlisle, 1970). In the process of mineralization, initially silicon and calcium concentrations rose congruently in osteoid tissue. In the more advanced stages of mineralization, silicon concentrations fell markedly while calcium concentrations approached proportions found in mature bone (Carlisle, 1970). Silicon appears to be involved in calcification through some effect on preosseous matrix. In vivo studies show that silicon hastens the rate of bone mineralization. The tibia of rats fed a high (250 ppm) silicon diet reached a higher degree of mineralization in a shorter time than the tibia from a medium (25 ppm) and low (10 ppm) silicon diet (Carlisle, 1970).

Glycosaminoglycan synthesis and structure. Formerly called mucopolysaccharides, glycosaminoglycans (GAGs) are isolated from vertebrate connective tissue. The structures of the different families of GAGs are similar, as each are hydrophilic, unbranched single chain polymers with repeating disaccharide units

(Lovekamp et al., 2005). Glycosaminoglycans, with the exception of hyaluronic acid, contain sulphate groups (Fraser et al., 1997). They are synthesized in the endoplasmic reticulum and Golgi bodies. Hyaluronic acid is the major GAG of synovial fluid (Scott et al., 2000). Unlike other GAGs, hyaluronic acid's primary structure does not contain a protein portion, a reflection of plasma membrane synthesis origin, and not Golgi body. Hyaluronic acid gives synovial fluid its viscosity, and is synthesized by cells in the synovial membrane. It appears to have several functions, which include lubrication, water homeostasis, filtering effects, and plasma protein distribution. Common thought has been that hyaluronic acid itself was the active ingredient in boundary lubrication of the joint, allowing the joint surfaces to reduce friction, thereby preventing wear. However, hyaluronic acid is not necessarily the lubricant of the joint, but rather the carrier of the lubricant to the binding site to facilitate boundary lubrication, as lubrication was not changed when hyaluronidase was applied to synovial fluid, even though viscosity was decreased (Hills and Monds, 1998). Furthermore, hyaluronic acid acts as a wetting agent, reducing the surface energy of the interface between synovial fluid and the hydrophobic articular surface. This could have an important role in promoting hydrodynamic lubrication, which is favored where load permits because it involves no contact on the sliding surfaces and, therefore, no wear (Hills and Monds, 1998). Hyaluronic acid concentration in the synovial fluid varies with species, with higher concentrations found in rabbits (3.6 mg/ml) (Price et al., 1996) than adult horses (approximately 0.5 mg/ml) (Persson, 1971). Bovine metatarso-phalangeal synovial fluid also contains approximately 0.5 mg/ml hyaluronic acid (Coleman et al., 1999). Age of

the subjects appears to play a role in hyaluronic acid concentration as the young human concentration of 3.8 mg/ml falls to 2.5 mg/ml in the elderly population (Balazs, 1982).

Results from several studies suggest that silicon may be important in glycosaminoglycan synthesis and composition. The total amount of hexosamines and the proportion of hexosamine in the comb were found to be greater in combs of chicks supplemented with silicon, as compared to combs of chicks on a silicon deficient diet (Carlisle, 1976). Additionally, articular cartilage from chicks supplemented with silicon contained greater amounts of GAG than cartilage from silicon deficient chicks (Carlisle, 1974). The same supplemented chicks also had greater water content in their tibial bones, which the author concluded was due to an increased GAG concentration as GAGs attract and bind water (Carlisle, 1976).

Fractionation procedures reveal that connective tissues yield high amounts of silicon (Carlisle, 1984). No less than 330 to 554 ppm bound silicon has been detected in chondroitin 4-sulfate, heparin sulfate and hyaluronic acid from umbilical cord (Schwarz, 1973). This could correspond to one atom of silicon to about 0.3 molecules of hyaluronic acid. However, hyaluronic acid from bovine vitreous humor contained negligible amounts of bound silicon. The silicon found in the GAGS exists not as free silicate ions or silicic acid, but as a firmly bound component of the polysaccharide matrix. Therefore the bound silicon could function in several ways. It could modulate molecular shape and establish a secondary structure by connecting different portions of the same chain (Schwarz, 1973). Instead of connecting the same chain, it could connect different polysaccharide chains together, thereby contributing to the high molecular size of GAGs (Schwarz, 1973). Additionally, silicon could connect polysaccharides and collagen

(Schwarz, 1973). Silicon could link the binding sites through a $R_1 - O - Si - O - R_2$ group. Thus silicon could aid in the architecture and stability of GAGs. As the Schwarz (1973) study appears to be the only study pinpointing silicon as an actual component of GAGs, a more current study utilizing current technologies should be conducted to confirm the findings.

Osteochondrosis. Supplemental silicon might impact osteochondrosis. Osteochondrosis (OC) is defined as a disturbance of cell differentiation in joint cartilage leading to altered endochondral ossification (Jeffcott, 1991). No specific cause of OC has been identified yet, but there are multiple factors that have been pinpointed including growth rates, nutrition, endocrinological factors, genetics, and trauma (Jeffcott, 1991). Regardless of the factor(s), the result remains the same – disturbed endochondral ossification. Key cellular events in the normal developing epiphysis include chondrocyte proliferation, extensive extracellular matrix creation, chondrocyte differentiation, vascular invasion and matrix calcification. In OC, vascular penetration of the distal region of the proliferative zone fails, which leads to a failure of the final stages of cartilage maturation and modification of the surrounding matrix, resulting in the accumulation of small rounded chondrocytes apparently trapped in the predifferentiation stage within the cartilage (Jeffcott and Henson, 1998). The early lesion of OC develops as a small retained core of cartilage extending into the subchondral bone.

Cartilage extracellular matrix alterations have been identified in naturally occurring OC in horses (Lillich et al., 1998). These included a loss of glycosaminoglycans, including chondroitin sulfate, in cartilage from OC lesions when compared to normal (Lillich et al., 1998). Proteoglycan production from chondrocytes

was decreased in explant cultures of cartilage from equine OC lesions (van den Hoogen et al., 1999). The authors hypothesized that the chondrocytes had lowered metabolism and decreased vitality, and that spontaneous regression of the lesions could not occur at that point. The disturbed proteoglycan synthesis was more likely to be a consequence than a primary cause of the disturbed ossification (van den Hoogen et al., 1999).

Silicon toxicity

The main problems with silicon toxicity occur in the lungs and kidneys. Inhalation of dust and silicates can cause silicosis, which stimulates a severe reaction in the lungs (Carlisle, 1984; Rimal et al., 2005). A subsequent increase in immunoglobulin production and lymphocyte production occurs (Moseley et al., 1988). After phagocytosis of the silica particle by the macrophages, the macrophages are killed, reingested by other macrophages, and the cycle continues. Collagen synthesis is then stimulated by the macrophage death (Carlisle, 1984). Additionally, inflammatory cytokines such as TNF-alpha are increased, causing damage to the lungs (Rimal et al., 2005).

In some instances, silica is deposited in the urinary tract, forming calculi (Carlisle, 1984). Eventually the calculi may become large enough that urine excretion is blocked, which could lead to death. There is little understanding of the formation of calculi, as efforts to create them in sheep and cattle, such as increasing silicon intake, have been unsuccessful. However, calculi were created in rats and dogs fed high levels of a silicon supplement, not silica (Carlisle, 1984).

Supplemental bioavailable silicon

Although abundant in the environment, silicon is usually not readily absorbed by animals. The naturally occurring form is silica (SiO_2) (Carlisle, 1982) which is present in grasses and cereal grains. However, silica is considered to be largely insoluble, and thus relatively unavailable. Silica would need to be broken down into soluble parts. Orthosilicic acid ($Si(OH)_4$) is formed by hydration of silica. Orthosilicic acid is the major form of silicon in drinking water and other liquid sources, including beer (Reffitt et al., 1999). Studies suggest that orthosilicic acid might be the only detectable, absorbable form of silicon (Jugdaohsingh et al., 2000) and has an approximate absorption rate of 53% in humans. Therefore, studies interested in supplementing silicon should use the bioavailable form, orthosilicic acid.

A study conducted on 60 calves demonstrated the high bioavailability of orthosilicic acid (Calomme and Vanden Berghe, 1997). The treated group received 377.5 mg of silicon, including basal diet silicon content and supplemental orthosilicic acid, while the control group received 360 mg of silicon through their basal diet. With just a 4.9% increase in dietary silicon intake from supplemental orthosilicic acid, the treated group had a 70% higher serum silicon concentration than control calves after 23 wks. Additionally, the treated calves had higher levels of collagen in the skin, indicating that not only was there increased absorption as evidenced by increased serum silicon, but that the silicon was able to illicit changes in extracellular matrix formation, suggesting bioavailability.

Increasing dietary silicon may have beneficial effects on bone health, as suggested by in vitro and in vivo work. Orthosilicic acid (absorbable form of silicon) at

physiological concentrations stimulates in vitro collagen type 1 synthesis in human osteoblast-like cells and enhances osteoblastic differentiation (Reffitt et al., 2003). Osteocalcin was used to determine osteoblastic differentiation, and was found to be increased by both 10 μM and 20 μM orthosilicic acid. Gene expression of osteocalcin was also increased in the presence of orthosilicic acid. Furthermore, alkaline phosphatase, a marker of bone formation, was increased by supplementation of orthosilicic acid (Reffitt et al., 2003). The precise method by which silicon enhances collagen type I synthesis is unknown.

The population sector that may reap the most benefits of silicon supplementation is that of post-menopausal women as well as people afflicted with osteoporosis. Silicon appears to alleviate the loss of bone mineral content or mass, common symptoms of osteoporosis and ones that normally accompany menopause. Rats that underwent an ovariectomy, and thereby experienced menopausal conditions such as a decrease in estrogen, did not show a loss of bone mass when supplemented with silicon, as compared to sham-operated controls (Rico et al., 2000). Not only did silicon appear to inhibit bone loss, it also stimulated bone formation in the ovariectomized rats. Silicon has also had positive effects in humans as well. Silicon supplementation in the form of monomethyl trisilanol at a dosage rate of 50 mg twice a week for a period of 4 months was associated with increased femoral bone density in post-menopausal, osteoporotic women (Eisinger and Clairet, 1993). Silicon may have a therapeutic effect on osteoporosis by the prevention of bone loss and stimulation of bone formation.

Sodium zeolite A. Sodium zeolite A (SZA) is an aluminosilicate that is hydrolyzed at low pH into silicic acid, amorphous aluminum silicates and aluminum (Thilsing-Hansen et al., 2002). Thus, by supplying SZA to the diet, an animal's gastrointestinal tract can break down the zeolite into orthosilicic acid. The orthosilicic acid is absorbed, thus providing the body with supplemental silicon. The extent of absorption of Al released from the zeolite is unknown. Zeolites are crystalline structures that have a high attraction for water and a large number of positively charged ions, such as K^+, NH_4^+, Ca^{2+}, and Mg^{2+}, which can be reversibly bound or released (Mumpton and Fishman, 1977). Multiple studies have been conducted in mammalian species with sodium zeolite A supplementation (Frey et al., 1992; Roussel et al., 1992; Nielsen et al., 1993; Lang et al., 2001b,c; Thilsing-Hansen et al., 2003).

An in vitro study conducted with avian osteoclasts concluded that sodium zeolite A can inhibit bone resorption (Schutze et al., 1995). When osteoclasts were treated with 100 μg/ml of SZA, the number of pits per osteoclast was reduced 3-fold at 24 hours after treatment. Thus, osteoclast-mediated resorption activity was reduced. Additionally, secreted cathepsin B enzyme activity was reduced 3-fold. The conclusion was that the actual structure of SZA is responsible for the effects as compounds used to mimic constituents of SZA failed to illicit the same responses (Schutze et al., 1995). Thus, inferences on the effect of SZA on bone resorption in vivo are limited, as the compounds in SZA are separated in the digestive tract, and therefore may be ineffective.

Studies conducted in horses produced inconclusive results on bone metabolism. Pyridinoline crosslinks (PYD) and carboxy-terminal pyridinoline cross-linked telopeptide region of type-I collagen (ICTP), markers of bone resorption, were measured in mares

from 1-d post-parturition to one month post-parturition (Lang et al., 2001b). Sodium zeolite A tended to decrease PYD in supplemented mares on d 30, but by d 45 the differences were gone. However, ICTP was not affected. Pyridinoline is not a bone specific marker, and can be affected by collagen breakdown in other tissues, such as the uterus (Christenson, 1997). The more specific bone marker, ICTP, was unaffected, implicating that SZA has little to no effect on bone resorption in broodmares. Alternatively, PYD was not affected in SZA supplemented yearlings, while ICTP was decreased on d 45 (Lang et al., 2001c). Not only are bone resorption results inconclusive, but bone formation results as well. Osteocalcin, a marker of bone formation, was not different between control and SZA supplemented yearlings (Lang et al., 2001c). However, supplemented broodmares tended to have higher concentrations of osteocalcin compared to control horses (Lang et al., 2001b). The effects of SZA on bone metabolism in vivo are uncertain, and should be clarified. Sodium zeolite A has been associated with reduced skeletal injuries in race horses. Fifty-three race horses entering race training at 18 months of age were divided into four groups at 6 mo of age: 0, 0.92, 1.86 or 2.00 % dietary SZA (Nielsen et al., 1993). The medium and high treatment groups had greater distances traveled before injury, and the medium group also had more cycles (strides) until injury. Fewer injuries occurred in the treated groups as well (Nielsen et al., 1993). Of thirteen control horses, 8 became injured during the study. Five horses (out of 13) in the 0.92% SZA group became injured, two horses (out of 9) in the 1.86 SZA% group were injured, and 4 (out of 12) horses in the 2.00% SZA became injured during the study. Furthermore, plasma Si concentrations correlated with training distance to failure

(Nielsen et al., 1993). However, the mechanisms by which SZA is associated with fewer injuries is unknown.

Sodium zeolite A has also been used in several ruminant studies, with marked effects on mineral metabolism, particularly that of calcium and phosphorus. Decreases in plasma phosphorus concentrations occurred in cows fed varying levels of SZA, from 0.5 to 1.0 kg daily (Thilsing-Hansen et al., 2003). Additionally, the incidence of parturient hypocalcemia (milk fever) was decreased in cows fed SZA (Thilsing-Hansen and Jorgensen, 2001; Thilsing-Hansen et al., 2003). Serum calcium concentrations were higher in treated cows than control cows following SZA supplementation, thereby preventing hypocalcemia. Cows also had increased serum calcium when fed 1.0 and 1.5% SZA of a diet consisting of 50% alfalfa hay as well as 1.5% SZA of a diet containing 50% corn silage (Roussel et al., 1992). An increase in calcium absorption has been shown in chickens fed 1.0% zeolite A for 7 d (Ballard and Edwards, 1988).

A single study investigating the effects of sodium zeolite A on tissue mineral composition was found. Swine fed 0.50% SZA had increased liver and bone zinc, but no accumulation of Ca, P, or Al in the bone (Ward et al., 1991). The pigs also had decreased serum Ca and P concentrations. However, silicon concentrations were not determined. As SZA appears to alter mineral metabolism, both in the blood and tissue, in-depth studies should be conducted to determine the extent of alteration, particularly that of silicon in tissues.

Silicon and aluminum interactions

There is a dearth of information pertaining to silicon and its interactions with other minerals, with the exception of aluminum. There appears to be a strong interaction between silicon and aluminum. Silicon may be the natural antidote to aluminum toxicity (Reffitt et al., 1999). An inverse relationship exists between silicon and aluminum in water supplies (Birchall and Chappell, 1989). Alzheimer's disease appears to be prevalent in areas with high concentrations of Al in drinking water supplies. The low incidence of Alzheimer's in areas with low aluminum concentration, and thus high silicon concentration, may be due to the excess silicon forming hydroxyaluminosilicates, and thus prevention of Al uptake. Supplementation of aluminum increased brain Al concentrations when rats were fed a low silicon diet, yet the increase was negated when rats were fed a silicon supplemented diet (Carlisle and Curran, 1987). Silicon has been hypothesized to be essential for collagen formation (Seaborn and Nielsen, 2002a,b), in which silicon deprivation decreased the amount of hydroxyproline. Aluminum appears to inhibit prolylhydroxylase activity; however, this inhibition is relieved when silicon is present.

Orthosilicic acid reduces Al absorption in humans (Edwardson et al., 1993). Silicate did not affect gastrointestinal uptake of ^{26}Al, but rather increased renal clearance almost two-fold over the first 24-hr post supplementation in humans (King et al., 1997). Bellia et al. (1996) found an increase in aluminum renal clearance in response to monosilicic acid in beer, and suggested that silicon and aluminum may form hydroxyaluminosilicates in the kidney, thereby decreasing aluminum reabsorption. Alternatively, Reffitt et al. (1999) found no increase in urinary Al in humans

supplemented with orthosilicic acid, and suggested that perhaps something in the beer besides silicon in the Bellia et al. (1996) study may be responsible for excretion of aluminum from body stores. The form of silicon also plays a role on its effect on aluminum metabolism. Monomeric silicon (orthosilicic acid) failed to prevent an increase in serum aluminum following ingestion of an aluminum supplement, while oligomeric silicon reduced serum aluminum by approximately 67% (Jugdaohsingh et al., 2000). Moreover, the oligomeric silicon also reduced urinary excretion of Al. To lend further evidence that oligomeric silicon interacts with aluminum in the gastrointestinal tract and prevents aluminum absorption, oligomeric silicon itself was not absorbed (Jugdaohsingh et al., 2000). They determined that 280 μmol Si/L is needed to chelate 8 μmol Al/L. Alternatively, monomeric silicon was easily absorbed and had no effect on aluminum metabolism. The form of silicon needs to be taken into account when designing studies to investigate the effects of silicon on aluminum metabolism.

The Si:Al ratio may also play a role in aluminum toxicity. When the Si:Al ratio dropped to 3.7 from 13.0 in a fish tank there was a significant increase in aluminum levels in the fish (Birchall et al., 1989). Aluminum levels in the fish continued to rise as the Si:Al ratio dropped below 1.0. An excess of silicon over aluminum appears to be necessary to reduce the effects of aluminum toxicity.

Aluminum and livestock

Excess exposure to aluminum is significantly associated with phosphate binding in the digestive tract, phosphate deficiency, and interference with phosphate metabolism (Krueger et al., 1985). The fact that high dietary aluminum interferes with phosphorus

metabolism in animals by forming unabsorbable complexes in the digestive tract has been known for a long time (Deobald and Elvehjem, 1935). A significant decrease in plasma phosphorus in response to supplementation of aluminum has been seen in lambs (Rosa et al., 1982), beef cows (Allen et al., 1986) and chicks (Hussein et al., 1990). The decrease can occur within 7 days post supplementation.

An increase in plasma calcium concentrations occurred when chicks were fed high amounts of aluminum (0.196% of the diet) (Hussein et al., 1990). This effect was seen as early as 7 days post supplementation. Increases in serum calcium concentrations were also observed in lactating beef cows receiving 1,730 mg Al/kg DMI (Allen et al., 1986). The increase in calcium may be as a result of homeostatic measures in response to the decreased phosphorus. Decreases in calcium concentration in the bone have been reported after high aluminum supplementation (Allen, 1984). When additional phosphorus was added to the diets, the increase in plasma calcium was less dramatic (Hussein et al., 1990). Changes in calcium and phosphorus metabolism have also been noted in humans with high aluminum intakes, including hypophosphaturia, hypophosphatemia, and hypercalciuria (Spencer et al., 1982). Hypercalciuria may be due to increased calcium absorption. An increase in calcium retention and availability was seen in non-lactating cows that were fed an additional 1000 mg Al/kg DMI (Robinson et al., 1984).

Aluminum interacts not only with phosphorus and calcium, but magnesium as well. Aluminum added to the rumen of steers decreased serum magnesium (Allen and Robinson et al., 1980), as did dietary aluminum (Allen et al., 1984; Allen et al., 1986). Chicks fed 0.392% Al for 21 days also had decreased magnesium blood concentrations

(Hussein et al., 1990). The same response was seen in sheep fed 2,000 mg Al/kg feed for 56 d (Valdivia et al., 1982) and tended to be seen in lambs fed 1,450 mg Al/kg feed for 76 d (Rosa et al., 1982). However, dairy calves approximately 64-d-old given 0.20% Al did not show a decrease in serum magnesium concentrations (Neathery et al., 1990a). The mechanisms by which aluminum interacts with magnesium are not as clearly defined as with phosphorus (Allen, 1984). Unlike with phosphorus, the effect of aluminum on magnesium does not appear to be from the formation of unabsorbable complexes (Allen and Fontenot, 1984). Although serum magnesium levels are depressed, there are no changes in magnesium absorption (Valdivia et al., 1982; Allen and Fontenot, 1984).

Very few studies exist on supplying aluminum to horses. One study found that there were no ill effects of feeding 1,370 mg Al/kg DMI but 4,500 mg Al/kg DMI decreased plasma phosphorus concentration, and increased plasma and urinary calcium concentration (Schryver et al., 1984). In the same study, the ponies on the high aluminum diet were found to have increased plasma hydroxyproline, indicating higher bone turnover perhaps due to altered calcium and phosphorus metabolism. Roose et al. (2000) found that feeding mature Thoroughbreds either 160 mg Al/kg DMI or 930 mg Al/kg DMI produced no adverse effects on phosphorus or calcium metabolism. Additionally, there were no changes in nutrient digestibilities such as dry matter, crude protein, fiber and mineral digestibilities.

There appears to be a threshold at which Al has negative effects on feed intake. Lambs fed 810 mg Al/kg DMI maintained feed intake (Thompson et al., 1959) as did steers fed 1,200 mg Al/kg DMI (Valdivia et al., 1978). Supplementation of 1,450 mg Al/kg DMI (Rosa et al., 1982) and 2,000 mg Al/kg DMI (Valdivia et al., 1982) decreased

feed intake in lambs. Young Holstein calves reduced their feed intake by 17% and had a 47% decrease in body weight gain after consuming a diet consisting of 0.20 % Al (Crowe et al., 1990). The decreases in feed intake may be a result of the binding of aluminum to phosphorus in the digestive system, essentially reducing dietary phosphorus to the animal. The animals then begin to exhibit classic signs of phosphorus deficiency, the most common deficiency in ruminants. These signs include decreases in both feed intake and milk production (Call et al., 1987), and unchanged concentrations of minerals in the milk (Underwood, 1981).

The negative effects of high aluminum intakes can be alleviated by supplementing dietary phosphorus. Increases in previously decreased plasma inorganic phosphorus concentration has been observed when phosphorus was supplied to the animals (Lipstein and Hurwitz, 1982; Hussein et al., 1990). Decreases in gain and feed intake in Al-supplemented lambs were reversed when the animals were fed additional phosphorus (Valdivia et al., 1977; Rosa et al., 1982). Lipstein and Hurwitz (1982) suggest that 0.76 g of supplemental phosphorus is required to offset the effect of 1 g of aluminum. Animals that are borderline phosphorus deficient would seem to be most at risk of the negative aluminum effects on phosphorus metabolism.

While the addition of dietary aluminum caused a decrease in plasma phosphorus concentration, and an increase in plasma calcium concentration, plasma aluminum concentration remained unchanged (Hussein et al., 1990). This might be a reflection on the thought that dietary aluminum is poorly absorbed and has "little effect on animal life" (McCollum et al., 1982). King et al. (1997) found that apparent gastrointestinal uptake of aluminum is between 0.1-0.3%, which was probably slightly underestimated. As plasma

aluminum levels are not indicative of tissue storage status (Krueger et al., 1985), it might be possible that plasma aluminum concentrations do not change because the aluminum is rapidly transported to the tissues. Several studies have investigated the effects of high aluminum on tissue mineral composition. Rosa et al. (1982) used 1,450 mg Al/kg DMI in lambs while Valdivia et al. (1982) used a slightly higher level of 2,000 mg Al/kg DMI, also in lambs. For ease of reporting results, Table 1 displays information on the organ responses to high aluminum intakes. Valdivia et al. (1982) found that at least a portion of dietary aluminum is absorbed and transported as there were increases in the liver, kidney and muscle. Therefore the opinion that dietary aluminum in essentially non-absorbable should be re-evaluated.

Table 1. Responses of tissue mineral composition to high dietary aluminum ($P < 0.10$)

Organ	Effect	Reference
Liver	Increased Al	Valdivia et al., 1982
	Increased Zn	Valdivia et al., 1978
	Decreased Mn	Valdivia et al., 1982
Kidney	Decreased Mg	Rosa et al., 1982; Valdivia et al., 1982
	Decreased P	Rosa et al., 1982
	Increased Al	Valdivia et al., 1982
	Increased Cu	Rosa et al., 1982; Valdivia et al., 1982
	Increased Zn	Valdivia et al., 1978; Rosa et al., 1982
Muscle	Increased Al	Valdivia et al., 1982
	Increased Ca	Rosa et al., 1982
	Increased Cu	Rosa et al., 1982; Valdivia et al., 1982
	Increased Mg	Rosa et al., 1982
Bone	Decreased P	Rosa et al., 1982
	Decreased Mg	Rosa et al., 1982
	Increased Mn	Valdivia et al., 1982

Aluminum toxicity

Aluminum is common in soils, and tends to cause problems with excess, rather than deficiency. Besides changes in feed intake and phosphorus absorption, high aluminum intakes can be toxic to both animals and humans. In livestock, the most common results of aluminum toxicity are phosphorus deficiency and grass tetany. Aluminum has been linked to grass tetany in cattle, a metabolic disorder characterized by hypomagnesaemia (Robinson et al., 1984). High intakes of aluminum depress serum magnesium levels (Rosa et al., 1982; Valdivia et al., 1982; Hussein et al., 1990).

Patients with chronic renal failure are often dosed with high levels of aluminum to reduce hyperphosphatemia (Krueger et al., 1985). However, oftentimes the patients develop vitamin D- and vitamin D metabolite-resistant osteomalacia, which is thought to develop from the aluminum (Krueger et al., 1985). Aluminum accumulates in the calcification portion of the bone, preventing calcium from entering the bone (Boyce et al., 1982). Subsequently, osteomalacia develops, and hypercalcemia occurs. Decreased bone remodeling, with decreased numbers of osteoclasts and osteoblasts, and high plasma and bone aluminum concentrations are signs of osteomalacia (Chazan et al., 1991). Besides osteomalacia, high aluminum intakes can cause adynamic bone disease, in which bone turnover ceases (Jeffery et al., 1996). Renal patients are not the only ones subject to bone problems following aluminum administration. Rats developed osteomalacia after accumulating aluminum in the bone (Goodman, 1986).

Chronic or high intakes of aluminum can cause anemia, mainly through the reduction of hemoglobin synthesis (Abreo et al., 1990) via iron deficiency (Jeffery et al., 1996). Rats supplemented with aluminum had decreased hematocrit, hemoglobin and

plasma iron concentrations (Guo et al., 2004). Aluminum likely interferes with iron metabolism. Aluminum can reduce intestinal iron uptake (Cannata et al., 1991). Additionally, the reduction of Fe^{3+} to Fe^{2+}, mediated by ceruloplasmin, is important for translocation of iron and for the release of iron from ferririn. Ceruloplasmin is inhibited by aluminum (Jeffery et al., 1996). However, the exact mechanisms by which aluminum interferes with iron metabolism are unclear, but it is likely through competition of binding sites.

Aluminum can bring about neurotoxic effects as well. In humans, aluminum toxicity has been associated with memory loss, tremor, jerking movements, impaired coordination, sluggish motor movement, loss of curiosity, and ataxia (Nayak, 2002). Rats fed 0.3% aluminum in their drinking water displayed specific cognitive impairment (Jope and Johnson, 1992). However, locomotor skills were not impaired. Aluminum has also been linked to Alzheimer's disease (AD) (Nayak, 2002). Elevated levels of aluminum have been found in the brains of AD patients (Yoshimasu et al., 1981), and AD incidence has been linked to aluminum in drinking water (Martyn et al., 1989). However, a more recent study concluded that aluminum in drinking water is not related to AD (Wettstein et al., 1991). Additionally, the paired helical filaments characteristic of AD were not found in dementia patients with elevated brain aluminum levels (Arieff, 1990). The link between aluminum and AD remains unclear, but ingestion of additional aluminum should be avoided just in case.

Aluminum can be toxic to the cardiovascular system as well as the hepatobiliary system, as both the heart and liver can accumulate aluminum (Nayak, 2002). Aluminum

is associated with arrhythmia and sudden death in hemodialysis patients (Birchall, 1991). Hepatocytes can be destroyed by large deposits of aluminum (Nayak, 2002).

Analyses

Silicon. Analysis of samples for silicon concentration is often regarded as being one of the most difficult procedures in trace mineral research for several reasons. The first is the presence of silicon in the environment. As the second most abundant element in the earth's crust, silicon is found almost everywhere. In the laboratory, contamination of samples by silicon-containing dust is a common problem. An even larger problem is that of silicon leaching from glassware into samples. This is a problem not only in the laboratory but also in the field, as blood collection into standard glass Vacutainers is discouraged due to contamination.

A second potential problem in silicon analysis is the ability to digest the samples so that total silicon is obtained, as silicon is found in tightly bound forms. It is resistant to most digestion substances, as it has a low solubility in acid media (Schwarz, 1973). Currently, there are two accepted methods for silicon digestion. One requires the use of hydrofluoric (HF) acid, the other tetra methyl ammonium hydroxide (TMAH) (Hauptkorn et al., 2001). The advantage of using the HF method is that it is strong enough to break silicon bonds, thereby allowing total silicon recovery. However, the HF is so strong it will dissolve glass (hence the reason for breaking silicon bonds), which could potentially harm analysis instruments. Therefore, a masking agent such as boric acid is needed to prevent the etching of glassware. In addition, the hazards to humans that come into contact with the HF are great. Lastly, HF mixtures are highly volatile,

thus care is needed to prevent silicon loss into the environment. Therefore, digestion with an alkaline mixture such as TMAH is beneficial, as losses to volatization are minimized and a masking agent is not needed (Hauptkorn et al., 2001). Additionally, biological samples need to undergo microwave digestion and the way silicon is embedded in the tissue sample dictates the amount of heat needed. For example, ascorbic acid and pork liver samples gave consistent results after heating to 60° C, while bovine liver and muscle needed to be heated to 80 and 125° C, respectively (Hauptkorn et al., 2001).

GAG concentration. In the past, glycosaminoglycan concentration of cartilage was determined in vitro using biochemical and histological analyses. Recently a method was developed that utilizes magnetic resonance imaging (MRI) to determine cartilage GAG content using the charged contrast agent gadolinium diethylene triamine pentaacetic acid ($Gd(DTPA)^{2-}$) (Burstein and Gray, 2003). This method, delayed gadolinium-enhanced magnetic resonance imaging of cartilage (dGEMRIC), is a sensitive and specific measure of GAG content that can be used for both in vivo and in vitro analyses. The dGEMRIC method works on the principle that cartilage GAGS have negatively charged carboxyl and sulfate groups that create a negatively fixed charged density to the cartilage matrix. Thus, ions that are distributed throughout the cartilage will respond to the local fixed charge density of the surrounding cartilage area. The $Gd(DTPA)^{2-}$ has a negative fixed charge as well. Thus, when the contrast agent is distributed throughout the cartilage it is repelled by the negatively charged GAGs (Burstein and Gray, 2003). Hence, there will be less $Gd(DTPA)^{2-}$ in areas of higher GAG

concentration, and greater amounts of $Gd(DTPA)^{2-}$ in regions of low GAG content. A quantitative number for GAG content in a cartilage region can be calculated by obtaining the $Gd(DTPA)^{2-}$ concentration of that particular region (Bashir et al., 1999).

The dGEMRIC procedure has been validated in vitro in bovine (Bashir et al., 1996) and human (Bashir et al., 1999) tissue. Cartilage samples were first placed in saline. After equilibration, T_1 of the sample was measured, and then the cartilage was placed into a solution containing saline with added $Gd(DTPA)^{2-}$ (Bashir et al., 1999) and allowed to equilibrate. T_1 was again measured on the cartilage plug. Tissue $Gd(DTPA)^{2-}$ concentration was calculated, followed by fixed density charge (FCD). Glycosaminoglycan concentration was calculated using the FCD and assuming -2 moles of charge per mole of disaccharide and a molecular weight of 502.5 g/mole (Bashir et al., 1999).

The samples were then digested and analyzed for GAG content using the standard biochemical method, the dimethylmethylene blue (DMMB) assay. There was a high correlation between using dGEMRIC and DMMB assay ($r^2 = 0.95$; slope = 1), thereby validating the use of dGEMRIC to quantify GAG concentration in cartilage samples (Bashir et al., 1999). The dGEMRIC results were also compared to a histological analysis, Toluidine blue staining. Similar to the biochemical comparison, there was a high agreement between dGEMRIC and histological analysis (Bashir et al., 1999). The dGEMRIC procedure was further validated in vivo in human subjects. $Gd(DTPA)^{2-}$ was injected intravenously into the subjects at a rate of 0.2 mM/kg BW (Bashir et al., 1999). The subjects then exercised for 10 minutes, to ensure adequate distribution into the tissues, as it was previously determined that exercise increased uptake into the joints

(Drape et al., 1993; Winalski et al., 1993). Approximately two hours post injection the subjects underwent MR imaging of their knee joints. The GAG concentration was then calculated. Total knee replacement surgery was performed on the subjects, and the removed patella and tibial plateau were imaged after equilibration with $Gd(DTPA)^{2-}$. There was good correlation between the in vivo and in vitro results, validating the use of dGEMRIC in vivo (Bashir et al., 1999). The dGEMRIC procedure appears to be an innovative and valid method of quantifying GAG content of cartilage, and would be of particular interest in in vivo studies.

Rationale for experiments

The major aim of these experiments was to explore the impact of sodium zeolite A on the physiology of different animals and further clarify several uncertainties surrounding the use of SZA. Specifically, the investigation of SZA's effect on osteochondrotic lesions will clear up anecdotal reports from the equine industry. Additionally, the issue of SZA and bone metabolism will be addressed not only by measuring bone markers of metabolism, but also by conducting mechanical stress tests and evaluating bone architecture. Finally, the determination of changes in mineral retention and absorption, and plasma and tissue mineral concentrations will provide a more complete picture on the effects of SZA on mineral metabolism. The impact of aluminum on the animals will also be investigated, as it has been largely ignored as a component of SZA. We hypothesize that SZA supplementation will decrease osteochondrotic lesion size in horses, enhance bone metabolism, and impact mineral metabolism, specifically that of calcium, phosphorus, silicon and aluminum.

CHAPTER 3

SILICON SUPPLEMENTATION AND OSTEOCHONDROTIC LESIONS IN TWO-YEAR-OLD STANDARDBREDS

SUMMARY

The objective of the study was to evaluate the effects of supplemental silicon on osteochondrotic (OC) lesion size in Standardbreds. Initial radiographs were taken on two-year-old Standardbreds (n=44) on private facilities to identify OC defects in the distal third metacarpus/tarsus or osteochondral fragments at the dorsal aspect of the joint and defects/fragments at the distal tibia and on the trochlear ridges of the talus. Only clinically sound horses were included. If lameness occurred, follow-up radiographs were taken and the horse was removed from the study. For sound horses, follow-up radiographs of lesions were taken at 120 days. Radiographs were digitized and the length and height of the OC defects/fragments were measured. Horses meeting study-inclusion requirements (n=8) were pair-matched by facility and affected joint(s) and assigned to a group: control (CO, receiving 200 g whole-grain flour) and Si supplemented (SS, receiving 200 g bioavailable Si source). Treatments were top dressed on the feed for 120 days. Four horses had lesions in one or more fetlock(s) and four had lesions in one or more hock(s) for a total of 12 affected joints. Due to the onset of lameness, four horses did not complete the 120-d study duration. There was no effect of treatment, time, or treatment*time on lesion length ($P>0.22$), height ($P>0.51$), or area ($P>0.67$). Silicon supplementation did not alter the radiographic appearance of OC lesions.

INTRODUCTION

Osteochondrosis (OC) is defined as a disturbance of cell differentiation in joint cartilage leading to altered endochondral ossification (Jeffcott, 1991). No specific cause of OC has been identified. Key cellular events in the normal developing epiphysis include chondrocyte proliferation, extensive extracellular matrix creation, chondrocyte differentiation, vascular invasion and matrix calcification. In OC, the vascular penetration of the distal region of the proliferative zone appears to fail, which disrupts the final stages of cartilage maturation and modification of the surrounding matrix, resulting in the accumulation of small rounded chondrocytes apparently trapped in the predifferentiation stage within the cartilage (Jeffcott and Henson, 1998). The early lesion of OC develops as a small retained core of cartilage extending into the subchondral bone. Cartilage extracellular matrix alterations have been identified in naturally occurring OC in horses (Lillich et al., 1998). These included a loss of glycosaminoglycans in cartilage from OC lesions when compared to normal (Lillich et al., 1998). Proteoglycan stimulation by chondrocytes was decreased in cartilage from equine OC lesions, suggesting that the chondrocytes had lowered metabolism and decreased vitality (van den Hoogen et al., 1999). It was suggested that spontaneous regression of the lesions could not occur at that point, and that the disturbed proteoglycan synthesis was more likely to be a sequence than a primary cause of the disturbed ossification.

Horse trainers have suggested a relationship between supplemental dietary silicon and spontaneous healing of osteochondrotic lesions. However, no scientific proof has been obtained. Results from several studies indicate that silicon is important in cartilage and bone metabolism. Chicks supplemented with silicon had increased

glycosaminoglycan concentrations in cartilage (Carlisle, 1976). Additionally, silicon deficiencies caused the amount of articular cartilage and glycosaminoglycan concentrations to be considerably less in chick tibias (Carlisle, 1980). Joints were less well formed and smaller when there was a silicon deficiency (Carlisle, 1976). Silicon has also been associated with hastened bone mineralization (Carlisle, 1982). Besides mineralization, the largest effect of silicon is on formation of the organic matrix (Carlisle, 1982). Silicon deficiency produced disturbed epiphyseal cartilage sequences resulting in defective endochondral bone growth in chicks (Carlisle, 1980). Therefore, supplemental silicon may reduce OC lesions by increasing glycosaminoglycan concentration, hastening bone mineralization, and improving altered endochondral bone growth.

We hypothesized that supplemental silicon would decrease the size of the lesions as they appear on radiographs. Our objective was to determine the effects of supplemental silicon on osteochondrotic lesions in the tarsi and fetlock joints of two-year-old Standardbred horses.

MATERIALS AND METHODS

Horses

Standardbred trainers in Southern Michigan with two-year-old horses were contacted via phone calls and farm visits to determine their interest in participating in the study. The horses remained on their respective facilities under the routine care of their caretakers during the duration of the study, and continued with their respective training programs as determined by their trainers. This study was approved by the Michigan State University All-University Animal Care and Use Committee.

Radiographs

Initial radiographs were taken to identify osteochondrosis lesions in the metacarpophalangeal, metatarsophalangeal (fetlock) and tarsocrural (hock) joints of the horses. Radiographs were taken by a veterinary radiologist, using a portable x-ray machine (MinXRay® HF100, Northbrook, IL) and conventional film-screen cassettes. Lateromedial projection images were taken of all four fetlocks to identify OC defects in the midsagittal ridge of the distal third metacarpus and metatarsus or osteochondral fragments at the dorsal aspect of the joint (60 KVp, 1.8 mA, 0.060 sec, 80 cm focal-film distance). Lesions in the palmar and plantar tubercles of the proximal first phalanx were excluded due to the controversial nature of the origin of these lesions (Dalin et al., 1993). Dorsaomedial to lateroplantar oblique projection images were acquired of both tarsi to identify defects or osteochondral fragments at the intermediate ridge of the distal tibia and on the trochlear ridges of the talus (65 KVp, 1.9 mA, 0.065 sec, 80 cm focal-film distance). These views were selected based upon frequency of the specific lesions of interest reported in previous literature (Alvarado et al., 1989; Grondahl, 1992). A metallic marker of known size was included on each radiograph to calculate image magnification. If horses had undergone prior joint surgery, the treated joint was excluded from the study while other joints on the same horse were included. Only clinically sound horses were included.

Treatments

Horses were assigned to two treatment groups, pair-matched by facility and affected joint(s). The control (CO) horses were fed a placebo of 100 g whole-grain flour,

top-dressed on their normal feed twice daily, so that each horse received 200 g per day for 120 days. The supplemental silicon (SS) horses received 100 g of sodium zeolite A (SZA), a bioavailable source of silicon, top dressed on their feed twice daily. Owners/trainers and the radiologist were blinded to the treatment groups. Horses were not to be fed additional supplements for the prevention or treatment of lameness (i.e., chondroitin sulfate, glucosamine).

Follow-up radiographs and lesion analysis

If lameness became apparent to the owner/trainer during the study, the date and signs of lameness were recorded, follow-up radiographs were taken, and the horse was removed from the study so that treatment could be sought. For all sound horses, follow-up radiographs of joints with OC lesions were taken at 120 days. The radiographs were digitized and an imaging software program was used to measure the length and width of the OC defects and osteochondral fragments. Area of the lesion was calculated using the formula for area of an ellipse. Using the metallic markers on the radiographs, adjustments were made on these measurements to account for image magnification.

Statistics

Due to the small numbers, lesions were grouped together and not analyzed by joint. The general linear model was used in SAS 8.2. The main effects were treatment, time and treatment*time with horse(treatment) as the error term.

RESULTS

Forty-four horses were radiographed to identify the presence of OC lesions. Thirteen horses had affected joints; however, five of those horses had plantar osteochondral fragments and were excluded. The remaining eight were placed on study. Of these eight horses, four horses had OC lesions in one or more fetlock(s) and four had lesions in one or more hock(s) for a total of 12 OC lesions in 12 joints (Table 2). At the onset of the study it was our intention to only use a trainer if they had more than one horse on study so that the horses could be pair-matched. However, due to the limited availability of horses, two trainers with one horse each were included. Due to either lameness or trainer reluctance during the trial period, four horses (three SS, one CO) did not complete the 120-d duration of the study.

Table 2. Detailed horse information with trainer, sex, gait, joints affected, treatment, and study duration.

			Joint(s) Affected			
Trainer #	Sex	Gait	Hock	Fetlock	Treatment	Duration (d)
1	Gelding	Trot		RF	SS	99
2	Horse	Trot		RH, LH	CO	120
3	Horse	Pace		RH	CO	120
2	Mare	Pace		RH, LH	SS	120
4	Horse	Trot	Both, DT		SS	62
1	Horse	Pace	Both, DT		CO	99
2	Horse	Pace	Left, LT		CO	120
5	Mare	Trot	Both, Lt		SS	53

RF = right fore; RH = right hind; LH = left hind; Both = left and right hock; DT = lesion on distal intermediate ridge of tibia; LT = lesion on lateral trochlear ridge

There was no effect of treatment (P = 0.32), time (P = 0.83), or treatment*time (P = 0.22) on length of the OC lesions (Figure 1). Lesion height (Figure 2) was also not affected by treatment (P = 0.96), time (P = 0.74) or treatment*time (P = 0.51).

Additionally, lesion area was not affected by treatment (P = 0.81), time (P = 0.67) or treatment*time (P = 0.87).

DISCUSSION

Silicon supplementation did not cause alterations in the radiographic appearance of the OC lesions in these two-year-old Standardbred horses. Fifty percent (4/8) of the trial horses did not complete the study. Two SS horses became lame and the trainers elected to remove the horses to seek treatment. The cause of lameness in the two SS horses was not determined; therefore the association between silicon supplementation and lameness was not investigated. The other two horses (1 SS, 1 CO) were from the same barn and were removed due to trainer reluctance. At the onset of the study, palatability of the SZA was a concern for several trainers; however, after a few days the horses became accustomed to it and the concerns dissipated. No negative effects of the SZA supplementation were observed.

Osteochondrosis is the disturbance of endochondral ossification by altered cell differentiation (Jeffcott, 1991). Additionally, cartilage matrix alterations such as a loss of glycosaminoglycans occur (Lillich et al., 1998). Silicon has been hypothesized to be necessary for proper endochondral ossification and glycosaminoglycan synthesis (Carlisle, 1976; 1980). A more appropriate study may have been investigating the effects of supplemental silicon on preventing lesions, rather than reducing the size of lesions that are already present.

Horses with plantar osteochondral fragments (POF) were not included in the study because there is debate over the etiology of these lesions. Many factors point

towards the fragments resulting from trauma, rather than OC. One such factor is that the classic characterization of OC, disturbance of endochondral ossification resulting in cartilage retention, is not present in the area near POF (Dalin et al., 1993). Additionally, the POF fragments are usually present in one joint of one type of Standardbred: the hind fetlock in trotters. Ninety-six percent of POF are found in the hindlimb, with 75% of them occurring in the medial region (Sandgren et al., 1993). If POF were the result of systemic mechanisms they would be found in multiple joints. A more likely cause of POF is trauma. More than 80% of Standardbreds show some degree of outward rotation of the hind legs (Dalin et al., 1993), increasing the strain on the medial proximal sesamoid bone. Minor tears in the insertion of the medial short sesamoidean ligament may occur, resulting in formation of an osteochondral fragment from osteogenic repairs (Dalin et al., 1993). Since it appears that trauma is the main cause of POF, and not failure of endochondral ossification, silicon will most likely not have an effect, so these lesions were excluded from this study.

Five out of 44 (11.4 %) horses were found to have POF. This is similar to the 11.5 % (89 out of 753) incidence reported in yearling Swedish trotters (Grondahl, 1992), but lower than the 21.5% (145 out of 674) incidence previously reported in Swedish Standardbred trotters around 18 months of age (Sandgren et al., 1993). As conformation and use appears to play a large role in the formation of POF, different genetics between Swedish Standardbreds and Michigan Standardbreds could account for the difference in incidence in the 18-month-old horses, as well as differences in sample size. Additionally, pacers were included in the current study, not just trotters as in the Swedish studies. Four of the 44 (9.1%) horses had lesions on the sagittal ridge of either the third

metacarpus/metatarsus, and only one horse had a lesion in more than one joint. This is close to the 15.7 % incidence found in previous literature (Grondahl, 1992). Of the five lesions found in the fetlocks, four were in the hindlimbs. This high prevalence in the hindlimbs was found in other Standardbreds (Grondahl, 1992). Of the 44 horses radiographed, 9.1% had lesions in their hocks. The majority of total lesions found were in the hocks (58.3%). These numbers are comparable to previous literature, in which Alvarado et al. (1989) found more lesions in the hock than in the fetlock and stifle in both yearling and adult horses. Additionally, most of the lesions were on the distal intermediate ridge of the tibia, compared to the lateral trochlear ridge (Alvarado et al., 1989). This was also the case in the current study as 57.1% of the lesions found in the hock were on the distal intermediate ridge of the tibia. Overall, 70.4% of the horses radiographed did not have radiographic evidence of flattening of the sagittal ridge, POF, or lesions on either the distal intermediate ridge of the tibia or lateral trochlear ridge. This is comparable to the 64.5% incidence found in both yearling and adult Standardbreds in Quebec (Alvarado et al., 1989).

Orally supplemented silicon did not alter the radiographic appearance of the OC lesions in two-year-old Standardbred horses. Silicon supplementation may be more appropriate at a younger age to prevent the development of osteochondrosis, rather than healing lesions in young adult horses in active training. Additionally, the small number of horses used in the study makes finding differences difficult. However, there is nothing in this study to support the anecdotal reports by horse trainers of lesion regression. The incidence rates found in the current study agree with previous literature in Standardbreds in Sweden and Quebec.

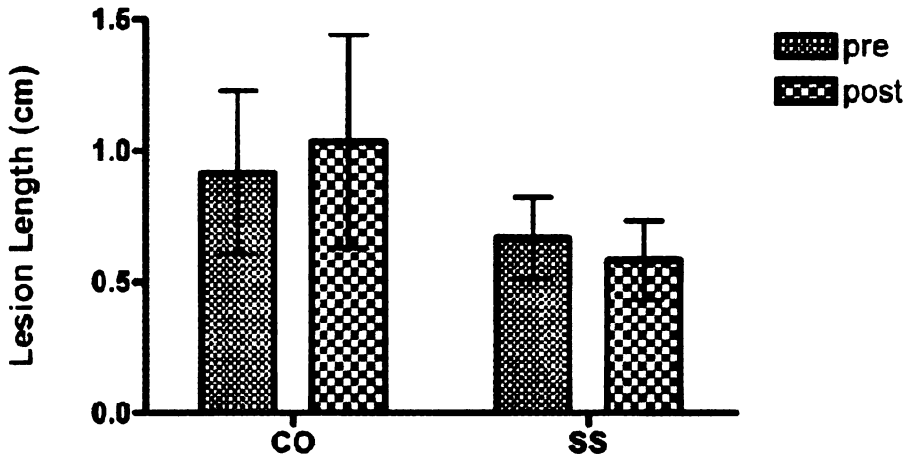

Figure 1. Lesion length (cm) in control (CO) and silicon-supplemented (SS) horses pre- and post-supplementation of placebo or silicon.

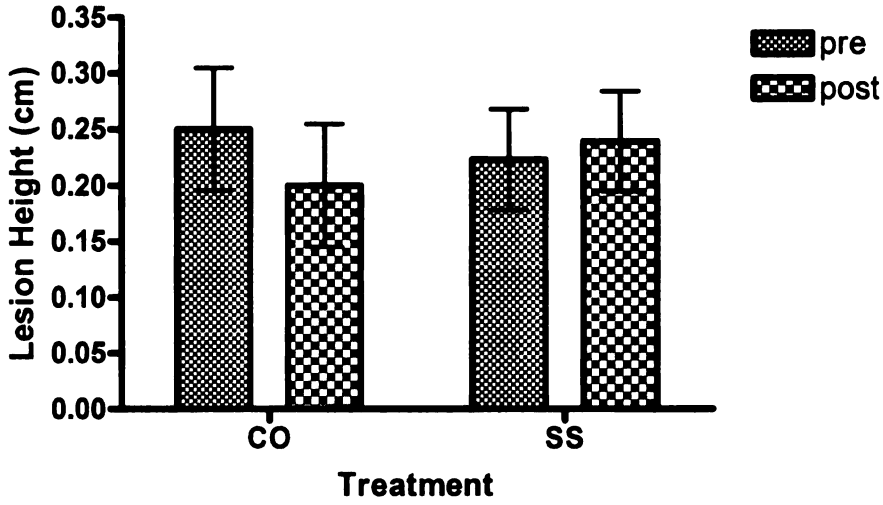

Figure 2. Lesion height (cm) in control (CO) and silicon-supplemented (SS) horses pre- and post-supplementation of placebo or silicon.

CHAPTER 4

SODIUM ZEOLITE A SUPPLEMENTATION TO LACTATING HOLSTEINS

SUMMARY

Twenty Holstein dairy cows in either their first or second parity were used to determine the effects of sodium zeolite A (SZA) on plasma and milk mineral concentrations. The cows were pair-matched by parity and milk production and placed into one of two groups – SZA supplemented (SS) or control (CO). Individual cow milk production and feed intake were recorded daily. All cows received their normal total mixed ration, but SS cows also were supplemented with SZA at a dosage level of 2% of the diet. Milk, blood, and feed samples were taken on d 0, 15, and 30 for mineral analysis. Blood was also used for osteocalcin and deoxypyridinoline determination. Treated cows decreased feed intake and milk production from d 15 to d 30 ($P<0.007$) and had lower feed intakes and milk production than CO cows on both d 15 and d 30 ($P<0.003$). On d 15, SS cows had higher plasma Si concentrations than CO cows ($P=0.01$) but there was no difference on d 30 ($P=0.71$). Overall, plasma Al concentration was increased by SZA (P=0.002). SS-cows had higher milk Al concentrations than CO cows (P=0.02). Milk P concentration decreased from d 15 to d 30 in SS cows ($P=0.007$). On d 15 and 30, milk P concentrations were lower in SS cows than CO cows ($P<0.03$). Treated cows had higher plasma Ca concentrations, and lower plasma P concentrations than CO cows ($P<0.0002$). There was no treatment*day effect on osteocalcin, DPD, or OC to DPD ratio ($P>0.24$). Sodium zeolite A decreased feed intake, milk production, and plasma phosphorus concentration, possibly via a phosphorus deficiency caused by the aluminum intake. Bone metabolism was not affected by the SZA.

INTRODUCTION

One method of preventing milk fever is by feeding low calcium diets prior to parturition; however, it is difficult to lower calcium in the diet. Alternatively, sodium zeolite A (SZA) supplementation to the diet may reduce or prevent milk fever by increasing plasma calcium levels prior to parturition (Thilsing-Hansen and Jorgensen, 2001). Several studies have reported changes in calcium metabolism due to SZA supplementation in dairy cows (Roussel et al., 1992, Thilsing-Hansen et al., 2002; Enemark et al., 2003). Differing hypotheses have been suggested for these changes, such as decreased calcium absorption (Enemark et al., 2003) or increased intestinal calcium absorption secondary to mobilization or resorption of phosphorus (Thilsing-Hansen et al., 2002). However, some of these studies have used small cow numbers (Enemark et al., 2003), reported flawed or ill-timed measurements (Thilsing-Hansen et al., 2002; Enemark et al., 2003) or supplemented SZA for unequal lengths of time (Thilsing-Hansen et al., 2002). Clarification of the effects of SZA on calcium metabolism, as well as phosphorus metabolism, is needed.

Few studies have looked into the effects of SZA on milk mineral concentrations. Calcium was increased in the milk of cows supplemented with 1% DMI SZA (Roussel et al., 1992). However, calcium was the only mineral analyzed in the milk. As a sodium aluminosilicate, SZA is broken down in the digestive system into aluminum and monosilicic acid, an absorbable form of silicon. As a component of SZA, aluminum must be taken into consideration as it can have neurotoxic effects in humans (Nayak,

2002). Increases in milk intended for human consumption should be a concern, and this possibility should be explored.

A secondary purpose of the current study was to investigate the effect of SZA on bone health of lactating dairy cows. Lameness in dairy cows is an animal health issue and an economic issue. Lameness decreases milk yield in cows by approximately 360 kg/cow per 305 d lactation (Green et al., 2002). In the same study, milk yields began to decrease up to 4 mo before the lameness was diagnosed, and continued to be depressed up to 5 mo after treatment. In another study, milk yields were found to decrease between 1.5 kg/d and 2.8 kg/d during the first two weeks after diagnosis (Rajala-Schultz et al., 1999). Dairy producers suffer economic losses not only from decreased production but also from the cost of treatment. Lameness treatment amounted to 27% of total health care costs, second behind mastitis, in a British study (Kossaibati and Esslemont, 1997). As clinical lameness affects milk yield and cow health, measures should be taken to reduce lameness.

Sodium zeolite A has been associated with reduced skeletal injuries in race horses, but the mechanism by which this occurred is unknown (Nielsen et al., 1993). Sodium zeolite A may have an effect on bone turnover. Sodium zeolite A tended to increase osteocalcin (marker of bone formation) concentrations in broodmares (Lang et al., 2001b), but had no effect on osteocalcin in yearlings (Lang et al., 2001c). Additionally, one marker of bone resorption was decreased in supplemented yearlings, while a second marker of bone resorption was unchanged in the same yearlings (Lang et al., 2001c). The effects of SZA on bone metabolism should be further explored in cows.

The hypothesis was that SZA would alter plasma and milk mineral concentrations as well as biochemical markers of bone metabolism in lactating Holsteins. The objectives of this study were to: 1) determine the effects of SZA on plasma and milk mineral concentrations in the lactating dairy cow, and 2) measure bone markers of formation and resorption to determine if SZA affects bone turnover in lactating dairy cows.

MATERIALS AND METHODS

Cows

The research was conducted at the Michigan State University Dairy Cattle Teaching and Research Center (DCTRC). The protocol was approved by the Institution's Animal Care and Use Committee. Twenty Holstein dairy cows aged 1,150 ± 50 d and with an average of 165 ± 9 d in lactation at the start of the study were used. Ten cows were in their first parity and 10 were in their second parity. The cows were pair-matched by parity and milk production and placed into one of two groups – SZA supplemented (SS) or control (CO). Weights were taken on the cows via weight tape at the start of the study (d 0). Weights also were taken at the end of the study (d 30). All cows were housed in tie-stalls and followed standard protocol of the DCTRC. Individual cow milk production and feed intake were recorded daily.

Diets

All cows received their normal total mixed ration (TMR). The SS-cows were supplemented with SZA at a dosage level of 2% of their diet, on a dry matter basis. The

SZA, in powdered form, was added to the TMR at the same time as all the other ingredients on a daily basis. This assured proper mixing within the ration. The control cows received a separately mixed TMR that contained the same ingredients as the treated cows, except for the SZA (Table 3). Silicon concentration of the CO diet was 341 ± 65 mg/kg, and of the SS diet was 1,222 ± 73 mg/kg. All cows were fed their ration once daily, and had free access to it throughout the day.

Table 3. Total mixed ration (TMR) formulation and mineral analyses for control (CO) and supplemented (SS) diets.

Item	CO TMR	SS TMR
Formulation[a]	%	%
Corn silage	45.2	44.3
Haylage	11.8	11.6
Corn, ground	9.7	9.5
Soybean meal, 48%	7.6	7.4
Cottonseed with lint	4.7	4.6
Vitamin-mineral mix	4.5	4.4
High moisture corn, bagged	3.9	3.8
Alfalfa hay	3.8	3.7
High moisture corn, silo	2.9	2.9
QLF TMR 20	2.7	2.7
16M7 UltEXT 24/36	2.4	2.3
Energy booster	0.8	0.8
Sodium zeolite A	_	2.0
Mineral analyses[b]	%	%
Calcium	0.62	0.61
Phosphorus	0.38	0.36
Silicon	0.03	0.1
Aluminum	0.007	0.3

[a]As-fed basis

[b]Dry matter basis

Plasticware

As silicon is a component of glass (Carlisle, 1972), leaching of silicon from glass may affect silicon analysis so plastic must be used in all aspects of sample collection (Lang et al., 2001a). Dust contamination also must be minimized. All plasticware, including pipette tips, collection tubes, scintillation vials, pipettes, and bags, were placed in 30% nitric acid bath overnight. Plasticware was then rinsed three times with dd H_20, placed into ovens to dry, put into acid-washed bags, and stored in a drawer to avoid dust contamination.

Sixty-six µl of 15% K_2EDTA was placed into 7-ml acid-washed plastic collection tubes. The tubes were capped with acid-washed conventional rubber stoppers off serum separator blood collection tubes (Vacutainer, Becton Dickinson, Franklin, NJ), and a vacuum pump with a hose and needle inserted into the cap created a vacuum in the tubes. These were used for collection of blood intended for plasma analysis.

Sample Collection

Milk was collected during both the morning and evening milkings on d 0, 15 and 30. A composite sample from all four teats flowed into a sample collection device during the cows' normal milking. This sample was then transferred to a 15-ml acid-washed scintillation vial and immediately frozen for mineral analysis at -20 °C. Prior to analysis, the milk samples were thawed and the morning and evening milking samples were combined to form one composite sample per cow per day.

Blood was collected from all cows at 0, 15, and 30 d prior to daily feeding. Ten ml of blood was collected into a 10 ml sample tube with no coagulant (Vacutainer, Fisher

Health Care, Chicago, IL) for serum collection, and 7 ml was collected into the specially-made acid-washed tubes containing EDTA plasma collection. The blood was spun at 2,000 x g for 20 min, and then serum was pipetted into microcentrifuge tubes and stored at -20° C for future analysis of osteocalcin (OC) and deoxypyridinoline (DPD). Plasma was pipetted into acid-washed plastic microcentrifuge tubes, within a plastic-lined box to minimize dust contamination, and stored at -20° C pending mineral analysis.

Feed samples from both treatment groups were collected at 0, 15, and 30 d by removing portions of feed from each cow's feed bin and placing the samples into their respective labeled acid-washed plastic bags for mineral analysis.

Sample analyses

Milk samples were digested via microwave digestion for calcium and phosphorus mineral analysis using a modified procedure by Shaw et al. (2002). Two grams of milk were placed into acid-washed Teflon-lined digestion vessels. Twenty ml of 40% nitric acid (70% trace-metal grade; Fisher Scientific, Pittsburgh, PA, USA) were then added to the milk sample and allowed to pre-digest overnight at room temperature. The vessels were sealed and placed in the microwave digestor (MARS-5, CEM, Matthews, NC, USA) and digested on a program consisting of a 10 min ramp up to 190 °C and 200 psi. This was held for 20 min and was followed by a 10 min cool down. The vessels were vented, and 2 ml of hydrogen peroxide (30%, H-1009, Sigma-Aldrich, St. Louis, MO, USA) was added to the digested samples and allowed to cool further. Upon cooling, the samples were transferred to acid-washed 25 ml volumetric flasks and brought up to volume with double-deionized water. The digests were diluted 50x with lanthanum chloride and

analyzed on an Unicam 989 atomic absorption spectrophotometer (Thermo Elemental Corp., Franklin, MA, USA) for calcium content. The digests were diluted 10x with double-deionized water and analyzed with a spectrophotometer (Beckman DU 7400, Beckman-Coulter, Palo Alto, CA, USA) for phosphorus content.

Plasma samples were diluted 25x and analyzed for calcium on the atomic absorption spectrophotometer. To determine plasma phosphorus, the plasma samples were precipitated with 12.5% trichloroacetic acid to remove interfering proteins. The supernatant was then analyzed on the spectrophotometer.

All samples were run in duplicate. Bovine liver standard (1577b; NIST, Gaithersburg, MD) was used to establish instrument accuracy. All glassware was soaked in 30% nitric acid overnight and rinsed five times with double-deionized water (Rincker et al., 2004).

Milk, plasma and feed samples were sent to an off-campus laboratory (London Laboratory Services Group, Trace Elements Laboratory, London, Ontario) to be analyzed for silicon and aluminum concentrations on a Finnigan MAT ELEMENT high-resolution inductively coupled mass spectrometer (ICP-MS). Approximately 0.3 g of sample was placed into a plastic tube. Five ml of 70% nitric acid were added to the tubes, which were then placed into a water bath at 103 °C for 20 min. The digest was diluted with water (3.5 ml) and 1 ml of 50% potassium hydroxide solution was added. The digests were then read on the ICP-MS.

Osteocalcin was determined by an ELISA kit (NovoCalcin®, Quidel Corporation, San Diego, CA, USA) following manufacturer's instructions. Serum dilutions were determined based on test runs of d 0 samples. Due to the variability between cows, each

sample was tested and a dilution was determined for each individual sample. Serum was diluted between 1:2 and 1:13 for individual samples. Dilutions for d 15 and d 30 samples were based off the individual d 0 values. Samples were tested in duplicate. The plates were read at 405 nm optical density on a Spectra Max 340 plate reader (Molecular Devices Corp., Sunnyvale, CA).

Total serum DPD was determined using a commercial competitive immunoassay kit (Metra DPD EIA) from Quidel Corporation (San Diego, CA). Samples were not diluted, and were run in duplicate. The plates were read at 405 nm optical density on a Spectra 340 plate reader (Molecular Devices Corp., Sunnyvale, CA).

Statistical analysis

There were no effects of lactation and treatment*lactation so the data were pooled. Data were analyzed for repeated measures using the mixed procedure in SAS 8.2. The main effects were treatment, time and treatment*time with cow(treatment) as the error term. When differences were detected on d 0, d 0 was used as a covariate. Data are displayed as least square means ± standard error of the mean. Differences were explored at $P < 0.05$, and trends were explored at $P < 0.1$.

RESULTS

Weight

There was a treatment*day effect on weight ($P = 0.03$). Control cows' weight remained unchanged from d 0 to d 30 (618 ± 3 vs 624 ± 3 kg; $P = 0.22$). Treated cows lost weight from d 0 to d 30 (577 ± 3 vs 567 ± 3 kg; $P = 0.03$).

Feed intake and milk production

There was an effect of treatment*time ($P < 0.05$) on both feed intake and milk production (Table 4). The overall treatment effects on feed intake and milk production are displayed in Table 5.

Table 4. Treatment*time effects (P < 0.05) on feed intake and milk production in control (CO) and SZA-supplemented (SS) cows

	Treatment	Day 0	Day 15	Day 30	SEM
Feed intake, kg DM	CO	36.9^{a}	41.7^{b}	41.4^{b}	1.09
	SS	36.6^{a}	$33.9^{b,z}$	$29.3^{c,z}$	1.09
Milk production, kg	CO	40.1	40.3	38.5	0.72
	SS	39.7^{a}	$33.3^{b,z}$	$27.9^{c,z}$	0.72

^{a,b,c} Means within rows with different superscripts differ (P < 0.05)

^{z}Means within columns within variable differ (P < 0.05)

Table 5. Overall effect of treatment between control (CO) and SZA-supplemented (SS) cows

	CO	SS	SEM	P-value
Feed intake, kg DM	41.5	31.7	1.4	0.0002
Milk production, kg	39.2	30.8	0.8	<0.0001

Minerals

Significant treatment*time effects (P < 0.1) on silicon and aluminum intake, plasma silicon and phosphorus concentrations, and milk phosphorus concentration are displayed in Table 6. Non-significant treatment*time effects are not shown. The overall effect of treatment on mineral intake, plasma concentration, and milk concentration is displayed in Table 7.

Table 6. Treatment*time effects (P < 0.1) on mineral intake and plasma and milk mineral concentrations in control (CO) and SZA-supplemented (SS) cows

	Treatment	Day 0	Day 15	Day 30	SEM
Si intake, g	CO	6.98	7.89	7.85	0.60
	SS	6.93^{a}	$23.0^{b,z}$	$19.9^{c,z}$	0.60
Al intake, g	CO	1.33	1.50	1.50	1.64
	SS	1.32^{a}	$57.4^{b,z}$	$49.7^{c,z}$	1.64
Plasma Si conc., ug/L	CO	518^{a}	496^{a}	445^{b}	17
	SS	501^{a}	$556^{b,z}$	437^{c}	17
Plasma P conc., mg/dl	CO	4.5	5.2	4.8	0.37
	SS	5.0^{a}	$0.62^{b,z}$	$0.99^{b,z}$	0.37
Milk P conc., mg/dl	CO	68.8^{a}	63.2^{b}	65.7^{b}	1.3
	SS	71.7^{a}	$59.7^{b,z}$	$54.3^{c,z}$	1.3

a,b,c Means within rows with different superscripts differ (P < 0.1)

z Means within columns within variable differ (P < 0.1)

Table 7. Overall effect of treatment on mineral intake and plasma and milk mineral concentrations in control (CO) and SZA-supplemented (SS) cows.

	CO	SS	SEM	P-value
Si intake, g	7.9	21.4	0.8	<0.0001
Plasma Si conc,, ug/L	470	496	20	0.37
Al intake, g	1.5	53.6	1.9	<0.0001
Plasma Al conc., ug/L	129	284	30	0.002
Milk Al conc., ug/L	9.9	15.5	1.6	0.02
Ca intake, g	138	114	4	0.0004
Plasma Ca conc., mg/dl	11.9	12.9	0.2	0.0002
Milk Ca conc., mg/dl	130	128	3	0.69
P intake, g	88.8	73.9	2.8	0.0004
Plasma P conc., mg/dl	5.0	0.8	0.2	<0.0001
Milk P conc., mg/dl	65.6	55.3	1.7	0.0006

Bone markers

There was no effect of treatment*day on osteocalcin ($P = 0.24$), DPD ($P = 0.81$), or OC to DPD ratio ($P = 0.51$). The effect of treatment on OC, DPD, and the OC to DPD ratio is displayed in Table 8.

Table 8. Overall effect of treatment on markers of bone metabolism in control (CO) and SZA-supplemented (SS) cows.

	CO	SS	SEM	P-value
Osteocalcin	50.8	50.9	4.1	0.99
Deoxypyridinoline	7.07	7.48	0.54	0.6
OC:DPD ratio	7.38	7.93	0.71	0.59

DISCUSSION

The high intake of Al by the SS cows most likely caused the decrease in feed intake and subsequent decreased milk production by impairing phosphorus absorption. The absorption of phosphorus was greatly reduced, thus leading to low blood phosphorus. The SS cows were hypophosphatemic with an average plasma phosphorus of 0.8 mg/dl, much lower than the normal range given for dairy cows (4-7 mg/dl) (Georgievskii, 1982). A decrease in blood inorganic phosphorus has been noted with high aluminum intakes for decades. This decrease has been seen in humans with chronic renal failure (Clarkson et al., 1972), chicks (Hussein et al., 1990), and lambs (Rosa et al., 1982). Karn (2001) suggested that ruminants will exhibit clinical signs of phosphorus deficiency when blood P levels fall below 2.0 mg/dl, as was the case in the current study. These include decreases in both feed intake and milk production (Call et al., 1987), and unchanged concentrations of minerals in the milk (Underwood, 1981). High aluminum intakes

decrease feed intake via lowering phosphorus absorption. Feed intake decreased when lambs were fed a diet containing greater than 2 g Al/kg feed (Valdivia et al., 1982). The SS-diet in the current study contained approximately 3 g Al/kg feed. Previous studies found conflicting results on the effect of SZA supplementation on feed intake, with it either decreasing (Johnson et al., 1988; Thilsing-Hansen et al., 2002) or increasing (Roussel et al., 1992). Comparisons of the studies revealed that the two studies in which feed intake decreases were found, as well as the current study, supplemented the cows with SZA at a rate approximate to 2% of the diet. The study finding an increase in feed intake fed SZA at levels less than 1.5% of the diet. There appears to be a threshold for aluminum intake suppressing feed intake, as steers supplemented with 1.2 g Al/kg feed suffered no ill effects (Valdivia et al., 1978) yet sheep consuming 2 g Al/kg feed had depressed appetites (Valdivia et al., 1982). The 1.5% of diet may have been below the threshold, while the 2% exceeded it.

The effects of high aluminum intakes, such as decreased plasma phosphorus, can be alleviated by additional dietary phosphorus. Chicks fed high levels of aluminum had decreased growth performance and bone breaking strength (Hussein et al., 1990). However, the effects were negated when dietary available phosphorus was increased. Increases in previously depressed plasma phosphorus occurred when animals were supplemented with phosphorus (Lipstein and Hurwitz, 1982; Hussein et al., 1990). Decreases in feed intake were also reversed with additional phosphorus (Valdivia et al., 1977; Rosa et al., 1982). Feeding additional phosphorus to lactating dairy cows in order to supplement SZA may be a viable option, but current environmental regulations on phosphorus render this option unfeasible for producers (Stallings and Knowlton, 2005).

The decrease in feed intake also could possibly have been due to a change in pH. A study supplementing SZA to cows found the treated cows to have a higher ruminal pH, although the authors concluded that the change in pH (0.2 units) may not be of physiological significance (Johnson et al., 1988). Two cows, one on the SZA supplement and one not on study, were culled and euthanized for reasons not related to this project. During the necropsies, visual inspections of the rumen, small intestine, and other parts of the digestive tract were performed by a licensed veterinarian to determine physiological differences between the supplemented and nonsupplemented cow. There was no accumulation of SZA in the supplemented cow. Rumen fluid was collected from both cows and the pH was determined using litmus paper. As there were only two cows, statistical analysis could not be performed on the rumen pH. The pH of the cow that never received SZA was 6.5, and the pH of the treated cow was 8.5. Thus, SZA may have decreased ruminal pH.

The SS cows had higher plasma silicon concentrations on d 15, compared to the CO cows. Plasma silicon concentrations increased in the SS cows from d 0 to d 15, but then decreased to below baseline on d 30. This is most likely due to the decreased feed intake in the SS cows, and therefore decreased silicon intake.

Plasma calcium concentration was higher in the SS cows (12.9 mg/kg) than the CO cows (11.9 mg/kg), and was slightly higher than the normal range previously given for dairy cows of 9.0-12.0 mg/kg (Georgievskii, 1982). An effective method to prevent milk fever is to feed a diet low in calcium prior to parturition, and then feed a high calcium diet during lactation. The low calcium diet then initiates homeostatic mechanisms prior to the sudden draw on calcium reserves at parturition. However,

formulating diets to be low in calcium is difficult as standard ingredients are often high in calcium. Sodium zeolite A has been an effective supplement to reduce milk fever (Thilsing-Hansen and Jorgensen, 2001) by increasing plasma calcium concentrations. Perhaps the binding of the aluminum in the SZA to phosphorus caused mobilization or resorption of phosphorus, resulting in a secondary mobilization or resorption of calcium, as suggested by Thilsing-Hansen et al. (2002). Plasma calcium concentration increases in lambs fed low phosphorus diets (Ternouth and Sevilla, 1990). The increase in plasma calcium may result from resorption of bone to acquire phosphorus. Additionally, an increase in plasma calcium may arise from the blocking of proper formation of hydroxyapatite by aluminum binding to phosphorus instead of calcium to phosphorus (Boyce et al., 1982). This would block calcium from bone deposition, thus increasing the plasma levels. This would explain the lack of an increase in DPD, which would be expected if bone resorption was occurring. This could also account for the lack of parathyroid hormone and 1, 25 dihydroxy vitamin D responses found in lactating cows given SZA (Enemark et al., 2003).

The treated cows were able to maintain stable milk calcium concentrations, which is a common occurrence in animals with phosphorus deficiency. Lactating animals respond to a deficiency in phosphorus by decreasing milk yield but maintaining mineral concentrations in the milk (Underwood, 1981). The milk calcium concentrations were within normal limits for cows (Georgievskii, 1982). Milk phosphorus concentrations were lower in the SS cows than the control cows. Because of the decreased phosphorus absorption, the homeostatic mechanisms were probably exhausted and the cows did not have enough available phosphorus to supply the milk. However, both groups of cows

had phosphorus milk values higher than that previously reported in cows (Georgievskii, 1982). Factors such as diet, stage of lactation, and milk production could create the discrepancy.

Osteocalcin, a vitamin-K dependent protein synthesized in osteoblasts, is a marker of bone formation (Allen et al., 1998). Sodium zeolite A tended to increase osteocalcin concentrations in supplemented Arabian mares, as compared to control mares (Lang et al., 2001b). There were no effects of SZA on osteocalcin concentration in the current study. Osteocalcin was also not affected in yearling horses on SZA (Lang et al., 2001c) Deoxypryidinoline, a marker of bone resorption, was also not affected by SZA supplementation in the cows. Similar to osteocalcin, effects of SZA on bone resorption markers have brought about mixed results. Two bone resorption markers were measured in yearlings (Lang et al., 2001c) and broodmares (Lang et al., 2001b), and each study showed a decrease (or trend) in one marker, and no change in the other. Due to the conflicting results, it is likely that SZA has little to no effect on bone metabolism. However, further research is needed to explore the effects of sodium zeolite A on calcium and phosphorus metabolism, as bone resorption appears to be unchanged.

In conclusion, sodium zeolite A decreased feed intake and milk production in lactating cows. Plasma phosphorus concentrations of the SS cows were below the level at which phosphorus deficiencies occur, most likely a result of the high aluminum intakes. Feeding additional phosphorus may alleviate the depression in feed intake and milk production, but is impractical with regards to environmental regulations. Additionally, the increase in milk aluminum concentration should be of major concern, as aluminum is linked to many neurotoxic conditions in humans, including Alzheimer's

disease. Sodium zeolite A had no effect on bone markers in the cows, thus its possible use as a lameness supplement is questionable. Supplementation of 2% SZA to lactating dairy cows is discouraged as it adversely affects feed intake and milk production and increases aluminum concentration in milk intended for human consumption.

CHAPTER 5

SODIUM ZEOLITE A SUPPLEMENTATION TO NEWBORN CALVES

SUMMARY

The objective of the study was to determine the effects of sodium zeolite A (SZA) on digestibility, glycosaminoglycan concentration, bone characteristics, mineral metabolism and tissue mineral composition in bull calves. Twenty calves were placed on study at three days of age, and were placed according to birth order into one of two groups: SS, which received 0.05% BW SZA added to their milk replacer and CO, which received only milk replacer. Blood samples were taken on d 0, 30, and 60 for osteocalcin (OC) and deoxypyridinoline (DPD) analysis. A total collection was done on d 30 for mineral metabolism, and on d 60 of the trial, the calves were euthanized and samples were taken from numerous organs for glycosaminoglycan, mineral, and bone quality analyses. There were no effects of SZA on digestibility. There were no differences in OC due to treatment ($P=0.12$), and CO calves had lower DPD concentrations than SS calves ($P=0.01$) but the OC to DPD ratio was not different between treatments ($P=0.98$). Glycosaminoglycan concentrations were not different in synovial fluid or cartilage. There was a trend for SS metacarpi to be longer than the CO metacarpi ($P=0.09$). No difference in bone architecture or mechanical properties was detected. Plasma Cu concentration was increased in SS calves, but P and Mg concentrations were decreased ($P<0.05$). Silicon concentrations were increased in the aorta, spleen, lung, muscle, and kidney of the SS calves, and Al was increased in all SS tissues but bone ($P<0.05$). Supplementation of SZA had very little effect on bone metabolism. However, it altered plasma and tissue mineral composition, specifically tissue Al.

INTRODUCTION

Sodium zeolite A (SZA) is an aluminosilicate that is hydrolyzed at low pH into silicic acid, amorphous aluminum silicates and aluminum (Thilsing-Hansen et al., 2002). Thus, by supplying SZA to the diet, an animal's gastrointestinal tract can break down the zeolite into orthosilicic acid. Silicic acid is absorbed, providing the body with supplemental silicon. The extent of absorption of Al released from the zeolite is unknown.

Although several studies have reported on the effects of SZA on blood mineral concentrations (Ward et al., 1991; Lang et al., 2001b,c; Thilsing-Hansen and Jorgensen, 2001), few, if any, have investigated long term (> 14 d) supplementation on mineral retention and absorption. Additionally, most studies have focused primarily on blood concentrations of calcium, phosphorus and silicon. The effects of SZA supplementation on other plasma minerals and mineral retention and absorption should be investigated.

Silicon has been suggested to be an essential mineral needed for proper bone growth. Among numerous roles, silicon may be involved in bone mineralization, endochondral bone formation, and glycosaminoglycan synthesis and composition (Carlisle, 1974; 1982). In horses, a study conducted on young racing Quarter Horses showed that supplemental SZA was associated with decreased skeletal-related injury rates and increased distances horses were able to train before being injured (Nielsen et al., 1993). A mechanism by which this occurred is unknown. Further research showed a trend for increased bone formation in broodmares (Lang et al. 2001b).

Studies have investigated the supplementation of SZA to horses (Frey et al., 1992; Nielsen et al., 1993, Lang et al., 2001b,c) and reported an increase in silicon concentration in plasma and milk. However, the level of tissue accumulation of silicon has not been investigated. Rat kidney, liver, bone, skin, spleen and lung were found to accumulate the greatest amounts of ^{31}-labeled silicic acid one hour after intracardiac injection of the labeled element (Adler et al., 1986). This appears to be the only study that investigated the effects of supplemental silicon on tissue uptake. Silicon supplemented through dietary means, and not via injection into circulatory system, might have different effects. Also, a livestock species such as the horse or cow, might have dissimilar tissue responses to supplemental silicon as the tissues might have different concentrations than the rat due to the livestock diet of grains, forage, and oftentimes, sand. The effects of long term supplementation of silicon are unknown as well.

Several studies have investigated the effects of high aluminum on tissue mineral composition in livestock. Rosa et al. (1982) used 1,450 mg/kg Al in lambs while Valdivia et al. (1982) used a slightly higher level of 2,000 mg/kg, also in lambs. Cattle fed 1,200 mg/kg Al were also studied (Valdivia et al., 1978). Very few studies have looked at aluminum accumulation in the tissues. Valdivia et al. (1982) found increases in the liver, kidney and muscle. Additionally, rats chronically supplemented with aluminum (15 wk) showed increases in kidney, spleen, and liver aluminum (Garbossa et al., 1998). However, no studies supplementing SZA have looked into subsequent tissue mineral composition. Sodium zeolite A, as an aluminosilicate, contains a substantial amount of aluminum, and tissue accumulation of aluminum should also be investigated along with silicon.

We hypothesized that supplemental SZA would increase bone turnover, bone strength, and glycosaminoglycan content, as well as alter mineral metabolism and tissue mineral composition. The purposes of this study were to determine the effects of SZA on: 1.) digestibility and mineral metabolism 2.) variables linked to soundness including markers of bone metabolism, glycosaminoglycan concentration, and bone mechanical properties and 3.) tissue mineral composition. To determine bone mechanical properties and tissue mineral composition, a terminal study was necessary. Therefore, we used the bovine neonate.

MATERIALS AND METHODS

Calves

The research was conducted at Michigan State University's Dairy Cattle Teaching and Research Center (DCTRC). The protocol was approved by the Institution's Animal Care and Use Committee. Twenty Holstein bull calves were placed on study at three days of age. The calves were individually housed in stalls bedded with straw. The calves were assigned to a treatment group based on birth order, with the first calf born placed into the control group (CO) and the second calf was placed into the group treated with supplemental sodium zeolite A (SS), and so on until 10 calves were in CO and 10 were in SS.

Treatment

At birth, calves received 2 doses of colostrum without SZA. From birth to 21 days of age calves received 2.4 l of milk replacer (Instant Cow's Match®, Land O'Lakes Animal Milk Products Co., Shoreview, MN, USA; Appendix C) twice daily. From 21 days of age until d 60 calves received 3.3 l of milk replacer twice daily. The CO group received their normal milk replacer only. The SS group received SZA at a dose of 0.05% BW. The SZA, in powder form, was added to the SS calves' milk replacer twice daily. Calves were weighed weekly so that silicon dosage could be adjusted. Mineral concentration of the milk replacer and SZA are in Table 9. The calves were allowed free access to water.

Table 9. Mineral concentrations (μg/g) of milk replacer and SZA fed to calves.

	Milk replacer	SZA
Al	117	130,325
Ca	7,429	93.4
Cu	7.58	0.49
Fe	54.7	74.1
Mg	1,282	ND
P	7,698	ND
Si	479	1,632
Zn	25	18

Analyzed by Grand Forks Human Nutrition Research Center
ND = Not detectable

Blood samples

Blood samples were taken on d 0, 30, and 60 of the study. Ten ml of blood from the jugular vein were collected into a Vacutainer with no coagulant to obtain serum. The serum was analyzed for bone markers osteocalcin (OC) and deoxypyridinoline (DPD),

and plasma was analyzed for mineral content. Since plasma was to be analyzed for silicon concentration, special care was taken to reduce contamination from glass. Seven-ml plastic tubes were acid-washed, then 66 µl of 15% K_2EDTA was added to the tube. The tubes were then capped with acid-washed rubber tops, and vacuumed. Blood was then collected into three of the 7-ml plastic tubes. Both plasma and serum tubes sat for one hour, and then were centrifuged for 20 min at 1,340 x g. Plasma and serum were removed and pipetted into microcentrifuge tubes and frozen at -20° C. To avoid contamination from silicon, plastic acid-washed pipettes and microcentrifuge tubes were used.

Total collection

On approximately d 30, calves were placed in stainless steel metabolism crates for three days for total collection of urine and feces. Urine and feces were collected every 6 h for the 3-d collection period. Urine flowed through wire mesh flooring, landed in a stainless steel tray, and then flowed through a plastic acid-washed tube which ended in a plastic acid-washed pitcher with a lid. Every 6 h, urine was poured from the pitcher into plastic acid-washed graduated cylinders, and the volume was recorded. Ten percent of the volume was then poured into a plastic acid-washed bottle and placed into a cooler and then frozen until future mineral analysis. An additional 10% of the volume was placed into a second plastic acid-washed bottle, 1.9 ml of 12 M HCl per ml urine was added, and then the urine was frozen until nitrogen analysis. For each individual calf, the urine collected over the three-day collection period for mineral analysis was thawed, pooled together, poured through three layers of cheesecloth, and then 250 ml was poured into a

bottle and frozen. This was repeated for the acid-treated urine samples intended for nitrogen analysis. To avoid contamination with urine, feces were collected in a canvas tail bag lined with a removable plastic bag. Three sides (sides and bottom) of the canvas bags were fitted to the calves' buttocks with Velcro™ and livestock glue, with the top attaching to the top of the calves' tails with arms. Every 6 h the canvas tail bag was removed from the calf and the inner plastic bag containing the feces was removed, weighed, and placed in a cooler. Feces collected from the three-day collection period were pooled together to form one sample per calf. Feces were thoroughly mixed together, then approximately 300 g of the pooled feces were frozen, freeze dried, and analyzed for nitrogen, ADF, NDF and mineral content. Approximately 4 g of feces were placed in tin containers in the oven to determine dry matter content. Samples were left in the oven at 105° C overnight until a constant weight was achieved. Dry matters were run in duplicates, and a CV < 2.5% was obtained.

Tissue collection

On d 60 of the study, calves were sedated with 0.5 ml xylazine intramuscularly and then euthanized via overdose of sodium pentobarbital into the jugular vein at a dosage of 0.22 ml/kg BW. Calves were determined to be dead by a lack of corneal response. The jugular vein and carotid artery were then cut and blood was drained from the calf. Synovial fluid was immediately collected from the hock and knee joints by a 3-ml syringe and then placed into two 7-ml plastic acid-washed scintillation vials. The vials were placed into a cooler on ice. Both front legs were disarticulated at the knee and placed into a cooler on ice. The carcass was then cut open and the liver, spleen, kidneys,

adrenal glands, heart, and pancreas were removed entirely. Total organ weights were recorded, and then approximately 25 g samples were taken from the organs and placed in the cooler, with the exception of the adrenal glands, which were kept intact as they weighed less than 25 g. The organs were then discarded. Twenty-five gram samples were also taken from the caudal portion of the right lung, mid-section of the trachea, aorta proximal to the bifurcation at the external iliac artery, and longissimus dorsi and placed into the cooler. The same individual removed the 25 g samples to ensure that the samples were taken from the same location on each animal. Upon return to the laboratory, the samples were taken out of the cooler and placed in a freezer at -20° C. The front legs were taken from the cooler and samples were taken of the mid-section of both the right and left superficial digital flexor tendon. Articular cartilage was collected from the weight-bearing regions of the carpi. Both left and right fused metacarpal bones (MC III and IV) were stripped down to the periosteum, and all samples were placed in a freezer at -20° C until further analysis.

Bone marker analyses

Serum OC concentration was determined using a commercial competitive immunoassay (Metra Osteocalcin EIA) from Quidel Corporation (San Diego, CA) (Hiney et al., 2004b). Serum samples were diluted with double-deionized water in a 1:30 ratio for d 0 and 30, and in a 1:20 ratio for d 60. Total serum DPD was determined using a commercial competitive immunoassay kit (Metra DPD EIA) from Quidel Corporation (San Diego, CA) (Hiney et al., 2004b). All samples were run in duplicate.

Digestibility analyses

Urine, ground dried fecal samples, and powdered milk replacer were analyzed for nitrogen by a LECO-FP-2000 (St. Joseph, MO) using AOAC method 990.03. Acid detergent fiber and NDF were determined using an approved protocol (Goering and Van Soest, 1970). Bomb calorimetery was used to determine energy content of the milk replacer and fecal samples. Nitrogen and energy retention was determined by subtracting output from intake, and ADF, NDF, nitrogen and energy digestibilities were determined by subtracting fecal output from intake, and then dividing the difference by intake.

Bone mechanical properties

The right fused third and fourth metacarpal bones were imaged with computed tomography (Marconi PQ 6000) at the level of the mid-diaphysis (120 Kv, 125 mA, 2 sec, slice thickness 10 mm). The transverse images were used to measure cross-sectional area of total (cortex + medullary canal) and cortical bone, cortical thickness at the dorsal, palmar, medial and lateral margins and total bone diameter. Bone length was determined using a ruler with 1 mm precision. Measurements were made in triplicate to ensure accuracy.

Three-point bending to failure was performed on the right fused third and fourth metacarpal bones using a universal testing instrument (Model 4202, Instron Corp., Canton, MA) according to ASAE Standards (2000) to determine peak fracture force, ultimate bending strength, and modulus of elasticity. A cross-head speed of 10 mm/min

was used with supports set at 9 cm apart and force data were logged every 0.25 mm. Ultimate bending strength (stress) was calculated by the following equation:

$\sigma = FLC/4I$

where

σ = ultimate bending stress, Pa

C = distance from neutral axis to outer fiber (m)

I = moment of inertia, m^4

L = distance between supports, m

F = applied fracture force, N

C for a hollow ellipse:

$C = D/2$

The moment of inertia for a hollow ellipse:

$I = 0.049[(B \bullet D^3) - (b \bullet d^3)]$

where

B = lateromedial (LM) bone diameter (outside major diameter), m

b = LM medullary diameter (inside major diameter), m

D = dorsopalmar (DP) bone diameter (outside minor diameter), m

d = DP medullary diameter (inside minor diameter), m

Apparent modulus of elasticity was calculated by using the data from the three-point bending test. The portion of the force/deformation curve between 10 and 90 % of

the peak fracture force was used to calculate the slope of the straight line (F/δ) via linear regression of those data. This portion was chosen because it represented the linear portion of the curve. Modulus of elasticity (E) was then calculated by the following equation:

$E = FL^3/48I\delta$

where

δ = deformation, m

Bone ash

The left fused third and fourth metacarpal bones were cut at the midshaft point by a bandsaw and a slice 1 cm wide was obtained. A second slice, 0.5 cm wide, was cut proximal to the carpus from the previous slice. The 0.5 cm slice was used to determine mineral content of the bone while the 1 cm slice was used to determine dry matter and percent ash. Fat was removed from the 1 cm slices via ether extraction. The slices were then dried at 150° C overnight and weighed. Following that the slices were placed in a muffle furnace at 600° C for 12 h and reweighed. The percent ash was then expressed on a dry fat-free basis.

Glycosaminoglycan concentration

Articular cartilage GAG concentration. Three cartilage samples per calf were sent to the Massachusetts Institute of Technology (MIT) to be analyzed for GAG content. Recently a method was developed that utilizes magnetic resonance imaging (MRI) to determine cartilage GAG content using the charged contrast agent gadolinium diethylene triamine pentaacetic acid ($Gd(DTPA)^{2-}$) (Burstein and Gray, 2003). This method,

delayed gadolinium-enhanced magnetic resonance imaging of cartilage (dGEMRIC), is a sensitive and specific measure of GAG content that can be used for both in vivo and in vitro analyses. The researchers at MIT were blinded to treatment. The cartilage samples were initially equilibrated in Hank's balanced salt solution (Invitrogen) (HBSS) to measure $T1_0$ (T1 without contrast agent). Each sample was imaged individually with HBSS in tubes. An 8.45T Bruker microimaging system was used. There was one T_1 weighted scan per sample. Imaging parameters are stated below:

Sequence: Saturation recovery (msme_vtr)

TR: 100, 150, 300, 400, 600, 900, 1400, 2000, 3200, 5000 msec.

TE: 15 msec

Averages: 1

Slice thickness: 0.5

Slice Number: 3 or 4 depending on sample size

Matrix Size: 128 x128

FOV: 128 x128

Coil size: 10 mm

For dGEMRIC imaging, the samples were re-equilibrated in HBSS containing 1mM $GdTPA^{-2}$ (Magnivist, Berlex Imaging, NDC 50419-188-02). Each sample was again imaged individually with the 1mM $GdTPA^{-2}$ solution in tubes. The imaging parameters were the same as above except the TRs were: 100,125, 175, 275,375,475,600, 900, 1800, and 2700 msec.

Tissue $Gd(DTPA)^{2-}$ concentration was calculated by the following equation:

$$[Gd^{-2}] = \frac{1}{R}\left(\frac{1}{T1_{Gd}} - \frac{1}{T1_0}\right)$$

where $T1_{Gd}$ = T_1 measured after equilibration of cartilage in saline and $Gd(DTPA)^{2-}$,

$T1_0$ = T_1 measured after equilibration of cartilage in saline only,

and R = relaxivity of $Gd(DTPA)^{2-}$, which is approximately equal to the relaxivity in saline (Bashir et al., 1999).

Fixed charge density (FCD) was then determined using the following equation:

$$FCD_t = -2[Na^+]_{bath}\left(\frac{\sqrt{[Gd^{-2}]_{tissue}}}{\sqrt{[Gd^{-2}]_{bath}}} - \frac{\sqrt{[Gd^{-2}]_{bath}}}{\sqrt{[Gd^{-2}]_{tissue}}}\right)($$

Glycosaminoglycan concentration was calculated using the FCD and assuming -2 moles of charge per mole of disaccharide and a molecular weight of 502.5 g/mole (Bashir et al., 1999). The equation is as follows:

$$[GAG] = FCD_t\left(\frac{502.5g/mol}{2}\right)*10^{-3}$$

Synovial fluid hyaluronic acid concentration. Hyaluronic acid concentration was determined in the synovial fluid using a commercial competitive ELISA kit (Echelon, Salt Lake City, UT). Synovial fluid samples were diluted in a 1:2,500 ratio with double-deionized water. Samples were run in duplicate.

Mineral analyses

Prior to mineral analysis, tissue samples (excluding bone and cartilage) were freeze-dried and then ground. Plasma, urine, feces, milk replacer, sodium zeolite A, water from the dairy farm, and the tissue samples were analyzed for Si, Ca, P, Al, Cu, Mg, Fe, and Zn at the Grand Forks Human Nutrition Research Center (Grand Forks, ND). For minerals other than Si, a nitric acid digestion was used. Approximately 0.5 g of

sample was weighed into Teflon tubes, and 10 ml of ultra-pure H_2NO_3 was added. The tubes were then capped, and placed into a heating block at 60 °C for two days. The tubes were then uncapped, and the temperature of the heating block was raised to 135 °C. Once the acid evaporated out, and a dry pellet at the bottom of the tube was obtained, 10 ml of ultra-pure H_2NO_3 was again added. This step was repeated until a pale yellow pellet was obtained, at which point 7 ml of H_2NO_3 and 3 ml of H_2O_2 was added to the pellet. This was also allowed to evaporate out. The final pellet was brought up to volume in 5 ml volumetric flasks with double-deionized water and then analyzed on an inductively-coupled argon plasma atomic emission spectrometer (PerkinElmer Optima 3100 XL). Samples were digested and analyzed in duplicate. A standard reference material (National Institute of Standards and Technology, Gaithersburg, MD), no. 1577b bovine liver, was used for quality control purposes.

For silicon analysis, an alkaline medium was used to digest the samples. Approximately 0.3 g of sample was weighed into 15 mL conical centrifuge tubes and 1 ml of 0.2 % (wt/v) of sodium dodecyl sulfate (SDS) was added to the tubes, followed by 9 ml of 40 % tetramethyl ammonium hydroxide (TMAH). The SDS was added to allow for better fat digestion. After the addition of SDS and TMAH, the tubes were capped and vortexed until all samples was suspended into TMAH mixture. The tubes were then placed on a rocker for 16 hours. All of the sample should be solubilized, but small amounts of undigested material may remain. The samples were centrifuged prior to analysis to remove any suspended particles. No additional dilutions were needed prior to analysis, thus reducing contamination. The samples were then analyzed by an inductively-coupled plasma atomic emission spectrometer (PerkinElmer 3300). Typical

Diet, standard reference material no. 1548a, was used for quality control purposes instead of bovine liver as the liver does not have a standard value for silicon.

Statistics

The mixed procedure for repeated measures in SAS was used to analyze weight, OCD, DPD and OC:DPD ratio data. The model tested for day, treatment, and treatment*day interactions. The remaining data were analyzed using the general linear model in SAS. Bartlett's test for homogeneity of variance was used in SAS. If variances were determined to be heterogenous, Welch's test was used for treatment differences, of which individual standard error of the means (SEM) were reported. If variances were homogeneous, then pooled SEMs were reported. Differences were explored at $P < 0.05$, and trends were explored at $P < 0.10$.

RESULTS

There was no effect of treatment*day on calf weights ($P = 0.19$) or average daily gains ($P = 0.89$) (Table 10).

Table 10. Body weights and average daily gain (ADG) of control (CO) and SZA-supplemented (SS) calves.

	CO	SS	SEM
Body weight (kg)			
Initial	46.9	49.9	0.94
Final	85.5	85.0	0.94
ADG	0.65	0.59	0.03

Digestibility variables

The average daily volume of urine excreted from the SS calves was greater than the CO calves (5,338 ± 317 ml vs. 4,348 ± 317 ml; $P = 0.04$). After accounting for differences in BW, there was a trend for SS calves to have a greater daily average volume of urine excretion on a per kg BW basis (81.0 ± 5.3 ml/kg vs. 66.8 ± 5.3 ml/kg; $P = 0.07$). There was no difference in average daily fecal excretion (SS: 0.62 ± 0.05 kg; CO: 0.58 ± 0.05 kg; $P = 0.61$) or on a kg feces/kg BW basis (SS: 0.93 ± 0.08 kg/kg BW; CO: 0.88 ± 0.08 kg/kg BW; $P = 0.68$). There was a greater fecal dry matter percent in SS calves compared to control calves (18.5 ± 0.6 % vs. 16.4 ± 0.6 %; $P = 0.03$).

There was a trend for ADF and NDF digestibility to be greater in the SS calves compared to the CO calves (P = 0.08; Table 11). There were no differences in the other digestibility variables (P > 0.4; Table 11).

Table 11. Digestibility variables in control (CO) and SZA-supplemented (SS) calves.

	CO	SS	SEM	P-value
ADF intake (g)	5.71	5.71	-	-
ADF fecal output (g)	11.2	6.52	1.76	0.08
ADF digestibility (%)	-96.2	-14.2	30.8	0.08
NDF intake (g)	12.1	12.1	-	-
NDF fecal output (g)	17.9	10.8	2.70	0.08
NDF digestibility (%)	-47.7	10.6	22.3	0.08
Nitrogen intake (g)	52.6	52.6	-	-
Nitrogen fecal output (g)	21.6	22.6	0.09	0.4
Nitrogen retention (g)	31.1	30	0.9	0.4
Nitrogen digestibility (%)	88.8	89.0	0.8	0.9
Energy intake (Mcal)	5.57	5.57	-	-
Energy fecal output (Mcal)	0.42	0.38	0.04	0.5
Energy retention (Mcal)	5.15	5.19	0.04	0.5
Energy digestibility (%)	92.5	93.1	0.6	0.5

Bone turnover, dimensions, and mechanical properties

There were no treatment*day effects on OC, DPD and OC:DPD ratios. Overall, CO calves had OC concentrations of 244 ± 13 ng/ml, and SS calves had concentrations of 275 ± 13 ng/ml but they were not significantly different ($P = 0.12$). Osteocalcin concentrations decreased from d 30 to d 60 (284 ± 12 vs 235 ± 12 ng/ml; P=0.003). Overall, SS calves had higher DPD concentrations than CO calves (12.4 ± 0.6 ng/ml vs. 10.2 ± 0.6; P=0.01) and there was no day effect (P=0.9). The OC to DPD ratio was not different between treatments (P=0.98). There was a trend for SS metacarpals to be longer than the CO metacarpals, and there were no differences in bone dimensions as measured on the CT images (Table 12). There were no treatment differences in the measured or calculated values of peak force, ultimate bending stress, moment of inertia, or modulus of elasticity of the metacarpals (Table 13).

Table 12. Length, diameters and cortical widths of MC III&IV in control (CO) and SZA-supplemented (SS) calves.

	CO	SS	SEM	P-value
Bone length (cm)	18.5	18.9	0.18	0.09
Diameters (cm)				
Dorsopalmar	1.98	1.98	0.03	0.93
Dorsopalmar medullary	1.26	1.27	0.04	0.99
Lateromedial	2.69	2.70	0.04	0.96
Lateromedial medullary	1.80	1.80	0.05	0.94
Cortical widths (cm)				
Dorsal	0.36	0.37	0.01	0.51
Palmar	0.34	0.35	0.01	0.72
Lateral	0.45	0.45	0.01	0.91
Medial	0.46	0.47	0.01	0.50

Table 13. Bone mechanical properties of MC III&IV from control (CO) and SZA supplemented (SS) calves.

	Peak Fracture Force (N)	Moment of Inertia (mm4)	Ultimate Bending Strength (MPa)	Modulus of Elasticity (MPa)
CO	4243	8.59E-09	113	0.45
SS	4340	8.64E-09	114	0.47
SEM	123	4.53E-10	6	0.04
P-value	0.58	0.93	0.85	0.68

Glycosaminoglycan concentration

Hyaluronic acid concentrations in the synovial fluid were not different between treatments with the mean of CO being 1,200 ± 78 μg/ml and the SS mean being 1,177 ± 78 μg/ml ($P = 0.84$). The addition of sodium zeolite A to the diet did not increase GAG concentration in the articular cartilage as determined by dGEMRIC ($P = 0.85$). Concentration in the SS calves was 57.9 ± 4.6 μg/mg wet wt and it was 56.5 ± 4.6 μg/mg wet wt in the CO calves.

Mineral analysis

Concentrations of the feces and urine from the calves are displayed in Table 14. The SS calves had a greater fecal concentration of Al, and tended to have a lower Cu fecal concentration ($P < 0.10$). There was no difference between treatments in urine mineral concentrations.

Table 14. Fecal and urine mineral concentrations in control (CO) and SZA-supplemented (SS) calves.

	CO	SS	SEM	P-value
Fecal (mg/g)				
Al	2.50	34.0	1.43	< 0.0001
Ca	37.5	36.6	2.55	0.80
Cu	0.02	0.15	0.002	0.07
Fe	0.82	0.72	0.05	0.11
Mg	8.21	7.40	0.51	0.28
P	20.1	20.0	1.30	0.93
Zn	0.29	0.23	0.04	0.34
Urine (μg/ml)				
Al	0.35	0.33	0.03	0.63
Ca	7.15	5.81	0.96	0.34
Cu	0.05	0.04	0.004	0.46
Fe	0.08	0.06	0.01	0.41
Mg	1.02	0.74	0.22	0.39
P	785	550	149	0.28
Zn	0.09	0.07	0.03	0.78

Data on mineral retention and absorption are shown in Table 15. The SS calves retained larger amounts of aluminum than the CO calves. Phosphorus apparent digestibility tended to be decreased in the SS calves.

Plasma mineral concentrations are displayed in Table 16. There was a day effect for calcium, iron, and phosphorus ($P<0.05$). Calcium concentration was highest on d 0 compared to d 30 and d 60 ($P<0.02$). Iron and phosphorus concentrations were lower on d 30 compared to d 0 and 60 ($P < 0.04$). There were treatment effects for copper, magnesium, and phosphorus concentrations with SS calves having higher plasma copper concentrations, and lower magnesium and phosphorus concentrations ($P < 0.05$). Values are within normal ranges previously reported (Georgievskii, 1982), except for phosphorus and zinc concentrations, which are higher than reported.

Table 15. Metabolism of minerals in control (CO) and SZA-supplemented (SS) calves.

	Intake (mg)	Urine (mg)	Fecal (mg)	Retained (mg)	Digested (%)
Aluminum					
CO	130	1.50	223	-93.7	-70.8
SS	4,445	1.66	3,752	692	15.7
SEM	70	0.11	169	143	38.2
P-value	< 0.0001	0.30	< 0.001	0.001	0.13
Calcium					
CO	8,510	30.5	3,393	5,087	60.1
SS	8,513	29.9	4,102	4,381	51.8
SEM	0.05	4.53	304	303	3.6
P-value	< 0.0001	0.91	0.12	0.12	0.12
Copper					
CO	8.44	0.20	2.03	6.21	75.9
SS	8.46	0.21	1.74	6.51	79.3
SEM	0.0003	0.02	0.29	0.30	3.5
P-value	< 0.0001	0.68	0.49	0.49	0.49
Iron					
CO	61.5	0.34	75.9	-14.8	-23.6
SS	63.9	0.31	78.7	-15.1	-23.1
SEM	0.04	0.06	5.13	5.12	8.2
P-value	< 0.0001	0.77	0.71	0.97	0.97
Magnesium					
CO	1,657	4.78	751	901	54.7
SS	1,657	3.87	814	839	50.9
SEM	.	1.22	52.7	52.7	3.2
P-value	.	0.61	0.41	0.42	0.42
Phosphorus					
CO	8,576	3,241	1,824	3,511	78.7
SS	8,576	2,850	2,231	3,494	73.9
SEM	.	524	163	578	1.9
P-value	.	0.61	0.09	0.98	0.09
Silicon					
CO	533	ND	ND	-	-
SS	587	ND	ND	-	-
SEM	0.88	-	-	-	-
P-value	< 0.0001	-	-	-	-
Zinc					
CO	28.1	0.36	27.8	-0.007	1.3
SS	28.7	0.34	24.5	3.89	14.7
SEM	0.01	0.11	4.71	4.79	16.9
P-value	< 0.0001	0.89	0.62	0.57	0.58

Table 16. Plasma mineral concentrations (μg/ml) in control (CO) and SZA-supplemented (SS) calves

	Day 0	Day 30	Day 60	SEM	Day*Trt P-value
Aluminum					
CO	0.40[a]	0.88[a,b]	0.97[b]	0.18	0.95
SS	0.68	0.83	0.72	0.18	
Calcium					
CO	143[a]	126[b]	115[b]	6	0.54
SS	131[a]	117[a,b]	115[b]	6	
Copper					
CO	0.66	0.69	0.67	0.05	0.82
SS	0.74	0.81[z]	0.80[z]	0.05	
Iron					
CO	1.82[a]	0.93[b]	1.77[a]	0.28	0.61
SS	1.57[a]	0.93[b]	2.04[a]	0.28	
Magnesium					
CO	22.1	22.1	20.4	1.02	0.79
SS	20.1	19.3[z]	18.8	1.02	
Phosphorus					
CO	89[a]	104[b]	85[a]	5	0.25
SS	78	87[z]	85	5	
Silicon					
CO	ND	ND	ND	-	-
SS	ND	ND	ND	-	-
Zinc					
CO	3.92	3.92	3.94	0.5	0.99
SS	3.74	3.91	3.85	0.5	

[a,b,c] Means within rows with different superscripts differ ($P < 0.1$)
[z]Means within columns within variable differ ($P < 0.1$)

Table 17. Percentage of body weight (%) of organs from control (CO) and SZA-supplemented (SS) calves.

	CO	SS	SEM	P-value
Adrenals (both)	0.009	0.009	0.001	0.85
Heart	0.64	0.65	0.02	0.72
Kidney (both)	0.57	0.59	0.02	0.45
Liver	1.96	1.82	0.09	0.24
Pancreas	0.07	0.09	0.01	0.15
Spleen	0.65	0.58	0.05	0.36

Organ weights, expressed as a percentage of body weight, are presented in Table 17. There were no differences between the two groups of calves ($P < 0.05$). Dry matter percentages of the organs and feces collected from the CO and SS calves are presented in Table 18. Dry matters were increased in the bone, heart and feces ($P < 0.03$), and tended to be increased in the muscle of the SS calves ($P = 0.08$).

Table 18. Organ and feces dry matter (%) from control (CO) and SZA-supplemented (SS) calves.

	CO	SS	SEM	P-value
Adrenal	21.0	21.8	0.4	0.20
Aorta	29.7	28.9	0.6	0.33
Cortical bone	87.9	88.1	0.05	0.01
Heart	21.4	23.2	0.4	0.008
Kidney	18.8	19.3	0.4	0.42
Liver	25.7	26.6	0.5	0.18
Lung	20.5	20.2	0.3	0.49
Muscle	23.1	24.2	0.4	0.08
Pancreas	24.4	24.9	1.0	0.72
Spleen	25.5	26.4	0.7	0.37
Tendon	29.5	28.8	0.4	0.28
Trachea	27.4	26.5	0.8	0.38
Feces	16.4	18.5	0.6	0.03

There was no difference (P = 0.44) in bone ash percentage between the treated group (66.5 ± 0.4 %) and control group (66.1 ± 0.4%). Mineral concentrations of the collected organs are presented in Table 19. Aluminum was increased in all organs of the SS calves ($P < 0.05$) except for bone. Sodium zeolite A increased adrenal Zn, aorta Ca, Mg, P and Zn, heart Mg and Zn, liver Ca and Mg, muscle Ca, pancreas Mg, and tendon Ca ($P \leq 0.05$). Additionally, SZA decreased kidney and liver Fe ($P \leq 0.05$). Silicon was increased in the kidney, lung, muscle and spleen of the SS calves ($P < 0.05$).

Table 19. Mineral concentrations (μg/g dry weight) of various tissues from control (CO) and SZA-supplemented (SS) calves.

	CO	SS	SEM	P-value
Adrenal				
Al	8.4	30.7	2.1	< 0.0001
Ca	411	445	16	0.17
Cu	9.59	9.42	0.7	0.86
Fe	189	174	12	0.4
Mg	564	594	16	0.2
P	12,492	13,123	435	0.32
Si	BDL	BDL	-	-
Zn	65.3	74.1	2.5	0.02
Aorta				
Al	1.68	3.15	0.32	0.005
Ca	333	372	8	0.003
Cu	0.98	1.19	0.10	0.14
Fe	49.8	39.9	6.9	0.32
Mg	174	186	4	0.05
P	1,954	2,169	52	0.009
Si	2.89	4.73	0.3	0.001
Zn	34.4	38.4	1.2	0.03

BDL = Below detectable limits

Table 19 (cont'd).

	CO	SS	SEM	P-value
Cortical bone				
Al	8.8	17.8	5.1	0.22
Ca	125,493	129,378	5,784	0.64
Cu	0.27	0.34	0.04	0.21
Fe	8.1	7.7	0.8	0.74
Mg	1,771	1,833	114	0.70
P	55,656	56,667	2,590	0.79
Si	3.82	4.46	0.26	0.11
Zn	40.5	44.0	2.8	0.39
Cartilage				
Al	2.15	4.30	0.73	0.05
Ca	1,134	1,171	209	0.9
Cu	0.50	0.27	0.12	0.18
Fe	4.26	4.09	0.47	0.81
Mg	96.7	88.4	5.0	0.26
P	690	632	101	0.69
Si	1.51	1.38	0.31	0.77
Zn	3.90	3.75	0.35	0.76
Heart				
Al	2.9	15.7	1.4	< 0.0001
Ca	166	180	5	0.1
Cu	14.9	14.6	0.2	0.36
Fe	171	161	6	0.25
Mg	1,045	1,223	58	0.04
P	11,425	11,325	218	0.75
Si	3.84	5.09	0.62	0.17
Zn	73.3	79.0	2.0	0.05
Kidney				
Al	7.4	55.4	4.7	< 0.0001
Ca	590	542	54	0.54
Cu	13.9	13.7	0.8	0.83
Fe	139	106	10	0.04
Mg	729	818	31	0.05
P	12,564	10,816	939	0.2
Si	8.1	44.2	8.3	0.007
Zn	81.9	86.6	3.6	0.37

Table 19 (cont'd).

	CO	SS	SEM	P-value
Liver				
Al	3.5	65.0	6.2	< 0.0001
Ca	147	185	7	0.0007
Cu	573	603	38	0.57
Fe	224	149	26	0.05
Mg	538	612	15	0.003
P	10,148	10,976	372	0.13
Si	1.43	2.08	0.49	0.36
Zn	141	151	15	0.64
Lung				
Al	9.4	28.2	2.7	0.0002
Ca	376	390	15	0.48
Cu	5.6	5.3	0.3	0.39
Fe	319	253	24	0.08
Mg	571	564	6	0.46
P	10,690	10,225	184	0.09
Si	5.36	7.58	0.48	0.005
Zn	81.3	80.1	1.2	0.5
Muscle				
Al	4.3	9.0	0.9	0.002
Ca	177	205	7	0.01
Cu	3.0	3.4	0.2	0.31
Fe	44.2	39.9	5.3	0.57
Mg	1,078	1,191	46	0.1
P	9,977	10,425	379	0.41
Si	4.85	7.63	0.95	0.05
Zn	86	92.8	4.74	0.33
Pancreas				
Al	4.0	12.6	1.0	< 0.0001
Ca	453	498	25	0.22
Cu	3.86	4.18	0.29	0.44
Fe	112	90	11	0.16
Mg	728	851	40	0.04
P	13,014	13,283	660	0.78
Si	7.80	7.34	1.14	0.78
Zn	195	295	37	0.07

Table 19 (cont'd).

	CO	SS	SEM	P-value
Spleen				
Al	3.4	22.6	3.4	0.001
Ca	127	115	20	0.66
Cu	2.68	3.11	0.29	0.3
Fe	3,406	6,600	1,938	0.26
Mg	370	388	35	0.73
P	6,601	6,728	396	0.82
Si	2.00	3.60	0.24	0.0002
Zn	61.1	65.5	4.2	0.48
Tendon				
Al	7.5	14.6	1.9	0.02
Ca	374	411	11	0.05
Cu	1.5	1.7	0.2	0.52
Fe	23.5	18.8	2.7	0.22
Mg	221	189	32	0.47
P	2,382	2,141	302	0.56
Si	15.9	22.1	4.3	0.29
Zn	47.1	49.3	5.8	0.77
Trachea				
Al	2.16	4.73	0.65	0.03
Ca	1,007	1,052	33	0.36
Cu	0.93	1.13	0.11	0.22
Fe	33.3	31.6	3.55	0.74
Mg	257	255	6	0.75
P	1,753	1,694	63	0.51
Si	5.28	6.84	0.89	0.23
Zn	18.2	18.0	1.1	0.94

DISCUSSION

Previously, studies using SZA have focused on the effects of silicon alone; however, with the high concentrations of not only Si but Al as well, both minerals should be considered when evaluating the effects of SZA.

Sodium zeolite A did not affect the weight of the calves, nor did it affect average daily gain. Horses fed varying levels of SZA also had similar weight gains or average daily gains compared to horses receiving no supplemental SZA (Frey et al., 1992). Similar results were seen in chickens (Ballard and Edwards, 1988), and pigs (Ward et al., 1991).

To determine the effect of the SZA on digestibility variables, total collection of feces and urine was attained. The SS calves had a greater average daily output of urine over the 3-d total collection period than the CO calves. Water intake was not recorded, but it may be that the SS calves drank more water than the CO calves. Fecal dry matter was also increased in the SS calves. It is also interesting that fewer SS calves were afflicted with scours (8 CO, 4 SS), as noted by the MSU Dairy Farm workers who worked with the calves on a daily basis and kept a journal of such occurrences. The addition of zeolites to the diets of ruminants, poultry, and swine can decrease the incidence of scours as well as other intestinal disorders (Mumpton, 1984). The exact role the zeolite plays in reducing scours is unknown. One suggestion has been that the effects are due to the zeolite's alkalinity and buffering capacity in the gastrointestinal tract (Mumpton, 1984). Or perhaps the effect is through silicon, as silicon impacts the immune system (Moseley et al., 1988; Seaborn et al., 2002) and thus might reduce scours and other intestinal problems through this route.

Sodium zeolite A did not affect nitrogen retention or digestibility, nor was the amount of energy retained or digested affected. However, there was a difference in ADF and NDF digestibility, with the SS calves tending to have greater fiber digestibilities although most of the values are negative. Previous literature has shown that natural

zeolite supplementation increases ADF digestibility (Sweeney et al., 1984) while synthetic zeolite supplementation did not affect ADF digestibility (Johnson et al., 1988). The different zeolite sources may have been the cause for the differing results, with the synthetic zeolite probably being most like the SZA used in the current study. While it appears that SZA increased fiber digestibility, more than likely an outside factor or a side effect of SZA supplementation affected the results. Although intake of milk replacer was identical in the two groups of calves (they consumed all that was given them), one factor that was unaccounted for was the intake of straw bedding. As significant straw consumption was unexpected, values for intake were not collected. The ADF and NDF values in the milk replacer were relatively low, while values in the fecal samples were much higher. However, the consumption of straw high in fiber might account for the higher fecal fiber values. The lower digestibility values in the CO calves could be due to higher straw intakes compared to the SS calves. It is difficult to determine if the differences in fiber digestibilities are from a direct effect of SZA in the digestive tract on fiber digestion, or from an indirect effect on feed intake. The increase in straw consumption may also contribute to the decrease in fecal dry matter in the CO calves. The straw would attract water, thus decreasing dry matter.

It is unknown if the desire to seek supplemental sustenance in the CO calves was increased above normal, or if it was depressed in the SS calves. A decrease in feed intake has been seen in cows supplemented with zeolites (Johnson et al., 1988; Thilsing-Hansen et al., 2002; Turner, Chapter 4, Dissertation). However, studies have also shown an increase in feed intake with zeolite supplementation (Roussel et al., 1992). The differences in results could be due to differences in amounts of SZA supplemented, as the

studies in which decreases were found fed SZA at levels approximately equal to 2% of the diet, while the increase in feed intake came after SZA supplementation at 1.5% of the diet. The SS calves on the current project were receiving approximately 3% of their diet in SZA on a dry matter basis, and it varied with their body weight. This might have been the cause of the potential decrease in foraging behavior (thus feed intake) in the SS calves.

Deoxypyridinoline, a marker of bone resorption, was higher in SS calves than CO calves. Despite the increase in DPD in SS, there were no treatment differences in the ratio of OC to DPD, indicating that as DPD increased in the SS calves, so did OC. This suggests that there was neither a net loss nor gain of bone, which is also reflected in the lack of differences seen in measured mechanical properties of the bone. Because DPD was higher in the SS calves but there was no difference in the ratio, this may signify the SS calves were experiencing more rapid bone turnover than the CO calves.

The SS calves tended to have longer metacarpi than the CO calves. This finding is consistent with studies in other species of animals. Carlisle (1980) found that chicks fed a diet supplemented with 250 mg Si/kg feed had greater tibial lengths compared to control chicks fed a basal diet containing 1 mg Si/kg feed. Furthermore, greater femoral lengths have been reported in growing rats supplemented with 35 mg/kg of Si versus control rats receiving only a basal level of 2 mg Si/kg feed (Nielsen and Poellot, 2004). Although longitudinal growth of the calf metacarpi was affected by silicon, radial growth was not. Length is obtained through endochondral ossification, while thickness is obtained through the apposition of new bone on both the endosteal and periosteal surfaces.

Endochondral ossification has been shown to be increased in animals on a silicon adequate diet, as compared to animals on a silicon deficient diet Carlisle, 1980).

Supplemental silicon did not affect metacarpi mechanical properties such as peak fracture force, ultimate bending stress and modulus of elasticity. This finding is consistent with a previous study in which supplemental silicon did not affect the same variables in the femurs of growing rats (Nielsen and Poellot, 2004). Therefore, the lack of an effect on bone breaking properties may indicate that silicon does not have a major effect on bone crystal formation or function. However, low dietary silicon decreased plasma osteopontin, and increased both plasma sialic acid concentration and urinary helical peptide excretion, indicating that silicon can regulate extracellular matrix protein synthesis (Nielsen and Poellot, 2004).

Bovine metatarso-phalangeal synovial fluid contains approximately 0.5 mg/ml hyaluronic acid (Coleman et al., 1999). The hyaluronic acid concentration (approximately 1.2 mg/ml) in the 60-d-old calves in the current study was a little higher than that of the previous study (Coleman et al., 1999). However, the age of the calves should be taken into account as younger subjects seem to have higher hyaluronic acid concentrations than older subjects (Balazs, 1982). Although silicon has been suggested as a component of hyaluronic acid (Schwarz, 1973) and is potentially involved in the synthesis of glycosaminoglycans (Carlisle, 1974), supplemental silicon did not increase hyaluronic acid concentrations in the SS synovial fluid compared to the CO synovial fluid, nor did it increase glycosaminoglycan concentrations in the articular cartilage. Increased glycosaminoglycan concentrations were found in chicks compared to chicks that were on a diet deficient in silicon (Carlisle, 1974). The CO diet, while lower in

silicon than the SS diet, was not deficient in the mineral. It would appear that silicon is not a limiting factor for glycosaminoglycan synthesis, specifically hyaluronic acid, when diets are not deficient in the mineral.

Total glycosaminoglycan concentration in the articular cartilage from the weight-bearing portions of the carpus and fused metacarpi also failed to increase with the addition of bioavailable silicon via sodium zeolite A. Perhaps GAG concentration did not increase because of an aluminum effect. The cartilage aluminum levels were increased in the SS calves. Following injection of an aluminum compound into rabbit knees, aluminum invaded the synovial cell layer and caused an apparent loss of proteoglycan in superficial zones of tibial and femoral cartilages (Chary-Valckenaere et al., 1994). Superficial zones of cartilage were the targets of interest in the current, and with the subsequent rise in cartilage aluminum content, perhaps possible increases in GAG concentration caused by additional silicon were negated by the additional aluminum.

Intake of most of the minerals was greatest in the SS calves, with the exception of Mg and P which had identical intakes between the groups. Magnesium and phosphorus were not detected in the SZA, and the calves ate identical amounts of milk replacer which would account for the identical intakes of those minerals. Except for aluminum and silicon, the difference between intakes of the other minerals is negligible and should not be taken into account.

Feeding SZA or aluminum to livestock has resulted in alterations to calcium and/or phosphorus metabolism, either by increasing calcium plasma concentrations (Allen et al., 1986; Turner, Chapter 4, Dissertation) and retention or digestibility

(Robinson et al., 1984), or by decreasing plasma phosphorus concentration (Thilsing-Hansen et al., 2003; Turner, Chapter 4, Dissertation) and retention or digestibility (Valdivia et al., 1982). In the present study calcium retention and digestion were not affected by additional SZA, but phosphorus digestion tended to be depressed. Additionally, a trend for greater amounts of phosphorus excreted in the feces was seen in the SS calves, indicating less phosphorus absorption. The phosphorus can bind to the aluminum, creating non-absorbable complexes (Krueger et a., 1985).

The SS calves had lower plasma phosphorus concentrations, averaged across all days, than the CO calves. This has been seen previously in cows fed SZA (Thilsing-Hansen et al., 2003; Turner, Chapter 4, Dissertation). Unlike the cows though, the plasma concentration did not drop below the normal range and the calves were not in a hypophosphatemic state (Georgievskii, 1982). The increase in Al intake decreased the phosphorus absorption, thus plasma phosphorus concentration was decreased. However, the accompanying increase in plasma calcium concentration seen previously in the lactating cows (Turner, Chapter 4, Dissertation), was not found in the current study, perhaps because the decrease in phosphorus was much smaller in the calves (about 10%) compared to the cows (about 84%). The larger decrease in the lactating cows resulted in subsequent drastic homeostatic mechanisms to maintain phosphorus levels. The calves did not have to go to such drastic measures, such as resorption of bone, which is supported by the similarity of calcium and phosphorus cortical bone content between the SS and CO calves. Therefore, the resulting increase in plasma calcium following bone resorption did not occur in the SS calves.

Previous literature has suggested that silicon is found in "unusually large quantities" in the connective tissues such as aorta, trachea, bone and tendon (Carlisle, 1972). The high silicon content of connective tissue is hypothesized to be mainly from its presence as an integral component of the glycosaminoglycans and their protein complexes that contribute to the structural framework of the tissues. In the current study, "unusually large quantities" were not noted in the connective tissues, and the connective tissues did not contain most of the silicon. Tendon did contain the highest amount of silicon, but the trachea was in the middle of the other tissues, and the aorta was towards the bottom of the group. The suggestion that the high silicon content in the connective tissues is due to its composition in glycosaminoglycans should be reevaluated, as the connective tissues in the present study did not contain the highest silicon concentrations.

Sodium zeolite A supplementation increased aorta, kidney, lung, muscle, and spleen silicon concentrations. The large increase in kidney silicon concentration (445%) could be due to high silicon in the urine. Alternatively, the high silicon concentrations could be indicative of silicon toxicity in the urinary tract. Silicon toxicity can cause calculi (Carlisle, 1984). Calculi were created in rats and dogs fed high levels of a silicon supplement, not silica (Carlisle, 1984). However, the formation of calculi is a relatively unclear process. Besides urinary problems, silicon toxicity also affects the lungs. Silicosis has been suggested to occur following inhalation of dust and silicate, which stimulates a severe reaction in the lungs (Carlisle, 1984). Based on the silicon accumulation in the lungs in the SS calves, it may be plausible that silicon deposited in the lungs from the blood may also contribute to silicosis, not just inhaled silicon.

The increase in silicon in the aorta most likely caused an increase in the zinc concentration. Tohno et al. (2002) found a significant correlation between silicon and zinc in the jugular vein. Additionally, aluminum, calcium, phosphorus and magnesium are directly correlated in the aorta (Minami et al., 2001). In the current study, the aluminum most likely increased first, thus increasing the concentrations of the three other minerals. The aorta was the tissue with the greatest number of changes in mineral concentrations.

There was no effect of SZA on plasma aluminum in the SS calves. After feeding high levels of aluminum, Hussein et al. (1990) also found that plasma aluminum remained unchanged. Previously, the lack of plasma response was thought to indicate that dietary aluminum is poorly absorbed and has little effect on animal life (McCollum et al., 1982). King et al. (1997) found that apparent gastrointestinal uptake of Al is between 0.1-0.3%, which was probably slightly underestimated. The SS calves in the current study retained approximately 15% aluminum.

Plasma aluminum concentrations likely do not change because the aluminum is rapidly transported to the tissues. Valdivia et al. (1982) found that at least a portion of dietary aluminum is absorbed and transported as there were increases in the liver, kidney and muscle of lambs following supplementation of 2,000 mg Al/kg feed. Additionally, rats chronically supplemented with aluminum (15 wk) showed increases in kidney, spleen, and liver aluminum (Garbossa et al., 1998). Although there was not an increase in plasma aluminum in SS calves, there were dramatic increases in aluminum in all SS tissues analyzed except cortical bone. The smallest increase was in the aorta, which increased 87.5%, and the largest was in the liver, with a 1,757% increase in aluminum.

The average increase was 384%. This reiterates the fact that the level of aluminum cannot be overlooked in sodium zeolite A, and must be considered when evaluating data.

While the extent of tissue damage by the aluminum accumulation is not known in the calves, several studies have indicated that the accumulation could be harmful. Decreased alkaline phosphatase activity in aluminum supplemented rats was concluded to be an indication of aluminum toxicity, as there was substantial hepatic aluminum accumulation (Guo et al., 2004). Hepatocytes can be destroyed by large deposits of aluminum (Nayak, 2002). The supplemented rats also had lower values of anemia-related variables such as hematocrit, hemoglobin, and plasma iron, most likely from aluminum interference with iron. Due to the high concentration of aluminum, future studies should determine the effects of sodium zeolite A supplementation on neurofunction of the brain. In humans, aluminum toxicity has been associated with memory loss, tremor, jerking movements, impaired coordination, sluggish motor movement, loss of curiosity, and ataxia (Nayak, 2002). Rats fed 0.3% aluminum in their drinking water displayed specific cognitive impairment (Jope and Johnson, 1992) Concern should be taken when feeding SZA to animals, particularly to animals producing a food product intended for human consumption, as there was a significant increase in the longissimus dorsi muscle of the SS calves. Aluminum has been linked to several disease syndromes in humans, not the least of which is Alzheimers Disease.

A decrease in plasma magnesium concentration occurred following supplementation with SZA. Although decreased, the plasma magnesium values were still within normal limits (17-25 μg/ml) for calves (Georgievskii, 1982). The decrease was so small that the effects are probably of little physiological value. Steers fed high aluminum

also had decreased magnesium blood levels (Allen and Robinson, 1980; Allen et al., 1984; Allen et al., 1986), as did chicks (Hussein et al., 1990) and lambs (Rosa et al.., 1982; Valdivia et al., 1982). The mechanisms by which aluminum interacts with magnesium are not as clearly defined as with phosphorus (Allen, 1984). Unlike with phosphorus, the effect of aluminum on magnesium does not appear to be from the formation of unabsorbable complexes (Allen and Fontenot, 1984). Although serum magnesium levels are depressed, there are no changes in magnesium absorption (Valdivia et al., 1982; Allen and Fontenot, 1984). In the current study, there was also not a change in magnesium retention or absorption. However, there were increases in heart, kidney, liver, muscle and pancreas magnesium concentrations. It appears that the magnesium is cleared from the plasma and deposited in the tissues of the SS calves.

Plasma copper concentrations were higher in the SS calves than the CO calves. However, there were no differences in retention or absorption. Moreover, copper tissue concentrations were not changed by SZA supplementation. The cause of the increase in plasma copper concentration in the SS calves is unclear, although similar results occurred in aluminum supplemented rats (Guo et al., 2004).

Concentrations of zinc, copper, and magnesium in the liver, kidney, heart, pancreas, spleen, and muscle were similar to values reported in Holstein bull calves (Neathery et al., 1990b). However, the iron concentrations of the tissues in both the SS and CO calves in the current study were approximately half that of values reported by Neathery et al. (1990b). Additionally, the calves were in a negative iron balance. Both groups of calves appeared to be releasing iron from their tissues and excreting it out. It is also interesting to note that on d 30, when the mineral balance collection was begun,

plasma iron was at its lowest point in both groups. The plasma then rebounded on d 60 and returned to baseline (d 0) levels, yet tissue iron was still below normal values. Aluminum decreases iron absorption and tissue levels (Han et al., 2000); however, the control calves had negative iron balances as well as the supplemented calves. As water comprised the majority of the calves' diets (powdered milk replacer was mixed into it), the water from the faucet used to distribute the calves' water was tested for mineral concentration, and was taken into account in the mineral balance. Thus, the occlusion of water minerals was not a factor. The low tissue iron may arise from low dietary levels. The iron requirement for calves on milk replacer is 100 mg Fe/kg feed; however, the "requirement" for veal calves is less than 50 mg/kg (NRC, 2001). Veal calves are purposefully fed well below their iron requirement so that maximal myoglobin formation and hemoglobin formation are decreased (Underwood and Suttle, 1999). The calves in the current study were being fed as veal calves, and the iron concentration of the milk replacer was approximately 55 mg Fe/kg feed, well below the true requirement (100 mg/kg) for calves. Thus, their tissue iron would be decreased because they would not have excess iron to store. The liver iron concentration of young animals depends on the content of the feed and may serve as an indicator of adequate supply to the animal (Georgievskii, 1982). The one tissue however that was not low in iron was the spleen, which contained the highest amount of iron. Iron is known to accumulate in the spleen due to the spleen's red blood cell (containing hemoglobin) storage function (Georgievskii, 1982).

With the exception of lung phosphorus, the only mineral to decrease in the tissues of the SS calves was iron. Decreases in tissue iron, particularly liver iron, have been

reported previously (Han et al., 2000). The decreases in iron may be a result of an interaction with aluminum. The mechanisms by which aluminum affects iron metabolism are debatable. Aluminum can compete with iron for absorption (Cannata et al., 1991; van der Voet, 1992; Han et al., 2000); however, retention and absorption of iron in the current study appeared to be unaffected by SZA supplementation. It may be that there is a higher affinity of aluminum for transferrin (Buys and Kushner, 1989; Guo et al., 2004), which might explain the increases in aluminum and the decreases in iron. However, transferring may be only 30% saturated with iron, which leaves ample open binding sites for aluminum (Buys and Kushner, 1989). The reduction of Fe^{3+} to Fe^{2+}, important for translocation of iron and for the release of iron from ferritin, is mediated by ceruloplasmin, which may be inhibited by aluminum (Jeffrey et al., 1996). Tissue ferritin levels were decreased in chicks supplemented with aluminum (Han et al., 2000). The cause of the decreased tissue ferritin was unknown, but the iron regulatory protein may have failed to accurately sense the iron status of the cell due to the presence of aluminum. Or perhaps the aluminum was competing with the iron for binding sites on the iron regulatory protein.

CONCLUSIONS

The addition of sodium zeolite A had little effect on digestibility variables, with the trend for increased ADF and NDF digestibility more than likely a result of unaccounted for straw intake. Previous studies have shown no effect on ADF digestibility by synthetic zeolite supplementation.

Since a trend for increased length was the only difference in the bovine metacarpal bones between the control and SZA supplemented animal, the means by which SZA supplementation was associated with a decrease in lameness in racing Quarter Horses (Nielsen et al., 1993) is still unknown. Based on the DPD and OC to DPD ratio data possibly revealing more active bone metabolism in the SS calves, our current hypothesis is that the mechanism in the racehorses was rapid repair of subclinical injuries, rather than prevention of damage. More research needs to be conducted to further elucidate the mechanism by which SZA decreases musculoskeletal injury rates.

Plasma mineral concentration responses to SZA, such as the decreases in magnesium and phosphorus, are in agreement with previous literature. The decrease in phosphorus apparent digestibility more than likely caused the decrease in plasma phosphorus; however, the cause of the plasma magnesium decrease is unknown. It appears though to be a result of clearance of the magnesium from the blood and deposition in the tissues. Sodium zeolite A increased silicon concentrations in the lungs, spleen, kidney, and aorta of the SS calves. There was an increase in aluminum retention, but plasma aluminum was unaffected. The excess aluminum was likely rapidly cleared from the blood and deposited into various tissues. Substantial increases in aluminum were found in all tissues except bone. Decreases in tissue iron could be linked to aluminum interactions, and the increases in zinc could be linked to the decreases in iron. Due to interactions of aluminum and silicon, to obtain a better understanding of tissue response to supplemental bioavailable silicon, a source lacking aluminum is necessary.

CHAPTER 6

CONCLUSIONS AND IMPLICATIONS

Supplemental sodium zeolite A had no effect on existing osteochondrotic lesion size as determined by radiographs. However, a better approach might be researching the effects of additional dietary silicon on prevention of osteochondrotic lesions, not treatment. One of the difficulties in a study such as the one investigating SZA on osteochondrotic lesion resolution is analysis sensitivity. Perhaps the SZA could have affected the lesions, but the effects were not visible yet because there were no differences in lesion size as determined using radiographs. Radiographs are useful in determining the actual location and size of the lesions, but offer little to no information on the metabolic state of the lesion or surrounding cartilage. The use of a technique such as delayed gadolinium-enhanced magnetic resonance imaging of cartilage (dGEMRIC) could be helpful in such a study by supplying information such as glycosaminoglycan concentration and location in the cartilage. This technique would be a more sensitive measure than radiographs, and could detect changes in the cartilage before radiographs could. It could reveal the current state of cartilage metabolism.

Feeding SZA to the lactating cows and young calves produced numerous effects on mineral metabolism, particularly that of calcium and phosphorus. The results were somewhat consistent between the two groups of animals, but the effects were not as dramatic in the calves as they were in the cows. The addition of 2% DMI SZA to the lactating cows drastically decreased feed intake, milk production, and plasma phosphorus concentration while increasing calcium plasma concentration. The decrease in plasma

phosphorus was substantial, and the concentrations were below levels determined to be hypophosphatemic. The calves, fed 0.05% BW SZA, had decreased plasma phosphorus and tended to have decreased phosphorus absorption. The decrease in plasma phosphorus was slight, and levels were still with normal ranges. Although the calves were only fed equal amounts of milk replacer, which was consumed entirely, fiber digestion data would indicate that the control calves consumed more straw bedding than the supplemented calves, although this was not recorded. Decreases in feed intake in adult cows have previously been reported (Johnson et al., 1988; Thilsing-Hansen et al., 2002). The calves had very little rumen development, determined by visual inspection during tissue harvesting, and were basically monogastrics. Perhaps this is the reason for the slightly decreased phosphorus absorption and plasma phosphorus concentration, while the cows had drastically decreased plasma concentrations. Additionally, the level of phosphorus in the diet may have played a role, as increasing dietary phosphorus when feeding high levels of aluminum alleviates the negative effects of the aluminum (Lipstein and Hurwitz, 1982; Rosa et al., 1982; Hussein et al., 1990). The cows were fed 0.36 % phosphorus, which is right at their requirement (NRC, 2001), while the calves' diet consisted of 0.76% phosphorus, which was approximately 8% above their requirement of 0.70% (NRC, 2001). There was no excess phosphorus in the cow diet, so the high aluminum had drastic effects on the phosphorus metabolism of the cows. The slight excess in the calf diet was marginally sufficient in preventing the decrease in phosphorus absorption as it was only a trend ($P = 0.09$).

Sodium zeolite A has relatively little effects on bone metabolism. There was no effect of SZA on osteocalcin in either the lactating cows or young calves. While the

cows displayed no changes in DPD concentration, the calves had increased DPD values. Several studies have measured bone markers of metabolism in SZA supplemented animals and found mixed results. Yearlings on SZA had no decrease in PYD concentrations, but ICTP concentrations were decreased (Lang et al., 2001c). Alternatively, SZA supplemented broodmares had no decrease in ICTP, but a trend for decreased PYD on d 30 existed; however, there was no difference on d 45 (Lang et al., 2001b). In the current study, there was no difference between OC and DPD ratios between the calf groups. As DPD was increased in the SS calves, it might signify that the calves had an increased rate of bone turnover, but no net loss or gain of bone compared to controls. Besides measurement of bone markers, the bones were mechanically tested and architecture was examined. There were no differences between the two groups of calves, validating the bone marker analysis that sodium zeolite A may have only marginally affected bone metabolism. The measurement of bone markers in a nutritional study utilizing SZA should be reconsidered as strong results are not obtained, and it seems to be unlikely that SZA has a large effect on bone metabolism.

There was a trend for a difference in length of the fused metacarpi bones between the SS and CO calves. The bones tended to be longer in the SS calves, as were femoral bones in rats supplemented with silicon (Nielsen and Poellot, 2004). This may be explained by the potential association of silicon with adequate endochondral ossification (Carlisle, 1980), the process for longitudinal growth. Lengths of other bones were not investigated, and measures such as wither and hip heights were not obtained. It is unknown if other long bones such as the femur were affected by the SZA supplementation. It would be interesting to further investigate the effects of silicon on

long bone growth. Specifically, would the overall height of the animal be increased, or was the process accelerated so that eventually the control animals would reach the same height. If the bones remained longer than the control animals, it could create positive effects on animals such as race horses. The longer bones could create longer stride lengths, and thus possibly faster times. Alternatively, if the silicon simply accelerated the process of growth, problems could be created. Many problems can arise when horses are on a higher plane of growth.

Marked changes in tissue mineral composition occurred following SZA supplementation. The tissue with the most changes was the aorta, with increases in aluminum, calcium, magnesium, phosphorus, silicon, and zinc. Calcium, phosphorus, magnesium and aluminum concentrations are correlated in the aorta (Minami et al., 2001), as are silicon and zinc (Tohno et al., 2002). The increase in silicon could prove to be an interesting find, in that the aorta may be strengthened, which could aid in reducing exercise-induced pulmonary hemorrhage in race horses, suggested to be caused by failure of the pulmonary capillaries (West et al., 1991, 1993; Manohar and Hutchens, 1993). Alternatively, the increase in calcium in the muscle may also be an interesting find. Increased calcium in muscle makes the meat more tender (Montgomery et al., 2004), and thus more palatable to humans. However, the tenderness arises from proteolysis (Koohmaraie et al., 1988), which might be harmful in an athletic animal such as the horse.

The accumulation of aluminum by most of the tissues is alarming, and should be of concern. The lack of an increase in plasma aluminum does not warrant a conclusion that the animal is not affected by the aluminum intake. In a performance animal such as

the horse, SZA supplementation may be detrimental to athletic performance as it is possible that aluminum may reduce hemoglobin and hematocrit (Guo et al., 2004).

The use of 2% sodium zeolite A as a dietary supplement to animals should be reconsidered. The substantial accumulation of tissue aluminum and therefore potential adverse consequences would preclude any benefits, regardless of whether or not the animal is intended for human consumption. The possibility of excess aluminum in human diets is a situation which should be avoided.

Determining the effects of sodium zeolite A on variables becomes slightly complicated as the effects of aluminum, silicon, and aluminum-silicon interactions must be taken into account. In this particular project, the high levels of aluminum appear to override the effects of bioavailable silicon. Perhaps glycosaminoglycan concentrations in cartilage would have increased if silicon acted alone, and did not have to overcome potential counter effects by aluminum. In addition, the distribution of supplemental silicon in tissues might have been different if not for the aluminum interactions. Therefore, when designing studies to evaluate the effects of bioavailable silicon, it would be best to use supplements without aluminum.

APPENDICES

APPENDIX A

MINERAL BALANCE IN HORSES FED SODIUM ZEOLITE A

SUMMARY

The purpose of the study was to investigate the effects of sodium zeolite A, a supplement high in aluminum and silicon, on mineral metabolism of adult horses. Eight mature Arabian geldings were randomly placed into one of two groups: control (CO) or supplemental silicon (SS). All horses received 1.5 kg of a commercial sweet feed and had ad libitum access to hay daily and the SS horses received an additional 200 g of sodium zeolite A per day. Following a 10-d adaptation period, the horses were brought into stalls and fitted with total collection devices. Feces and urine were collected for 3 consecutive days. Hay intake was recorded. Blood samples were taken on d 13 for mineral analysis. The addition of sodium zeolite A to the horses' diets did not affect their weight ($P = 0.95$). There was no difference ($P = 0.30$) in hay intake between SS horses and CO horses. Intakes of Al and Si were greater in the SS horses ($P < 0.03$). Sodium zeolite A increased plasma Si concentration and increased both fecal and urine Si excretion ($P < 0.05$). Although plasma Al concentrations were not changed, Al retention tended to be greater in SS horses ($P = 0.08$). Calcium retention and apparent absorption were increased by the SZA supplementation ($P < 0.05$), and there were no effects on phosphorus absorption. Supplementation of sodium zeolite did not affect weight and feed intake of the horses. Calcium retention and absorption were increased, and Al retention tended be increased, but P was unaffected. Caution should be used when supplementing horses with SZA as a lack of a plasma response does not indicate tissue uptake, and the combination of high Al intake and retention could lead to adverse effects.

INTRODUCTION

Silicon, as the second most abundant element found in the earth's crust, is found in many substances such as dust, soil, and glass. Although silicon is found throughout the environment, it is not readily absorbable in the gastrointestinal tract. A bioavailable form of silicon, sodium zeolite A (SZA) has been used in numerous studies to determine the effects of supplemental silicon. As a sodium aluminosilicate, SZA is broken down in the digestive system into aluminum and monosilicic acid, an absorbable form of silicon (Thilsing-Hansen et al., 2002).

Sodium zeolite A has been used in both ruminant (Johnson et al., 1988; Roussel et al., 1992; Thilsing-Hansen et al., 2002; Turner, Chapter 4, Dissertation) and equine studies (Frey et al., 1992; Nielsen et al., 1993; Lang et al., 2001a,b). However, the results of the studies have yielded different results between the species. Several, if not most, studies in which cows were supplemented with SZA described decreases in feed intake (Johnson et al., 1988; Thilsing-Hansen et al., 2002; Turner, Chapter 4, Dissertation). However, no adverse effects have been reported in the equine studies. The decreases in feed intake seen in the ruminants are most likely a result of a phosphorus deficiency caused by the high intake of aluminum from SZA. Significant changes in both phosphorus and calcium metabolism have been reported in cows supplemented with SZA (Enemark et al., 2003; Thilsing-Hansen and Jorgensen, 2001; Thilsing-Hansen et al., 2003). While mineral metabolism studies have been conducted in cows supplemented with SZA, no such studies have been done in the horse. By investigating the effects of SZA supplementation on mineral metabolism, specifically calcium, phosphorus, aluminum and silicon, perhaps the cause for the difference between equine and ruminant

reactions to SZA may be elucidated. Our hypothesis was that calcium and phosphorus metabolism would not be affected by supplemental SZA in horses. Our objective was to determine the effects of sodium zeolite A supplementation on mineral metabolism of horses.

MATERIALS AND METHODS

Horses

Eight mature Arabian geldings with an average body weight of 468 ± 7 kg were used for the study. The horses were housed on dry-lots during a 10-d diet adaptation period. The protocol was approved by the Institution's Animal Care and Use Committee.

Treatment

The geldings were pair-matched by weight and randomly placed into one of two groups: control (CO) or supplemental silicon (SS). All horses received 1.5 kg of a commercial sweet feed and had ad libitum access to hay daily. The SS horses received an additional 200 g of SZA per day, top-dressed on their sweet feed. Mineral concentrations of the sweet feed, hay, and SZA are in Table 1A. The total basal diet (CO) contained approximately 75 mg/kg silicon and 115 mg/kg aluminum. With the addition of SZA the total SS diet had a silicon concentration of approximately 87 mg/kg and an aluminum concentration of approximately 2837 mg/kg. These numbers are based on the average hay intake of the two groups, and are therefore approximations. During the diet adaptation period the horses were individually fed their rations. Horses were weighed on d 0 and again on d 13.

Table 1A. Mineral concentrations (mg/kg dry wt) of the diet

	Al	Ca	P	Si
Sweet feed	97.7	5,850	5,080	68.7
Hay	113	9,640	3,330	72.6
SZA	133,000	132	38.7	646

Total collection

Following the 10-d adaptation period, the horses were brought into stalls and fitted with total collection devices, or nappies (Equisan, Melbourne Victoria, Aust.). Feces and urine were collected for 3 consecutive days. The nappies were emptied every 8 h, and total feces and urine were weighed, and the weight was recorded. Then 10% of the feces and urine was saved and placed into a cooler. The remaining portions were discarded. At the end of the total collection period the saved urine and feces were pooled so that each horse had one urine and one fecal sample for the 3-day period. The composite urine and feces were stored in the freezer until mineral analysis.

During the collection period the hay intake of the horses was recorded. Samples of the hay, sweet feed, and sodium zeolite A were taken for mineral analysis.

Blood collection

Blood samples were taken on d 13 of the study, the last day of the total collections. Since plasma was to be analyzed for silicon concentration, special care was taken to reduce contamination from glass. Seven-ml plastic tubes were acid washed, then 66 μl of 15% K_2EDTA were added to the tube. The tubes were then capped with acid-washed rubber tops, and vacuumed. Blood was then collected into three of the 7-ml plastic tubes. The tubes were centrifuged for 20 min at 1,340 x g. Plasma was removed

and pipetted into microcentrifuge tubes and frozen at -20° C. To avoid contamination from silicon, plastic acid-washed pipettes and microcentrifuge tubes were used.

Mineral analysis

The fecal samples were freeze-dried, and then ground in a mill. The samples of sweet feed and hay were ground as well. Subsamples of the feces, hay, sweet feed, and SZA were reserved and placed in a drying oven to obtain dry matter data. Approximately 0.5 g of feed, SZA, hay, and feces were placed into plastic tubes. Approximately 1 ml of plasma and 2 ml of urine were placed into plastic tubes as well. Five ml of 70% nitric acid were added to the tubes containing feed, SZA, hay and feces, and 2 ml of 70% nitric acid were added to the tubes containing urine and plasma. The tubes were then placed into ovens at 90 °C and allowed to sit overnight. Then 10,000 μg/ml cesium (ionization synchronizer) and 100 ppm yttrium (Y) were added to all tubes so that the final concentrations were 500 ppm cesium and 1 ppm Y. Samples were then transferred to volumetric flasks (25 ml for hay, feed, SZA and feces, 10 ml for plasma and urine), brought up to volume with double-deionized water, and poured into plastic conicles. The diluted samples were then analyzed with an inductively-coupled plasma mass spectrometer at the Diagnostic Center for Population and Animal Health at Michigan State University (East Lansing, MI, USA). Samples were run in triplicate.

Statistical analysis

Data were analyzed with the general linear model in SAS 8.2. Bartlett's test for homogeneity of variance was used in SAS. If variances were determined to be

heterogenous, Welch's test was used for treatment differences, of which individual standard error of the means (SEM) were reported. If variances were homogeneous, then pooled SEMs were reported. Differences were explored at $P < 0.05$, and trends were explored at $P < 0.10$.

RESULTS

The addition of SZA to the horses' diets did not have an effect on their weight ($P = 0.95$). There was no difference ($P = 0.30$) in hay intake between SS horses (8.57 ± 0.81 kg DM) and CO horses (7.27 ± 0.81 kg DM). Fecal dry matter was higher in SS horses than CO horses (21.7 ± 0.8 % vs. 17.7 ± 0.8 %; $P = 0.01$).

Plasma, fecal and urinary mineral concentrations are displayed in Table 2A. Sodium zeolite A increased plasma Si, fecal Al, and urinary Si concentrations, decreased fecal Ca and tended to decrease fecal P concentration.

Table 2A. Plasma, fecal, and urine mineral concentrations in control (CO) and SZA-supplemented (SS) horses.

	CO	SS	SEM	P-value
Plasma (µg/ml)				
Al	0.24	0.23	0.08	0.95
Ca	119	119	2	0.94
P	89.3	86.6	1.8	0.34
Si	0.53	1.02	0.12	0.03
Fecal (mg/kg)				
Al	655	5,955	767	0.003
Ca	9,590	8,071	446	0.05
P	6,124	5,690	273	0.3
Si	72.2	99.1	10.4	0.12
Urine (µg/ml)				
Al	0.19	0.26	0.05	0.46
Ca	2,377	1,829	452	0.42
P	7.17	4.99	0.7	0.07
Si	28.6	46.6	3.3	0.008

Information such as intake, absolute mineral amounts in feces and urine, retention, and absorption is included in Table 3A. Intakes of Al and Si were greater in the SS horses ($P < 0.03$). There was a greater fecal Al and Si output and a greater urinary Si output from the SS horses ($P < 0.02$). Calcium retention and apparent digestion and aluminum retention were affected by the SZA supplementation ($P < 0.10$).

Table 3A. Metabolism of minerals in control (CO) and SZA-supplemented (SS) horses.

	Intake (g)	Urine (g)	Feces (g)	Retained (g)	Digested (%)
Aluminum					
CO	0.95	0.001	2.49	-1.55	-172
SS	26.6	0.002	23.5	3.16	11.8
SEM	0.09	0.0006	1.55	1.59	71.6
P-value	< 0.0001	0.28	< 0.001	0.08	0.12
Calcium					
CO	77.6	16.7	36.5	24.4	52.9
SS	90.2	16.2	33	41.0	62.9
SEM	7.86	4.50	2.87	4.81	1.92
P-value	0.30	0.93	0.42	0.05	0.01
Phosphorus					
CO	31.2	0.05	23.1	7.6	24.0
SS	35.6	0.04	23.4	11.7	33.1
SEM	2.7	0.008	1.6	1.8	3.78
P-value	0.30	0.61	0.92	0.15	0.14
Silicon					
CO	0.62	0.19	0.27	0.15	54.7
SS	0.84	0.40	0.40	0.03	51.3
SEM	0.05	0.04	0.03	0.06	6.4
P-value	0.04	0.006	0.02	0.22	0.73

DISCUSSION

Sodium zeolite A is an aluminosilicate that is hydrolyzed at low pH into silicic acid, amorphous aluminum silicates and aluminum (Thilsing-Hansen et al., 2002). The

high amounts of aluminum in the SZA as compared to silicon necessitates evaluating SZA as a source of aluminum as well as bioavailable silicon. The SZA significantly increased the intake of silicon and aluminum in the horses.

Previous studies found conflicting results on the effect of SZA supplementation on feed intake in cows. Cows supplemented with 2% SZA decreased their feed intake (Johnson et al., 1988; Thilsing-Hansen et al., 2002; Turner, Chapter 4, Dissertation) while cows supplemented with less than 1.5% SZA increased their intake (Roussel et al., 1992). Additionally, steers supplemented with 1,200 mg Al/kg feed suffered no ill effects on intake (Valdivia et al., 1978) yet sheep consuming 2,000 mg Al/kg feed had depressed appetites (Valdivia et al., 1982). There appears to be a threshold at which aluminum or SZA begin to adversely affect animals.

The decreases in feed intake may be a result of the binding of aluminum to phosphorus in the digestive system, essentially reducing dietary phosphorus to the animal. High dietary aluminum interferes with phosphorus metabolism in animals by forming unabsorbable complexes in the digestive tract (Deobald and Elvehjem, 1935). A significant decrease in plasma phosphorus in response to supplementation of aluminum has been seen in lambs (Rosa et al., 1982), beef cows (Allen et al., 1986) and chicks (Hussein et al., 1990). The decrease usually occurs approximately 1 wk after supplementation. The animals then begin to exhibit classic signs of phosphorus deficiency, the most common deficiency in ruminants. The most common sign of phosphorus deficiency is a reduction in feed intake (Call et al., 1987).

Alternatively, decreases in feed intake in previous equine studies on SZA supplementation have not been reported (Nielsen et al., 1993; Lang et al., 2001b,c).

Subsequently, SZA did not affect feed intake in the current study. Phosphorus deficiency is rare in horses, and there is not much literature on the subject. However, no mention of a decrease in feed intake has been indicated in association with phosphorus deficiency in horses. Additionally, in contrast with ruminant literature, phosphorus absorption in the horses was not affected by the SZA. Moreover, a decrease in plasma phosphorus concentrations was also not seen in the horses, and the values were slightly higher than the range previously given for horses of 30-70 mg/kg (Georgievskii, 1982).

The discrepancies between equine and bovine studies may come about because of digestive tract differences. Besides a P requirement for the whole animal, there is an additional P requirement for rumen microbes in ruminants which must be met for optimal microbial activity (Bryant et al., 1957). As horses lack rumens, this would be a non-issue in equine studies. However, it has been suggested that while a decrease in microbial digestion occurs due to P-deficiency, the decrease in feed intake may actually be due to a non-digestive tract disturbance such as a disturbance of intracellular metabolism due to low blood P levels (Milton and Ternouth, 1985). If this were true, although horses do not have rumens and would therefore not have ruminal microbial activities to maintain, they might still have disturbed intracellular metabolism due to low P levels in the blood.

Perhaps the different responses of horses and ruminants to dietary SZA are due to the level of phosphorus in the diet. The negative effects of high aluminum intakes can be alleviated by supplementing dietary phosphorus. Increases in previously decreased plasma inorganic phosphorus has been observed when phosphorus was supplied to the animals (Lipstein and Hurwitz, 1982; Hussein et al., 1990). Decreases in gain and feed intake in aluminum-supplemented lambs were reversed when the animals were fed

additional phosphorus (Valdivia et al., 1977; Rosa et al., 1982). It has been suggested by Lipstein and Hurwitz (1982) that 0.76 g of supplemental phosphorus is required to offset the effect of 1 g of aluminum. Animals that are borderline phosphorus deficient would seem to be most at risk of the negative Al effects on P metabolism. The phosphorus requirement for the light working horses in the study is 18 g (NRC, 1989). They were receiving 27-39 g on a dry matter basis, well above their requirement. However, the cows in the previous study (Turner, Chapter 4, Dissertation) had a requirement of 0.36% P (NRC, 2001), and were receiving approximately 0.36% P in their diet. That may have caused them to be more susceptible to high aluminum intakes because they had less "to spare" then the horses. Therefore, the Al would have bound up the cows' phosphorus and caused a deficiency while the horses were able to overcome the binding.

Few studies have focused on the effects of aluminum in horses. One study found that there were no ill effects of feeding 1,370 mg Al/kg feed, but 4,500 mg/kg decreased plasma phosphorus, and increased plasma and urinary calcium concentration in ponies (Schryver et al., 1984). Roose et al. (2000) found that feeding mature Thoroughbreds either 160 mg Al/kg feed or 930 mg Al/kg feed produced no adverse effects on phosphorus or calcium metabolism. Additionally, there were no changes in nutrient digestibilities such as dry matter, crude protein, fiber and mineral digestibilities.

The SZA increased plasma concentrations of silicon in the SS horses which is a common finding (Nielsen et al., 1993; Lang et al., 2001b,c; Mazella et al., 2005). The SS horses had greater amounts of total Si excreted in both the urine and feces. The major route of silicon excretion was through the urine, as is the case in humans (Reffitt et al., 1999). However, there was no difference in either retention or absorption between the

CO and SS horses. The specific reason for the lack of an increase in Si absorption is unknown. Perhaps there was not a need for the additional silicon, and therefore it was not absorbed. Or perhaps some of the silicon bound to Al in the digestive tract, forming a nonabsorbable compound, and thus the Si was excreted in the feces with the Al. A relationship exists between silicon and aluminum such that the presence of silicon will reduce aluminum absorption and toxicity. Orthosilicic acid reduces aluminum absorption in humans (Edwardson et al., 1993).

The aluminum intake was quite high in the SS horses, but plasma aluminum concentration in the SS horses was unaffected by the increase. The SS horses also tended to retain more aluminum than the CO horses, although there was no difference in digestibility. However, it cannot be assumed, since plasma aluminum levels and apparent digestibility did not increase, that the ingested aluminum from SZA does not adversely affect the horse. Plasma aluminum concentrations are not indicative of tissue storage (Krueger et al., 1985), and increases in plasma aluminum are not always seen following aluminum ingestion (Hussein et al., 1990). Calves supplemented with SZA did not have an increase in plasma aluminum nor was there a difference in absorption, yet 12 of 13 tissues investigated had a 384% average increase in aluminum (Turner, Chapter 5, Dissertation). Further research, such as tissue mineral metabolism effects, should be conducted in the horse before concluding that aluminum from SZA does not adversely affect horses.

Calcium metabolism can also be affected by high dietary intakes of aluminum. In the current study, the addition of SZA increased retention and digestibility of calcium. This effect has been seen in ruminants. Robinson et al. (1984) found an increase in Ca

retention and availability when non-lactating cows were fed an additional 1000 mg/kg Al in their feed for 20 days. Increases in serum Ca were also observed in lactating beef cows receiving 1,730 mg Al/kg feed (Allen et al., 1986). Unlike in the previous lactating cow study (Turner, Chapter 4, Dissertation), there was not an increase in plasma calcium in the horses. The increase in blood Ca may be as a result of homeostatic measures in response to the decreased P. Decreases in Ca concentration in the bone have been reported after high Al supplementation (Allen, 1984). However, when additional phosphorus was added to the diets, the increase in plasma Ca was less dramatic (Hussein et al., 1990). Therefore, in the current study, unlike in the cow study, there was an abundance of dietary phosphorus, thus blood calcium failed to increase. The plasma calcium values were within normal ranges previously reported for the horse (Georgievskii, 1982).

Fecal dry matter was greater in SS horses than CO horses. This has been seen in calves as well (Turner, Chapter 5, Dissertation). There are numerous reports on the addition of zeolites to the diets of ruminants, poultry, and swine decreasing incidence of scours as well as other intestinal disorders (Mumpton, 1984). The exact role the zeolite plays in reducing scours is unknown. One suggestion has been that the effects are due to the zeolite's alkalinity and buffering capacity in the gastrointestinal tract (Mumpton, 1984). Or perhaps the effect is through silicon, as silicon impacts the immune system (Moseley et al., 1988; Seaborn et al., 2002) and thus might reduce scours and other intestinal problems through this route.

In conclusion, the supplementation of 200 g of SZA to the horses did not significantly affect phosphorus metabolism under the current study conditions.

Specifically, the drastic changes seen in ruminants, particularly that of phosphorus blood concentration, did not occur in the horses. However, there was an increase in calcium retention and digestion. Consideration should be given to phosphorus levels in the diet, as the overabundance of P in the horses' diets could have overridden the potential ill effects of the aluminum. Conclusions about negative effects of aluminum on horses cannot be made as plasma aluminum concentration and absorption are not indicative of tissue mineral composition.

APPENDIX B

Although the calf nutritional study (Chapter 5) failed to produce differences in bone mechanical properties, an interesting effect of exercise was found. The calves received no formal exercise, and left their stalls only to be weighed once per week. During the walk to the scale, which was approximately 75 m, the calves were allowed to go at their own pace. Some calves ran while others walked the entire time. There was one calf in particular that was much more active than the other calves, as noted by the researchers. Although most calves were not named, the high activity level of this calf earned him the name "Spitfire" within three days of his birth. It was interesting to note that Spitfire had the greatest peak fracture force, ultimate bending stress and modulus of elasticity of the MC III & IV. The calf with the second highest peak fracture force and ultimate bending stress, and one of the highest modulus of elasticity was a calf that had escaped from his stall for the day and had free access to the dairy farm. He was found at the far end of the farm about 450 m away. The bone dimensions and mechanical properties of the two calves with the highest activity levels were compared to the other calves to identify other possible differences (Table 1B).

Spitfire and Runaway had shorter lateromedial and lateromedial medullary diameters, but their lateral and medial cortical widths were slightly larger, if not the same, as the other calves. Additionally, the medullary areas of Spitfire and Runaway's bones were substantially less than that of the other calves, even though the cortical area was not that different (Runaway's is practically identical). The total area also appears to be less in the two active calves compared to the others. The percent cortical area of total

area was highest in Spitfire and Runaway. Therefore, it appears that exercise gives the calves a more circular than oval medullary cavity and smaller total area with a greater percentage of cortical area to total area. Thus, this yields bones with greater peak fracture force, ultimate bending stress, and modulus of elasticity. Similar data were seen in horses and calves. Hiney et al. (2004a) found that young horses sprinted 82 m/d for 5 d/wk for 56 d had decreased dorsopalmar medullary cavities and increased dorsal and medial cortical widths compared to group-housed and stalled horses. In addition, young calves exercised for a short duration had smaller medullary cavities and a larger percentage of cortical bone area than calves confined to stalls (Hiney et al., 2004b). The changes in bone led to a trend for increased fracture force of the bones. Therefore the changes in bone exhibited by the two very active calves in the present study more than likely caused the higher fracture forces.

The osteocalcin and deoxypyridinoline comparisons were also interesting Table 2B). Spitfire had less of a decrease in osteocalcin compared to the others, particularly during the first 30 d. Therefore, he possibly had greater bone formation than the others. It is also interesting to note that he had a larger decrease in OC during d 30-60 than the rest of the calves. It may be due to the fact that Spitfire was getting larger, and therefore had less space to jump around in his stall and his activity level had to decrease. The two active calves also had substantially greater decreases in DPD than the other less active calves, indicating less bone turnover. The comparisons made between the two active calves and the other calves reinforce the idea that exercise is extremely important to bone development, particularly early in life. It is also intriguing that 60 d of nutritional

supplementation failed to produce results, while bouts of sporadic exercise seemed to affect the bone.

Table 1B. Differences in bone dimensions and mechanical properties between two highly active calves and the other calves.

	Spitfire	Runaway	Others (n = 18)
Diameters (cm)			
Dorsopalmar	1.86	1.91	2.00 (1.77-2.15)
Dorsopalmar medullary	1.10	1.16	1.28 (1.06-1.45)
Lateromedial	2.45	2.56	2.72 (2.45-2.90)
Lateromedial medullary	1.49	1.60	1.83 (1.49-2.07)
Cortical widths (cm)			
Dorsal	0.40	0.39	0.37 (0.32-0.43)
Palmar	0.35	0.39	0.34 (0.29-0.39)
Lateral	0.47	0.49	0.45 (0.37-0.53)
Medial	0.49	0.53	0.46 (0.40-0.54)
Areas (mm2)			
Cortical area	279	307	306 (269-338)
Medullary area	128	140	175 (128-232)
Total area	407	447	481 (404-538)
% cortical of total area	68.5	68.7	63.8 (56.8-68.7)
Mechanical properties			
Peak fracture force (N)	5085	4715	4224 (3630-5085)
Ultimate bending stress (MPa)	145	145	110 (87-145)
Modulus of elasticity (MPa)	0.64	0.55	0.44 (0.30-0.64)

Table 2B. Comparisons of osteocalcin and deoxypyridinoline between two highly active calves and the other calves.

Bone markers (ng/ml)	Spitfire	Runaway	Others (n = 18)
OC d 0	337	527	407 (100-738)
OC d 30	294	412	284 (128-412)
OC d 60	202	312	233 (152-324)
DPD d 0	18.2	20.4	16.1 (11.4-24.6)
DPD d 30	11.0	14.0	11.3 (6.4-16.2)
DPD d 60	10.4	11.9	11.3 (7.4-17.0)
Net changes			
OC from d 0 to 30	-43	-115	-123 (-440-150)
OC from d 30 to 60	-92	-100	-51 (-113-117)
OC from d 0 to 60	-135	-215	-174 (-427-144)
DPD from d 0 to 30	-7.2	-6.4	-4.8 (-13.5-1.8)
DPD from d 30 to 60	-0.6	-2.1	0.0 (-2.1-2.4)
DPD from d 0 to 60	-7.8	-8.5	-4.8 (-14.2-2.8)

APPENDIX C

Milk Replacer Data for Calf Study

Instant Cow's Match® NT Medicated
Manufactured by: Land O'Lakes Animal Milk Product Co., Shoreview, MN

Active drug ingredients

Oxytetracycline	100 g/ton
Neomycin base (from neomycin sulfate)	200 g/ton

Guaranteed analysis

Crude Protein	MIN 28.00%
Crude Fat	MIN 20.00%
Crude Fiber	MAX 0.15%
Calcium	MIN 0.75%
Calcium	MAX 1.25%
Phosphorus	MIN 0.70%
Vitamin A	MIN 10,000 IU/lb
Vitamin D_3	MIN 2,500 IU/lb
Vitamin E	MIN 50 IU/lb

Ingredients

Dried whey, dried why protein concentrate, dried whey product, dried skimmed milk, dried milk protein, animal fat (preserved with ethoxyquin), lecithin, polyethylene glycol (400) monooleate, dicalcium phosphate, calcium carbonate, methionine supplement, l-lysine, vitamin A acetate, vitamin D_3 supplement, vitamin E supplement, thiamine mononitrate, riboflavin, niacin supplement, calcium pentothenate, biotin, ascorbic acid, pyridoxine hydrochloride, folic acid, vitamin B_{12} supplement, choline chloride, roughage products, calcium silicate, manganese sulfate, zinc sulfate, ferrous sulfate, copper sulfate, cobalt sulfate, copper sulfate, cobalt sulfate, ethylenediamine dihydriodide and sodium selenite.

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VITA

Kari Krick Turner was born on March 27th, 1978 in West Palm Beach, FL to Peter and Sara Krick. Upon graduation from Wellington High School in 1996, Kari attended the University of Florida. She obtained her Bachelor of Science degree in Animal Science and a Minor in Agribusiness Sales and Management in 2000. Kari then received a John Lee Pratt Fellowship in Animal Nutrition to attend Virginia Polytechnic Institute and State University for a Master of Science degree with Dr. David Kronfeld. Her research focused on immunoglobulins in endurance horses as affected by exercise, and a possible selective IgA deficiency which had previously not been identified in horses. After completing her M.S. degree in Animal Science in 2002, Kari began her doctoral work at Michigan State University under the direction of Dr. Brian Nielsen. Her research focused on sodium zeolite A and its effects on mineral metabolism and bone/joint physiology in dairy cows, calves and horses. Following completion of her Ph.D. Kari will begin work at the University of Georgia as an assistant professor of equine science.