

Cathepsin K Inhibition In Bone And Bone Marrow In Horses

Dissertation

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By

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## **Abstract**

Cathepsin K (CatK), a cysteine protease, has been implicated in the process of bone resorption and inflammation. Selective inhibitors of CatK are promising therapeutic agents for the treatment of diseases associated with excessive bone loss and osseous inflammation, such as osteoarthritis, rheumatoid arthritis, periodontitis, osteoporosis, and multiple myeloma. Multiple reports have emerged over the last several years demonstrating the effect of different CatK inhibitors on osteo-inflammatory conditions. Therefore, the study of CatK inhibition as a target to prevent bone loss and inflammation, and influence bone marrow osseous progenitor cells, in a large animal model, is the subject of this dissertation. The horse was selected as the large animal model because this species suffers from ailments of adaptive bone remodeling in their sport performance and CatK inhibitors may serve as therapeutics in this species as well as serve as a large animal model for human applications.

In the first phase of this work, we determined an optimal dose and dose interval for a CatK inhibitor (CatKI), VEL-0230, in healthy adult horses. Plasma pharmacokinetic

(PK) and bone resorption biomarker [carboxy-terminal cross-linking telopeptide of type I collagen (CTX-1)] analyses were performed following single and multiple oral dose protocols of a CatKI (VEL-0230) in horses. Weekly administration of VEL-0230, at a dose of 4 mg/ kg body weight, provided effective inhibition of bone resorption in young exercising horses that returned to baseline within 7 days after drug withdrawal even after multiple doses.

In the second phase of this work, we evaluated bone structure and turnover in healthy young exercising horses receiving repeated oral dosing of a CatKI in a randomized, controlled, double-blinded, prospective, sufficiently powered clinical trial. With the objectives of: 1. To determine whether repeated dosing of a CatKI produced a desired inhibition of the bone resorption biomarker CTX-1, 2. To determine the effect of repeated dosing of this CatKI on bone homeostasis as evaluated by quantitative imaging, bone histomorphometric measurements, immuno-staining of bone CatK and the quantitative analysis of bone turnover related gene expression. We found that repetitive dosing for 4 weeks of this CatKI transiently and repetitively inhibited plasma CTX-1 (reflecting inhibition of bone collagen resorption) and increased bone plasma osteocalcin levels (reflecting increased bone formation), however, over the 4 week administration time period, CatKI did not prevent normal adaptive bone remodeling to exercise as both the control and CatK-treated groups demonstrated radiographic evidence of remodeling. Bone morphology, and density did not differ between groups by all intensive measures in these normal animals over this 4 weeks period. We concluded that this CatKI had minimal negative influence on adaptive remodeling in normal young exercising animals, permitting

endogenous mechanical and biologic signals of exercise to control bone density and mineralization.

In the third phase of this work, we compared the effects of *in vivo* CatKI administration for 4 weeks to control vehicle administration for 4 weeks on equine bone marrow-derived stem and progenitor cells. Bone marrow was harvested from the sternum of six horses (3 assigned to receive CatKI and 3 assigned to receive control vehicle) prior the start of the study and from twelve horses 24 hours after the fourth weekly dose of CatKI (n=6) or vehicle (n=6). Bone marrow was cultured in monolayer to select/deselect bone marrow derived-mesenchymal stromal cells (BMD-MSCs) and cell characterization by flow cytometry and differentiation capacities were studied. We hypothesized that neither exercise nor repeated oral administration of VEL-0230, a potent and selective CatKI, would affect the characterization or differentiation capacity of equine bone marrow derived-stem and progenitor cell, specifically chondrogenic, osteogenic or osteoclastogenic tri-lineage differentiation or expression of stem cell markers. Bone marrow cells from both groups were multi-potent and showed strong capacity to differentiate into chondrogenic, osteogenic and osteoclastic pathways with no difference in staining scores between the CatKI and control groups. There was no significant difference in expression of CD90, CD34, CD11b, MHCI, and MHCII molecules from before exercise to after exercise in either the control or CatKI groups. There was no difference in viability or differentiation capacity between control and CatKI groups as determined by 7AAD, Toluidine blue, von Kossa, Alizarin red, and Tartrate-resistant acid phosphatase (TRAP) staining. Cathepsin K

inhibition for 4 weeks did not alter the regenerative ability of bone marrow, specifically for bone formation (osteoblastogenesis) or remodeling (osteoclastogenesis).

In the fourth phase of work, we have studied two inflammatory models in equine bone marrow nucleated cells (BMNCs); the Lipopolysaccharide (LPS) and the unmethylated CpG stimulation with the following objectives: 1. To determine whether CatK inhibition will alter the cytokine secretion by stimulated BMNCs; specifically IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , and 2. To determine the changes in BMNCs surface markers' expression and MHC molecules under CatK inhibition. Equine BMNCs were exposed to VEL-0230 at concentrations of 0, 1, and 10  $\mu$ M in cell culture media with and without LPS (1  $\mu$ g/ml) and unmethylated CpG (5  $\mu$ g/ml). Cathepsin K inhibition promoted BMNC viability and reduced cell apoptosis. Moreover, CatK inhibition significantly decreased cytokine secretion of either naïve or stimulated BMNCs, and decreased their MHC molecules expression. In conclusion, CatK inhibition in horses did affect BMNCs other than mature osteoclasts rendering them hypo-responsive to both TLR4- and TLR9-induced inflammation, predicting a proteolytic activity for CatK within the MyD88 pathway and/or the following proteolytic events required for the cytokines secretion.

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## Publications

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## Field of Study

Major Field: Comparative and Veterinary Medicine.

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# **Chapter 1**

## **Introduction**

Cathepsin K (CatK), a cysteine protease, has been implicated in the process of bone resorption and Inflammation. Selective inhibitors of CatK therefore could be promising therapeutic agents for the treatment of diseases associated with excessive bone loss and inflammation such as osteoarthritis, rheumatoid arthritis, periodontitis, osteoporosis, and multiple myeloma. Multiple reports have emerged over the last several years demonstrating the effect of different CatK inhibitors on osteo-inflammatory conditions. Therefore, illustrating the evidence of CatK inhibition as a target to prevent and treat inflammation and bone loss in equine is the subject of this dissertation.

The most commonly used antiresorptive drugs to treat bone resorption disorders act to inhibit osteoclast-mediated bone resorption. Osteoclasts are multinucleated giant cells that locally attach and demineralize bone, followed by the enzymatic degradation of organic bone matrix. The demineralization is achieved through the



secretion of acid onto the bone surface where the enzymatic action, mainly the cysteine protease CatK, can degrade the bone organic matrix (mainly type 1 collagen) (1). CatK is the most abundantly expressed cysteine protease in osteoclasts and exhibits collagenolytic activity only under acidic conditions (2).

Several antiresorptive drugs are available for the treatment of bone resorption disorder such as osteoporosis and osteoarthritis, but all have limitations. Hormone replacement therapy (HRT) is a major treatment for post-menopausal osteoporosis. However, several reports revealed increased incidence rates of breast cancer, heart attacks, stroke and vascular pathology (3), necessitating the Food and Drug Administration (FDA) to release safety warnings against the use of HRT (4). Bisphosphonates, another commonly used class of antiresorptive drugs, accumulate on the bone surface, which necessitated the development of new preparations to be administered only once a year. The bisphosphonates act by inducing apoptosis in osteoclasts and, thus, the reduction of osteoclast-mediated bone resorption and have also been used in cancer patients suffering of bone metastases with osteolytic activity (5-7). A number of severe side effects have been associated with the long-term use of bisphosphonates; the most severe one is jaw osteonecrosis. An estimate of 1-7% of cancer patients on bisphosphonates has been reported to experience debilitating jaw necrosis (8). In addition, studies indicated that bisphosphonates at high doses interfere with angiogenesis in bone leading to multiple tissue necrosis (9). Other common therapies include selective estrogen receptor modulators (raloxifene), calcitonin and parathyroid hormone, RANKL antibodies such as (Denosumab), chloride channel inhibitors, c-src kinase inhibitors, and several CatK

inhibitors are being also investigated in attempt to provide safe efficient alternative anti-resorptive therapies (10-14).

Inhibition of CatK is an attractive new target, since the discovery of the effect of its genetic deficiency in humans, pycnodysostosis, where it was found to be restricted to the skeleton causing failure of resorption (15). Several CatK inhibitors have been evaluated as potential therapeutics to prevent bone loss. They have been found to induce potent inhibition of resorption (16-19). Although CatK inhibitors have been developed as antiresorptive agents, there is growing evidence of an anabolic activity for cathepsin K inhibitors (20).

Human and animal experiments have implicated pro-inflammatory cytokines; such as interleukin (IL)-1, tumor necrosis factor- $\alpha$ , and interleukin-6, as primary mediators of accelerated bone loss (21-23). Increased production of pro-inflammatory cytokines has been associated with increased osteoclastic activity in a number of disease states including rheumatoid arthritis (24), periodontitis (25), and multiple myeloma (26) as well as estrogen withdrawal in post-menopausal women (27). Multiple studies have also shown that other pro-inflammatory cytokines are capable of stimulating osteoclastic bone resorption such as IL-11, IL-15, and IL-17 (28-30). Selective inhibitors of cathepsin K therefore could be promising therapeutic agents for the treatment of such diseases characterized by excessive bone loss and inflammation.

Cathepsin K has shown an unexpected role in innate immunity. Inhibition of CatK resulted in impaired secretion of pro-inflammatory cytokines by Toll-like receptor - 9 (TLR9), which is an intracellular TLR specifically recognizing unmethylated CPG motifs of microbes that have been phagocytosed by cells (31). Decreased secretion of pro-inflammatory cytokines by dendritic cells in turn was shown to attenuate the induction of T-helper 17 cells, an osteoclastogenic T cell subset known to play an important role in the inflammatory bone resorption (32). Moreover, CatK also plays an important role in autoimmune inflammatory disorders such as encephalomyelitis and chronic colitis (31 & 33). In summary these studies support an anti-inflammatory effect of CatK inhibition in bone and supports its potential use in osseous inflammatory disorders such as stress fractures, arthritis, bone metastases.

In the equine athlete, the accelerated physical activity during training in young growing horses is mainly responsible for marked changes in cortical and trabecular bone. The effect of increased loads on bones in exercising horses is well documented and has shown to increase cortical bone thickness; Treadmill-exercised horses have shown an increased cross sectional area, mineral density and elastic modulus of cortical bone of the third metacarpal bone (MCIII) (34). Other studies have also emphasized the relationship between exercise and trabecular bone structure, especially in the distal condyles of the MCIII, a common site of fracture in the Thoroughbred racehorse (35-37). The prolonged, heavy intensity exercise has also been found to increase infection risk in human and animals and aggravate the inflammatory response (38). However, the precise nexus between physical activity,

inflammation, and immunity has not been fully described.

Stress-related injuries in racehorses are common and occur as a result of repetitive mechanical stress, which causes a severe periosteal inflammatory response. This increases the release of pro-inflammatory cytokines to abnormal levels (39). This increase in the pro-inflammatory cytokine secretions results in an increase in osteoclast number and activity. The result of this pathological process is an osteo-inflammatory condition in which the bone is weakened due to increased resorption. One very common example of stress-related injuries in equine is the dorsal metacarpal disease and the associated Bucked shin syndrome. Bucked shins involves both abnormal bone turnover and an enhanced inflammatory reaction that together result in accelerated bone loss. Such osteo-inflammatory responses are salient features of many equine bone diseases including, but not limited to, periostitis (40).

In the following chapters, we investigated the anti-resorptive and anti-inflammatory properties of a CatK inhibitor, VEL-0230, in several *in vivo* and *in vitro* equine experimental studies. Previous efficacy studies in humans, non-human primates, and rats, demonstrated that VEL-0230 eliminated inflammation and restored bone homeostasis. VEL-0230 is effective in animal models including rat adjuvant-induced osteo-inflammatory disease where it prevented bone erosion and inflammation (31 & FreeStride Therapeutics, personal communication). In a similar and more robust collagen-induced model in rats, VEL-0230 decreased disease burden, bone resorption, C-reactive protein levels (a biomarker of inflammation)

and improved bone health. Moreover, it inhibited bone loss and prevented bone resorption as demonstrated in other *in vivo* models, including the drug-induced estrogen depletion osteopenia model, the surgical ovariectomy model in monkeys, as well as the low-calcium diet model and surgical ovariectomy model in rats (FreeStride Therapeutics, personal communication). From these studies, we predicted that VEL-0230 would affect bone turnover in horses and could be a potential therapeutic in equine osteo-inflammatory conditions.

## **Chapter 2**

# **Pharmacokinetics And Bone Resorption Evaluation Of A Novel Cathepsin K Inhibitor (VEL-0230) In Healthy Adult Horses\*.**

\*This research has previously been published;

Hussein, H., Ishihara, A., Menendez, M., & Bertone, A. (2014). Pharmacokinetics and bone resorption evaluation of a novel Cathepsin K inhibitor (VEL-0230) in healthy adult horses. *Journal of veterinary pharmacology and therapeutics*, 37(6), 556-564.

### **SUMMARY:**

Plasma pharmacokinetic (PK) and bone resorption biomarker [carboxy-terminal cross-linking telopeptide of type I collagen (CTX-1)] analyses were performed following single and multiple oral dose protocols of a Cathepsin K inhibitor (VEL-0230) in horses. Outcomes included plasma and urine drug and CTX-1 concentrations. In the dose-range study, 2, 4, and 8 mg/kg body weight (b.w.)

doses were administered in a Latin-square design to three mares and evaluated for 1 week. Based on the PK characteristics of VEL-0230, 4mg/kg b.w. was selected for the dose interval study in which 3.25 days (d) and 7d dose-intervals were evaluated over three administrations using four exercising horses in a Latin-square design. The 3.25d and 7d dose intervals provided a rapid inhibition of bone resorption based on plasma CTX-1. CTX-1 inhibition prior to next dose administration was not different from baseline in the 3.25d and 7d protocols, and for the first 3 days but the sustained CTX-1 inhibition in the 7d protocol along with the cost and logistic benefits for weekly administration made the 7d protocol preferable. Weekly administration of VEL-0230 may provide effective inhibition of bone resorption in young exercising horses that returns to baseline within 7 days after drug withdrawal even after multiple doses.

## **INTRODUCTION**

The drug, VEL-0230, alternatively named NC-2300, is a highly specific, irreversible inhibitor of bone Cathepsin K. The drug suppresses osteoclast-mediated bone resorption *in vitro* and *in vivo* in rat models (1). The drug binds to intracellular and extracellular Cathepsin K and interferes with the Cathepsin K-mediated bone resorption by osteoclasts. Cathepsin K is highly expressed in osteoclasts and is involved in degradation of bone matrices, mainly type I collagen (2). For more than 15 years, Cathepsin K remained a potential therapeutic target for the treatment of bone diseases in human and several animal disease models in which osteoclast

activity is increased, such as osteoporosis and autoimmune arthritis (3-6). Several studies have also investigated the effect on both Cathepsin K as well as its inhibition on bone resorption in horses. For instance, Gray *et al.* (6) investigated the localization, activity, and effect of inhibition of Cathepsin K within the equine osteoclast. Years afterward, Vinardell *et al.* (7) suggested that Cathepsin K also plays a role in the pathogenesis of equine osteoarthritis by degrading collagen type II within the articular cartilage.

In horses, the effect of some anti-resorptive drugs, such as a cysteine proteinase inhibitor (E-64) and the bisphosphonate pamidronate, on inhibiting osteoclast-mediated bone resorption *in vitro* has been studied (6 & 8). Moreover, multiple reports exist on using anti-resorptive drugs in equine musculoskeletal disorders, which are associated with increased bone resorption activities. Examples included the use of tiludronate as a therapeutic agent for navicular disease (9), or for the treatment of lesions of the thoracolumbar vertebral column (10), and zoledronate for the treatment of bone fragility disorders (11). The pharmacological effect of some anti-resorptive agents has been studied in healthy adult horses, such as gallium nitrate (12), tiludronate (13), and zoledronate (14).

Unlike members of bisphosphonate anti-resorptive drugs (e.g. tiludronate and zoledronate) which led to over-suppression of bone turnover (15 & 16), VEL-0230 was found to induce a rapid, short-acting inhibitory effect of bone resorption as proven by efficacy studies in humans, non-human primates, dogs and rats (1 &



unpublished data). Studies reported by Asagiri *et al.* demonstrated that Cathepsin K sits at the nexus of both musculoskeletal system (bone) and immune system. It mediates both bone resorption as well as the production of pro-inflammatory cytokines mediated by Toll-like receptor 9 signaling of dendritic cells. These findings suggest that VEL-0230, with its dual anti-resorptive and anti-inflammatory properties, could target osteo-inflammatory disorders in a specific manner (1).

The reduction in biochemical markers of bone resorption has been correlated with an increase in bone mineral density and was frequently used to predict the effect of several anti-resorptive drugs (17-19). Serum and plasma carboxy-terminal cross-linking telopeptide of type I collagen (CTX-1) was proven to be a reliable biomarker of bone resorption in horses (20 & 21). Furthermore, CTX-1 was used to evaluate the anti-resorptive effect of tiludronate (22) and zoledronate (14) in horses.

We aimed to determine an effective oral dose and dose interval of VEL-0230 in horses. Two experiments were conducted with the objectives to select an optimal dose, effective dose interval, and to investigate our research hypothesis. Our hypothesis was that VEL-0230 will produce a dose-dependent inhibition of a pre-validated bone resorption biomarker in horses, CTX-1. The outcomes were assessed by evaluating plasma and urine VEL-0230 concentration, plasma CTX-1 concentration and percent inhibition, and applying pharmacokinetic (PK) analysis on the acquired data.

## **MATERIALS AND METHODS**

### ***Study Design:***

All experimental protocols were pre-approved by the Institutional Animal Care and Use Committee (IACUC) of the Ohio State University.

#### **A. Dose range study**

This study was conducted to determine an optimal dose of VEL-0230 in horses. Three Thoroughbred mares (3 years of age), healthy on physical exam, were used in this study. Before the drug (VEL-0230) administration, the horses were acclimated for 2 weeks in the stalls at the Veterinary Medical Center, The Ohio State University. Horses were dosed orally on day 0 with one of three VEL-0230 doses [2, 4, or 8 mg/kg body weight (b.w.)] each week based on a Latin-square design as shown in figure (Fig.) 1. The dose range was chosen based on the reported effective doses in other species. The study was conducted for 3 consecutive weeks and samples were collected according to the schedule shown in Table. 1. A baseline blood sample (0 hour) was collected prior to the drug administration, then at 15, 45, and 60 minutes, 2, 4, 6, 8, 12, 24 hours (h), and 2 days (d) and 5d after drug administration. Urine samples were collected via urinary catheter before drug administration (0 hour), 6h, 9h, 24h, 2d and 6d after drug administration. Data from

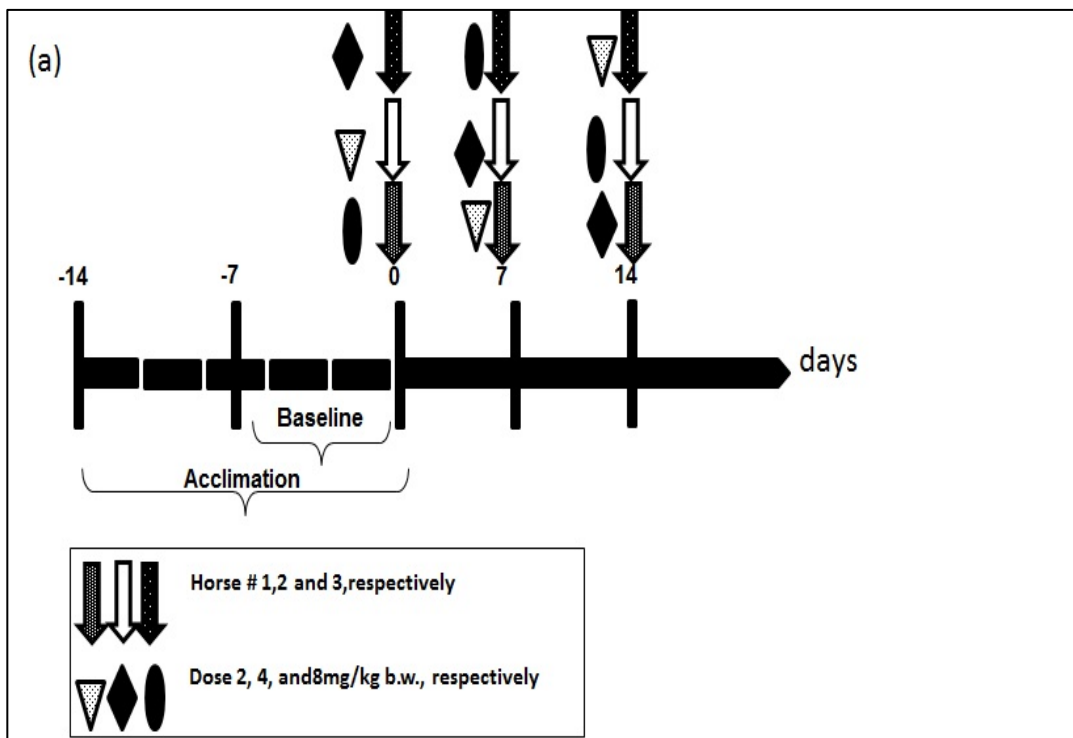
this dose range study was used to select one dose (4 mg/kg b.w.) for further investigation in the dose interval study.

#### B. Dose interval study

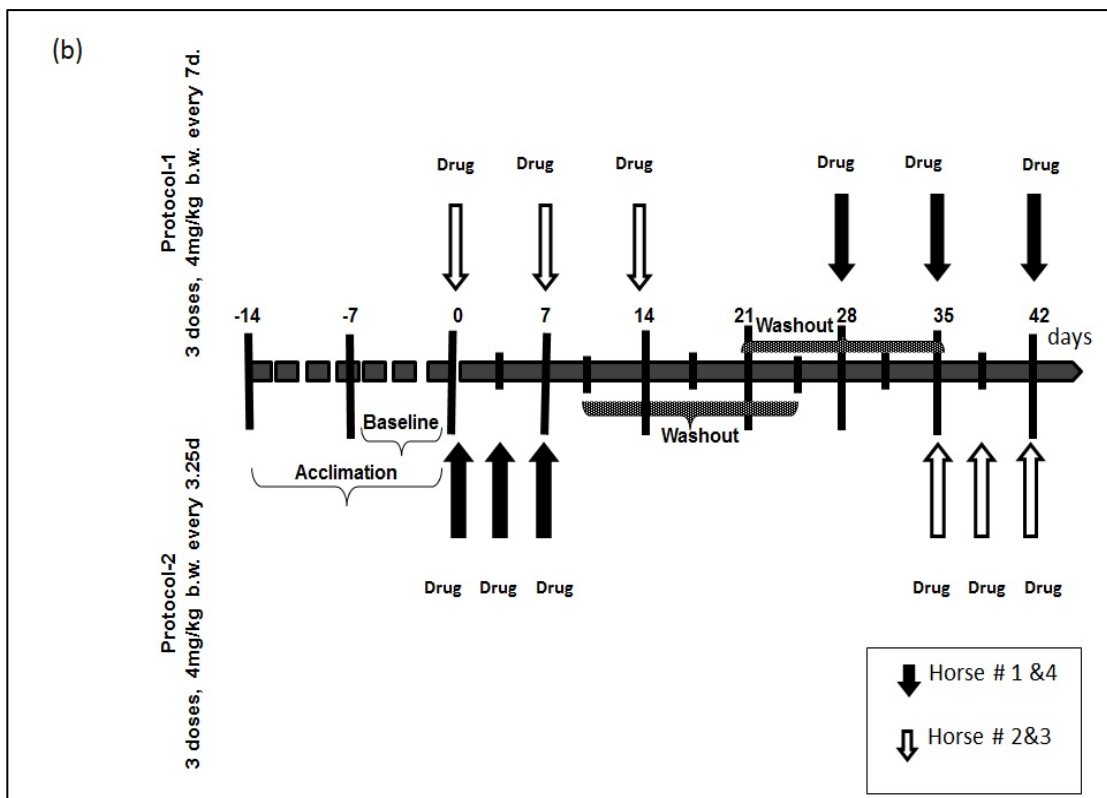
This study was conducted to determine an effective dose interval of VEL-0230 in horses. Four mature horses, three racing Thoroughbreds and one racing Standardbred, two males and two females, aged 2-5 years were used in this study. Horses were randomized at the start of the study to receive three dose administrations on one of two dose intervals; either an every 7 days dose interval (7d; Protocol 1), or on an every 3 days dose interval (3.25d; Protocol 2). After a two weeks washout period, each group was switched to the other protocol in a Latin square manner. (Fig.2). The horses had to be normal on physical examination to be included in the study. Horses were acclimated to the treadmill at the Veterinary Medical Center of The Ohio State University during day -14 to day -7, and exercised twice weekly to mimic a training schedule. The training schedule included 5 minutes of warm-up at the walk (5 mph), 5 minutes at the trot (10 mph), 5 minutes at the gallop (20 mph), and finally 5 minutes cool-down at the walk (5 mph). All baseline data were collected between day -7 and day 0. Horses were dosed on day 0 according to the assigned protocol, and were maintained in exercise for the duration of the study (6 weeks) in order to mimic sport horse training.

Sampling schedule for the dose interval study was summarized in Table 1. For Protocol 1, horses had baseline blood and urine sample obtained on day 0, received a drug dose, and had blood obtained at 1h, 2h, 4h, 8h, 12h, 24h, 2d, 3d, 4d, 5d, 6d

followed by a second and third dose 7 days apart. Urine samples were obtained at d3 and d6 with similar sampling after the second and third doses. For Protocol 2, horses had blood and urine sampled at day 0 (0h), received a drug dose, had blood sampled at 1h, 2h, 4h, 8h, 12h, 24h, 2d and 3d followed by a second dose and third dose 3.25 days apart, had urine sampled at d1 and d3, and the same sampling protocol after the second and third doses. Urine was also collected during the washout period.



**Figure 1:** The experimental design of the dose range study.



**Figure 2:** The experimental design of the dose interval study.

<b>Study</b>	<b>Pre-treatment sampling</b>	<b>During treatment sampling</b>	<b>Post-treatment sampling</b>
<b>Dose range study</b>	Baseline blood and urine samples	Blood: 15, 45, and 60 minutes, 2h, 4h, 6h, 8h, 12h, 24h, 2d, and 5d after drug administration and prior to next dose. Urine: 6h, 9h, 24h, 2d and 6d after drug administration and prior to next dose	NA
<b>Dose interval study Protocol 1</b>	Baseline blood and urine samples	Blood: 1h, 2h, 4h, 8h, 12h, 24h, 2d, 3d, 4d, 5d, and 6d after each drug administration. Urine: 3d & 6d.	Blood: 21d post first dose administration Urine: In the washout period, 13d, and 20d post last treatment
<b>Dose interval study Protocol 2</b>	Baseline blood and urine samples	Blood: 1h, 2h, 4h, 8h, 12h, 24h, 2d, and 3d after each drug administration. Urine: 1d & 3d.	Blood : 10d post first dose administration Urine: In the washout period, 3d, and 20d, and 20d post last treatment

**Table 1:** Sampling schedule (blood and urine) for dose range and dose interval studies.

***Data and samples collection:***

Blood samples for measuring the drug and bone resorption biomarker, CTX-1, in plasma were obtained by venipuncture of the jugular vein into sterile 5 ml Vacutainer glass tubes containing heparin as an anticoagulant. Plasma was then separated by centrifugation (1500 g at room temperature for 15 minutes) and stored at -20°C within 1 hour of sampling. For the urine collection, a temporary Foley catheter was introduced aseptically into the horse's urinary bladder with a sterile urine collection bag attached to the end of the catheter. One urine sample was collected each time according to the sampling protocol. The urine bags were gently shaken before taking representative portions to be stored at -20°C until the analysis of VEL-0230 concentrations in urine. Horses were physically examined and body weight recorded weekly. Automated complete blood count (CBC) data was obtained pre and post study <sup>a</sup>.

***VEL-0230 Administration:***

Horses were held off feed for 8 hours prior to each dose administrations. The horse's mouth was washed out with water immediately before drug administration. The drug dose was administered and the horse's head was held from under the chin in an upright position for 15 seconds to ensure the swallowing of the whole dose. For the dose-range study, the drug was in a water-soluble solution and shipped by



the manufacturing company ready to administer in syringes. The assigned drug doses were administered orally at the same time for the three mares. Based on the dose-range study, a dose of (4 mg/kg b.w.) was selected for use in the dose interval study. For the dose interval study, the drug and materials were shipped packaged and contained vials of powdered drug and vials of gel vehicle packed separately and stored at room temperature. The drug was formulated by mixing these at the stable-side. Drug mixing was performed as per manufacturer's instructions. In brief, the vehicle was mixed with the powdered drug and the resultant gel drawn up into a 60 ml plastic dose syringe. The syringe was refrigerated for at least one hour before administering the drug using a pre-calculated volume based on body weight to achieve the selected dose. The drug was administered orally according to the study design schedule.

***VEL-0230 assay:***

Plasma and urine samples were packed frozen on dry ice and shipped to Pharmoptima Laboratories <sup>b</sup>, Portage, MI. Plasma and urine concentration of VEL-0230 were measured on the 2 and 4 mg/kg doses, and the 3.25d and 7d dose interval protocols by a GMP-validated HPLC procedure. The lower limit of quantification (LLOQ) was 1 ng/ml. The samples for the 8 mg/kg doses were not analyzed and were stored frozen at -20C.

***CTX-1 assay:***

The biologic effect of VEL-0230 was studied using plasma CTX-1 concentration, percent CTX-1 inhibition from baseline, and applying pharmacokinetic parameters into the CTX-1 inhibition data. The plasma samples were thawed to room temperature and CTX-1 was quantified using Serum Crosslaps<sup>®</sup> ELISA kit <sup>c</sup>. The kit is an enzyme immunologic assay for the quantification of the degradation product of the C-terminal telopeptides of type I collagen in human serum and plasma. The kit has been validated for assaying CTX-1 in equine plasma in previous equine publications (20 & 23). All samples were assayed in duplicate. Plasma CTX-1 concentrations (ng/ml) were calculated for each time point. Results were expressed as raw concentration (ng/ml), and percent inhibition from baseline CTX-1 value (CTX-1 inhibition).

***PK and CTX-1 inhibition analyses:***

All data were analyzed using SAS<sup>®</sup> for Windows <sup>d</sup>. The VEL-0230 data were modeled in a non-compartmental model, and VEL-0230 plasma concentrations versus time plots were generated for PK analysis. Basic pharmacokinetic parameters were calculated from standard equations within the SAS software for maximum plasma drug concentration ( $C_{max}$ ), time to reach maximum concentration ( $T_{max}$ ), terminal half-life (terminal  $t_{1/2}$ ), and elimination rate constant ( $K_e$ ). The area

under the plasma concentration-time curve from time 0 to the last sampling time (AUC 0-t), and the area under the plasma concentration-time curve from time 0 to infinity AUC (0-∞) were also calculated by the linear-log trapezoidal rule method. Similar analyses were performed for the plasma CTX-1 inhibition values, as the maximum inhibition value ( $C_{imax}$ ), time to reach the maximum inhibition ( $T_{imax}$ ), and the half-life of inhibition recovery ( $t_{i1/2}$ ) were calculated for the CTX-1 inhibition of each examined dose in the dose range study.

***Statistical analysis:***

The statistical analyses were run using SAS software<sup>d</sup>. Descriptive statistics were generated for all the data of CBC, CTX-1 values, VEL-0230 concentrations, and PK parameters of both VEL-0230 and CTX-1 inhibition. Plasma VEL-0230 concentrations were analyzed for the repetitive dose administrations in the 3.25 d and 7 d protocols by analysis of variance for repeated measures and were not statistically different among the first, second or third dose within the dose interval protocols. Therefore, the three dose administrations within each dose interval protocol were averaged and graphed as mean plasma VEL-0230 for a 24 hours period. PK parameters were compared between two doses (2 and 4 mg/kg) and between two dose interval protocols (3.25d and 7d) by two- group comparison with t-tests for normally distributed data or Mann-Whitney U rank test for skewed data. The normal distribution within the previous variables was investigated using Shapiro-Wilk test and the significance was determined when  $P < 0.05$ .

The effect of dose (2 and 4 mg/kg b.w.) or dose interval (3.25 d or 7d) and time on VEL-0230 concentrations and drug PK data were determined by two-way repeated measures ANOVA using the SAS GLM. Selected comparisons using LSD post-test were performed to compare relevant time points. Similarly, the effect of dose (2, 4, and 8 mg/kg b.w.) or dose interval (3.25 d or 7d) and time on CTX-1 inhibition was also determined. Spearman's correlation coefficient analysis was applied to investigate the correlation between the dose (2, 4, and 8 mg/kg b.w.) and the plasma CTX-1 inhibition. CTX-1 inhibition analysis data for the dose-range study was compared among the 4 and 8 mg/kg doses using Student's t-test.

## **RESULTS**

All of the horses completed the studies, all samples were obtained, and all horses maintained normal health status for the duration of the study based on physical examination. The VEL-0230 was easy to administer, appeared palatable, with minimal to no loss of drug during oral administration. None of the horses showed adverse effects after the drug administration at any time point. The CBC data were within reference ranges <sup>a</sup> and showed no significant difference between pre and post-study values for either the dose range study (baseline and day 21) or the dose interval study (baseline and day 42). Urine concentrations for VEL-0230 were less than the LLOQ, which was 1 ng/ml, for all the time points except at the 1d sampling time point in the 3.25 protocol.

#### A. Dose-range study

VEL-0230 was detected within 15 minutes in plasma samples of both doses (2 and 4 mg/kg b.w.). The plasma VEL-0230 values peaked within a mean of 45 minutes ( $T_{max}$ ), at a concentration ( $C_{max}$ ) of mean  $\pm$  SD  $74.5 \pm 16.7$  and  $96.2 \pm 36.4$  ng/ml for the 2 mg and 4 mg doses, respectively. VEL-0230 values were below the limit of detection at the 24h time point in both examined doses. As expected, the (4 mg/kg b.w.) dose provided greater plasma VEL-0230 values than the values of the (2 mg/kg), however, this difference between the two examined doses across all time points was not statistically different ( $P > 0.05$ ). The non-compartmental PK parameters are shown for each individual horse for the two selected doses in Table 2. None of the PK parameters ( $C_{max}$ ,  $T_{max}$ , terminal  $t_{1/2}$ ,  $K_e$ , and  $AUC_{0-\infty}$ ) was statistically different between the two doses.

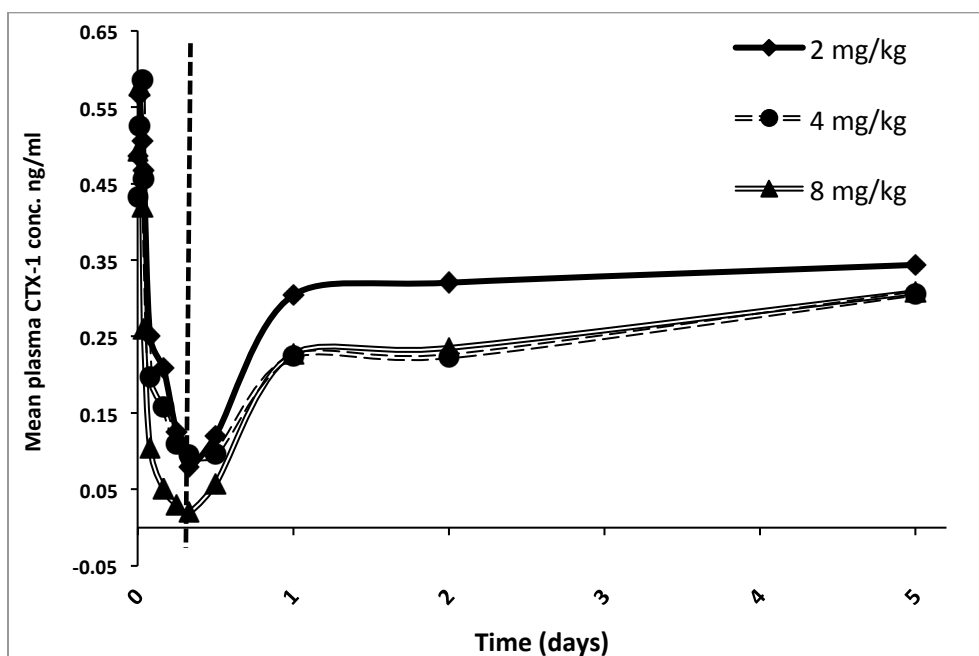
Horse#	C <sub>max</sub> (ng/mL)	T <sub>max</sub> (hr)	K <sub>e</sub> (1/hr)- Elimination rate constant	t <sub>1/2</sub> (hr) – Terminal Half-life	AUC(0-t) (ng•hr/mL)	AUC(t-∞) (ng•hr/m L)	AUC( 0-∞) (ng• hr/m L)
<b>Dose: 2mg/kg b.w.</b>							
1	68.2	0.75	1.211231	0.57	148	1	150
2	116	1.0	0.810329	0.66	254	3	258
3	61.9	0.75	0.54554	1.27	98	5	103
<b>Mean</b>	<b>82.0</b>	<b>0.83</b>	<b>1.010780</b>	<b>0.83</b>	<b>167</b>	<b>3.0</b>	<b>170</b>
<b>SD</b>	<b>29.6</b>	<b>0.14</b>	<b>0.283481</b>	<b>0.38</b>	<b>79.7</b>	<b>2.0</b>	<b>79.5</b>
<b>Dose: 4mg/kg b.w.</b>							
1	101	0.75	0.652995	1.06	196	8	203
2	67.8	1	0.93972	0.74	229	5	235
3	146	1.0	0.218836	3.17	295	40	335
<b>Mean</b>	<b>105</b>	<b>0.92</b>	<b>0.603850</b>	<b>1.66</b>	<b>240</b>	<b>17.7</b>	<b>258</b>
<b>SD</b>	<b>39.2</b>	<b>0.14</b>	<b>0.362946</b>	<b>1.32</b>	<b>50.4</b>	<b>19.4</b>	<b>68.9</b>

**Table 2:** Plasma pharmacokinetic parameters of VEL-0230 following oral administration of two doses (2 and 4 mg/kg b.w.) in 3 horses.

After oral administration of VEL-0230, plasma CTX-1 concentrations fell precipitously ( $p < 0.05$ ) within 1 hour, reaching a nadir after 8 hours and then began to increase (Fig. 3). Equivalent reductions were seen at the 2 and 4 mg/kg doses while the 8 mg/kg dose showed somewhat lower values at few time points. However, no significant differences were seen between these doses.

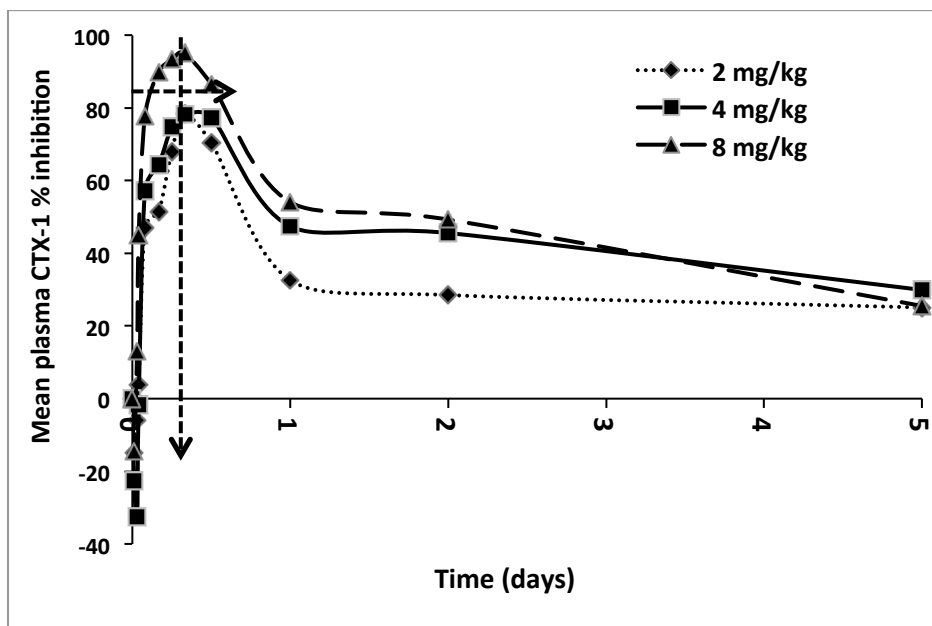
Inhibition of plasma CTX-1 occurred within 1 hour of drug administration and peaked at approximately 80% within 8 hours for all doses (Fig. 4). The inhibition persisted for up to 5d in all dose cohorts. For the 4 and 8 mg/kg doses, the CTX-1 inhibition remained at 40-50% until day 5 when they fell somewhat with the mean CTX-1 inhibitions being  $25.1 \pm 11.0$ ,  $29.9 \pm 22.7$ , and  $25.5 \pm 15.1$  for the 2, 4, and 8 mg/kg doses, respectively. There was no significant difference between the three doses (2, 4, and 8 mg/kg b.w.) in the plasma CTX-1 inhibition values at different time points. Of note, a significant difference was detected in the  $C_{i\max}$  between the 4 and 8 mg/kg doses (Table. 3) with the latter being higher. Correlation coefficient analysis revealed a strong positive correlation ( $r^2 = 0.74$ ,  $P = 0.023$ ) between VEL-0230 dose amount and plasma CTX-1 inhibition.

A comparison of plasma VEL-0230 levels and the inhibition of CTX-1 showed that the drug was rapidly taken up and cleared but had a prolonged inhibitory effect on CTX-1 levels (Fig. 5). At 2 and 4 mg/kg, VEL-0230 peaked at roughly 1 hour and was essentially absent from the plasma within 6–8 hours. In contrast, CTX-1 inhibition peaked at 3 to 8 hours and gradually dropped over the ensuing 5 days.



**Figure 3:** Mean plasma CTX-1 concentrations after oral administration of VEL-0230 at three doses (2, 4, and 8 mg/kg b.w.) demonstrating maximum reduction of CTX-1 (vertical dashed line -----) at an average of 8 hours and sustained reduction for 5 days similar to the CTX-1 inhibition data.

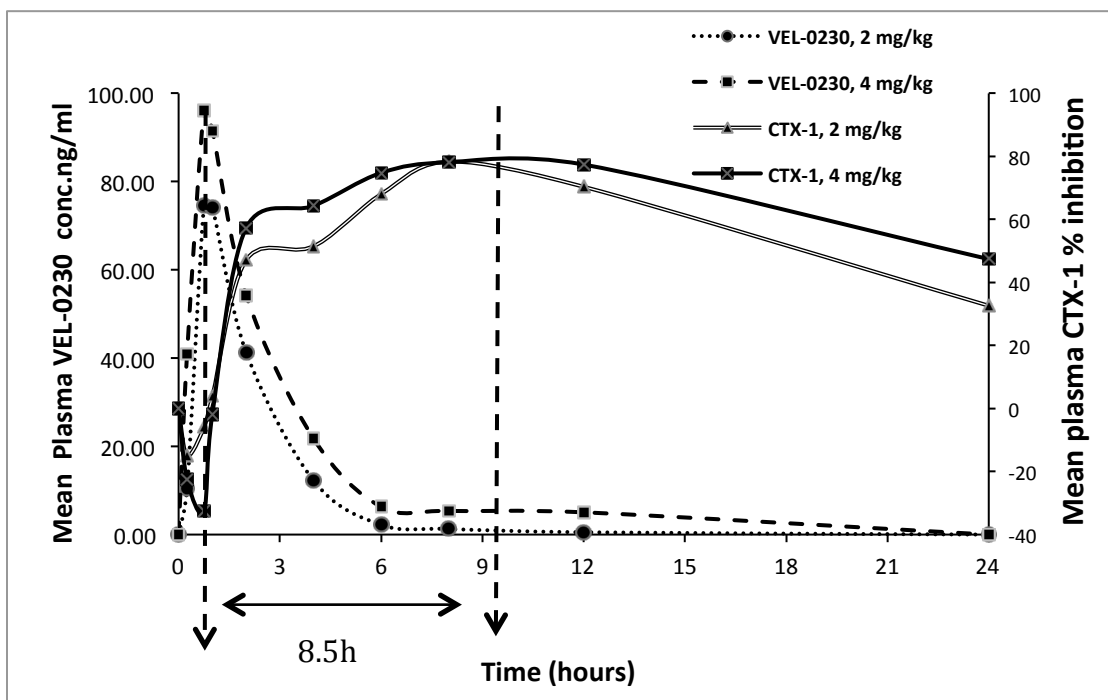




**Figure 4:** Mean plasma CTX-1 inhibition in all doses of the dose-range study. Maximum CTX-1 inhibition ( $C_{\text{imax}}$ ) was an average of 85.3 % for the 3 doses (horizontal dashed line -----), detected at an average ( $T_{\text{imax}}$ ) of 8.7 hours ((vertical dashed line -----), and sustained at an average inhibition of 27.0 % at 5 days post VEL-0230 oral dose administration.

Dose Horse#	2mg/kg b.w.			4mg/kg b.w.			8mg/kg b.w.		
	C <sub>imax</sub> %	T <sub>imax</sub> h	t <sub>i1/2</sub> h	C <sub>imax</sub> %	T <sub>imax</sub> h	t <sub>i1/2</sub> h	C <sub>imax</sub> %	T <sub>imax</sub> h	t <sub>i1/2</sub> h
1	59.48	8	7.92	83.98	8	26.27	95.77	6	37.6
2	93.61	8	28.1	71.97	12	55.95	98.21	8	59.3 2
3	83.79	12	46.2	86.58	8	80.81	94.21	8	34.4 4
Mean	<b>78.96</b>	<b>9.33</b>	<b>27.41</b>	<b>80.84</b>	<b>9.33</b>	<b>54.34</b>	<b>*96.06</b>	<b>7.33</b>	<b>43.7 9</b>
SD	17.57	2.31	19.15	7.79	2.31	27.31	2.02	1.15	13.5 4

**Table 3:** Plasma CTX-1 inhibition parameters (C<sub>imax</sub>, T<sub>imax</sub>, and t<sub>i1/2</sub>) or oral doses of VEL-0230 at 2, 4, and 8mg/kg b.w.



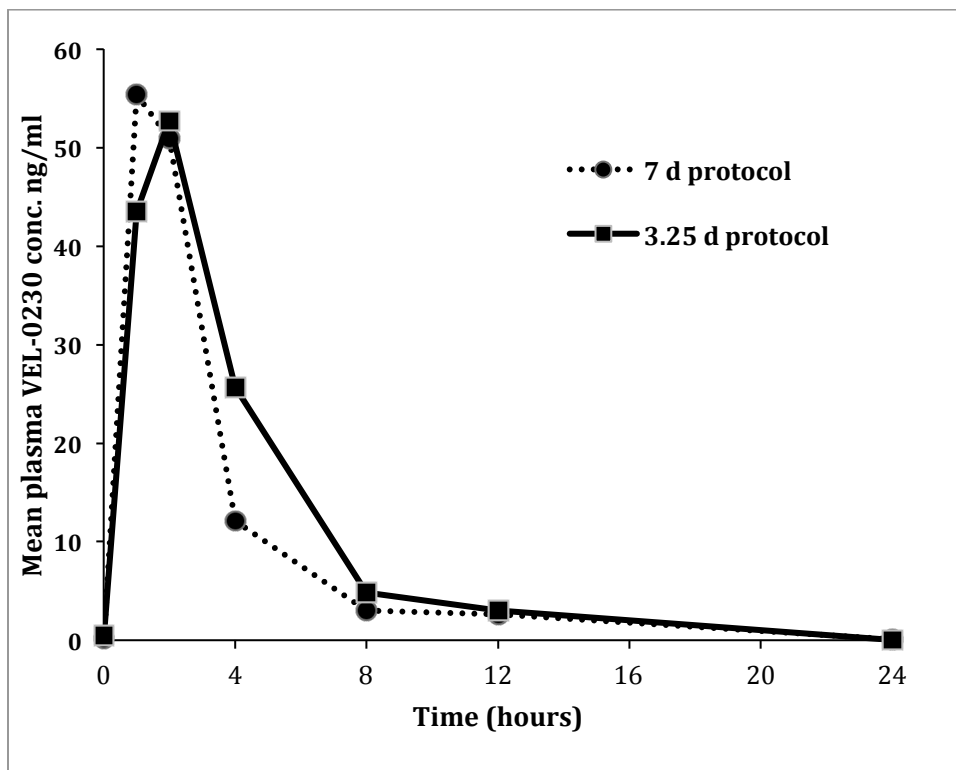
**Figure 5:** Composite graph for mean plasma VEL-0230 concentrations and CTX-1 inhibition of the 2 and 4 mg/kg doses for up to 24 h period. Peak plasma VEL-0230 (left vertical dashed line) was an average of 93.5 ng/ml and occurred 8.5 h before peak plasma CTX-1 inhibition (right vertical dashed line).

## B. Dose interval study

Analysis of VEL-0230 concentrations in the 3.25d and 7d protocols demonstrated a similar pattern to those seen in the dose range study (Fig. 6). VEL-0230 was rapidly detected in the plasma samples. Peak plasma concentrations were seen at the 2h time point, except for the samples of the first dose in the 7d protocol in which the peak plasma drug was detected within 1.5 h after drug administration. The drug was rapidly cleared from plasma and all values were below the LLOQ at the 24h time point. No statistical differences were detected between the two dose interval protocols in the drug plasma concentrations. Table 4 shows the PK parameters, which were calculated based on a non-compartmental model for each dose within each protocol. None of the PK parameters ( $C_{max}$ ,  $T_{max}$ ,  $t_{1/2}$ , and  $AUC_{0-\infty}$ ) was statistically different between the two dose interval protocols.

Mean plasma CTX-1 concentrations over three successive dose administrations for the 7d protocol and the 3.25d protocol are shown in Fig. 7. The data exhibited a rapid decrease in plasma CTX-1 concentrations within 1 hour of administration in all doses for both the protocols (3.25d and 7d). Plasma CTX-1 concentrations at baseline (time 0 prior to first dose), washout plasma samples, and plasma samples taken prior the protocol switch were not statistically different. Peak CTX-1 inhibition was detected at the 4h time point (mean  $\pm$  SEM;  $71.0 \pm 2.95$  in the 7d protocol, and  $70.55 \pm 0.79$  in the 3.25d protocol) (Fig. 8). Although a greater CTX-1

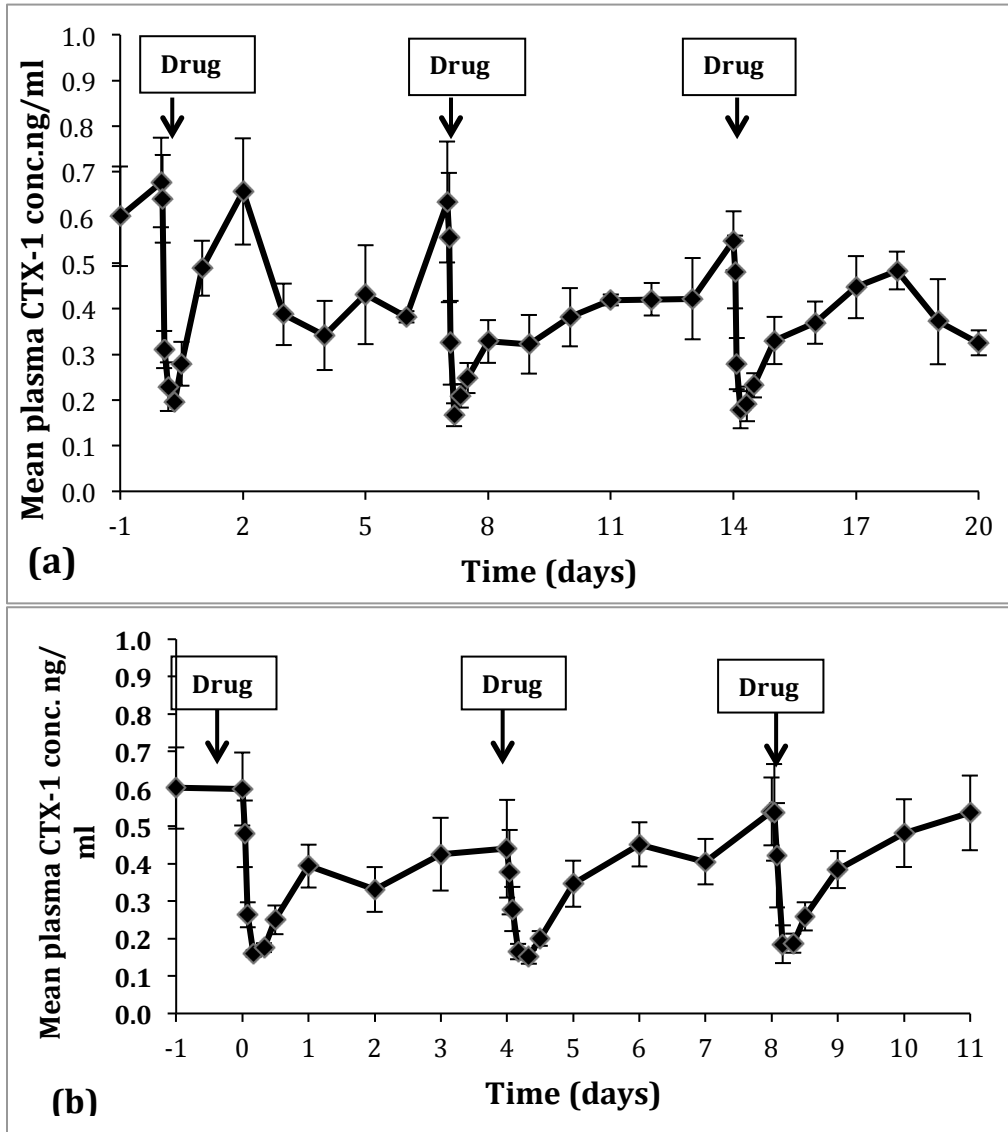
inhibition value was observed at the 3d time point in the 7d protocol, there was no significant difference between the two dose protocols. The inhibition in the 7d protocol remained at mean  $\pm$  SD 42.5%  $\pm$  3.5% at 6d. There was no significant difference in CTX-1 inhibition at day 7 prior to next dose administration and baseline prior to the first dose administration.



**Figure 6:** Mean plasma VEL-0230 concentrations after drug administration in the 7d and 3.25d protocols. Different doses within each protocol were averaged for graph simplification. Peak plasma drug concentration was detected at 2h and 1h for the 7 d and 3.25 d, respectively. A rapid clearance of the drug from plasma was observed within 24 hours in both protocols. No statistically significant difference was observed between the two protocols in plasma VEL-0230 concentration similar to the finding of the PK analysis.

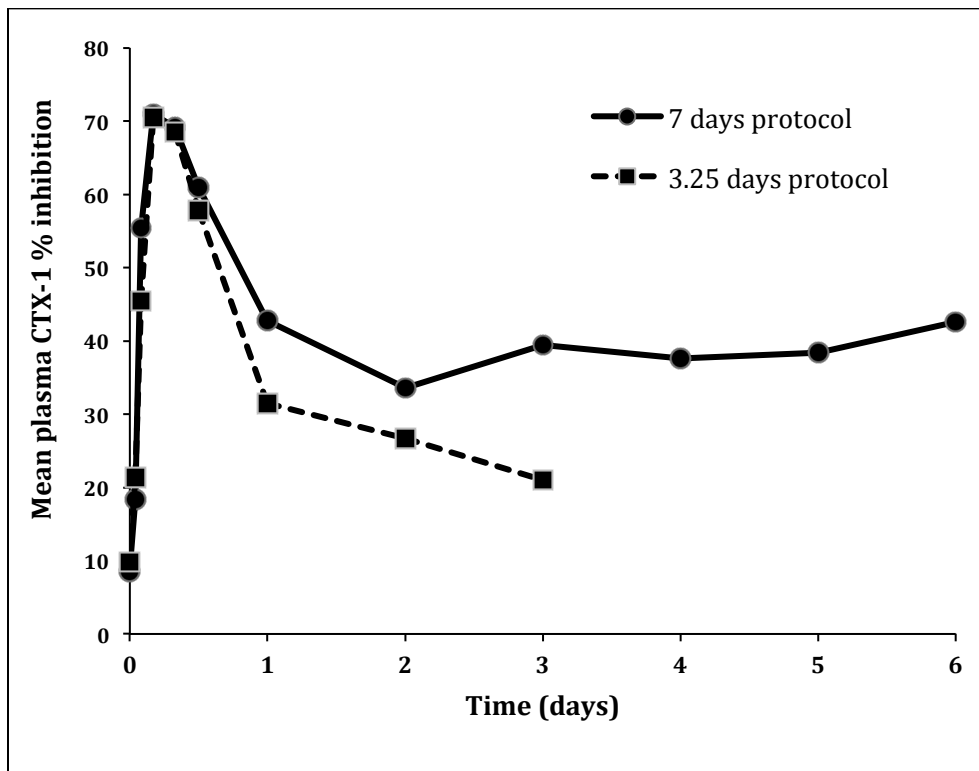
Dose #	T <sub>max</sub> (hours)	C <sub>max</sub> (ng/mL)	Terminal t <sub>1/2</sub> (hours)	AUC <sub>0-last</sub> (ng*h/mL)	AUC <sub>0-infinity</sub> (ng*h/mL)
<b>3.25d</b>					
<b>Dose 1</b>	1.75± 0.5	70.4± 52.6	4.7± 4.2	246.5± 213	268.8± 202
<b>Dose 2</b>	1.75± 0.5	48.65± 37	2.9± 0.52	206.5±165	218.5±168
<b>Dose 3</b>	2±1.4	61.8±27.8	4.6± 3.74	222.3±119	243.5±118
<b>7d</b>					
<b>Dose 1</b>	1.25± 0.5	83.4± 25.2	6.57±3.8	226.0 ± 92	243.3± 83
<b>Dose 2</b>	1.5± 0.5	59.0± 29	4.9± 2.0	160.5± 89	179.3 ± 84
<b>Dose 3</b>	1.5± 0.6	61.1± 39.0	4.9± 2.0	175.2± 110	198.9± 100

**Table 4:** Mean ±SD values for PK parameters of the dose interval study, for the two dose interval protocols; 3.25d and 7d.



**Figure 7:** Mean  $\pm$  SEM plasma CTX-1 concentrations (ng/ml) before and after oral administration of three consecutive doses of VEL-0230 at a dose of (4mg/kg b.w.) for the (a) 7 days protocol, and (b) 3.25 days protocol.





**Figure 8:** Mean values of plasma CTX-1 inhibition from baseline for the 7d and 3.25d protocols. Different doses within each protocol were averaged for graph simplification. There was no statistical difference in CTX-1 inhibition between the two dose interval protocols, including a similar maximum inhibition (71%) at 4 hours post-dose administration. Sustained CTX-1 inhibition until 6 days at an average of 43% was observed in the 7d protocol.

## DISCUSSION

VEL-0230 is a highly specific Cathepsin K inhibitor and thus inhibits bone resorption. The drug acts through the selective binding of the Cathepsin K active site leading to loss of its enzymatic function. Recently, Asagiri *et al.*, (1) demonstrated a role for Cathepsin K in the release of pro-inflammatory cytokines that has been disrupted in the presence of VEL-0230. The drug inhibited the production of IL-12 and IL-6 by dendritic cells when challenged by unmethylated CpG DNA. As a result, VEL-0230 was a powerful inhibitor of both bone loss and inflammation. We hypothesized that VEL-0230 would be a possible treatment for osteo-inflammatory diseases in horses which are known to be associated with an increase in bone resorption and inflammation such as dorsal metacarpal disease (acute periostitis), navicular disease (navicular bursitis), and osteoarthritis. We determined an effective oral dose and appropriate dose interval regimen for VEL-0230 by evaluating the pharmacokinetic characteristic and the biologic effect of VEL-0230 in young sport horses. We observed that the drug is rapidly taken up and cleared in exercising horses indicating that plasma levels were easily regulated. VEL-0230 markedly inhibited bone resorption as determined by the plasma CTX-1 biomarker. This inhibitory effect occurred within 6–8 hours and was sustained at approximately 50% of peak value for 5 days. Dose range and dose interval studies indicated an optimal dosage of (4 mg/kg b.w.) administered weekly.

VEL-0230 was rapidly absorbed,  $T_{\max}$  was at 1–2 hours, and was rapidly cleared from the plasma within 24 hours following either single or repetitive doses. The drug was detected in urine samples until the 1d time points, but thereafter, samples were negative (i.e., below the LLOQ of 1 ng/mL). These data demonstrated that the majority of VEL-0230 was eliminated within 24 h. It is apparent that VEL-0230 is rapidly cleared from the blood with renal clearance confirmed as a pathway. Other clearance pathways, such as hepatic clearance are currently not known. VEL-0230 was not detected in urine for out to 2 weeks suggesting it is not stored in other tissues including bone. Moreover, the terminal half-life of the plasma VEL-0230 in all experiments did not exceed 7h confirming that the drug was short-lived in the body. Comparison of plasma VEL-0230 concentrations between the 3.25d and 7d dose intervals showed equivalent plasma concentration profiles of VEL-0230 when dosed at 4 mg/kg and no significant differences in PK characteristics between the two protocols. Another class of compounds, bisphosphonates, is used to inhibit bone resorption. In contrast to VEL-0230, the terminal half-life of bisphosphonates was reported to be several days with values ranging between 2 hours to 11 days depending on the type of bisphosphonate, method and study design (24-26). Regardless of the different drug formulation in each experiment, VEL-0230 had similar plasma profiles and PK data in both dose range and dose interval studies. Interestingly, VEL-0230 displayed a nonlinear pharmacokinetic behavior that might indicate slower absorption or lower bioavailability of VEL-0230 with increasing drug concentration (27). This observed nonlinearity favored the use of the 4 mg dose with the 7d interval over the 8mg with either 3.25d or 7d intervals.

VEL-0230 induced a rapid, significant inhibition of plasma CTX-1 in both dose range and dose interval experiments. Oral administration of VEL-0230 induced more than 70% plasma CTX-1 inhibition from baseline. In the dose range experiment, the  $C_{\text{imax}}$  was 79%, 81% and 96% for doses at 2, 4, and 8 mg/kg, respectively, indicating an effective, dose dependent suppression of bone resorption. These results confirmed and extended previous studies where plasma CTX-1 proved to be a reliable and reflective biomarker of bone resorption in horses (13, 20, 21, 22, & 28). Moreover, two equine studies demonstrated that a plasma CTX-1 inhibition in the range of 50-70 % following intravenous injection of tiludronate was biologically relevant (13 & 22). Likewise, Asagiri *et al.*, (1) reported that VEL-0230 markedly suppressed bone resorption in a rat adjuvant-induced arthritis model, which further strengthens and supports the validation of CTX-1 as an excellent biomarker of bone resorption.

All doses and dose interval protocols following VEL-0230 administration showed similar profiles of plasma CTX-1 inhibition. There is an initial, rapid peak inhibition followed by a prolonged plateau of inhibition at approximately 50% of peak value. The initial peak inhibition was significantly higher at 8 mg/kg, but the plateau-phase was equivalent at all doses. There are no significant differences between the 2 and 4 mg/kg doses as the variability among horses in the measured plasma CTX-1 concentrations, as well as the rapid clearance of VEL-0230 from plasma, reduced the ability to detect statistical differences. That said, it appeared that plateau-phase suppression occurred around 2 mg/kg. We doubled that to assure we were not on the ascending limb of the concentration-effect curve and used 4 mg/kg as the effective dose, administered weekly.

The biphasic (peak/plateau) appearance to the inhibition of CTX-1 and the more prolonged inhibition of CTX-1 appears to be specific to horses. Other mammals (rats, dogs, non-human primates and humans) showed a single phase of inhibition (~70%) that occurred 8 to 12 hours after VEL-0230 administration, necessitating the twice daily dosing, and returned to baseline plasma CTX-1 levels within 2–3 days without evidence for enterohepatic circulation (data not shown, FreeStride Therapeutics, personal communication). The reason for the inhibition-plateau and longer inhibition of plasma CTX-1 after VEL-0230 administration in the horse is unknown. It may be that the unique architecture and density of equine bone creates a bone interstitial compartment that retains VEL-0230. In this scenario, VEL-0230 could continue to act on Cathepsin K within the space until it was locally degraded and thus undetectable when excreted. Alternative explanations include a slower Cathepsin K production or distribution into bone in horses compared to the other species. This is an area of active investigation.

We conclude that VEL0230 is a powerful, relatively short acting, inhibitor of bone resorption in young, exercising horses. It was effective when administered orally at (4 mg/kg b.w.) on a weekly dosing schedule with no observed side effects. VEL-0230 effects were rapid and it was quickly cleared from the body allowing precise control of dose and dose interval. Coupled with its anti-inflammatory effect that has been detected in other animal disease models, VEL-0230 has the potential to be an effective therapy in equine osteo-inflammatory diseases.

## **FOOTNOTES**

- a. Clinical Hematology laboratory, Veterinary Medical Center, The Ohio State University.
- b. Pharmoptima Laboratories , Portage, MI, USA.
- c. CTX-1, Serum Crosslaps® ELISA Immunodiagnostic system Inc, Fountain Hill, Ariz.

## **Chapter 3**

# **Repeated Oral Administration Of A Cathepsin K Inhibitor Significantly Suppresses Bone Resorption And Increases Bone Formation In Young Exercising Horses With Evidence Of Maintained Bone Remodeling**

### **SUMMARY:**

The objectives of our study were 1. To determine whether repeated dosing of a Cathepsin K (CatK) inhibitor, VEL-0230, produced a desired inhibition of the bone resorption biomarker (CTX-1) in horses, and 2. To determine the effect of repeated dosing on bone homeostasis, structure and dynamics of bone resorption and formation. Twelve young exercising horses were randomized in a prospective, controlled clinical trial and received 4 weekly doses of a CatK inhibitor or vehicle. We performed baseline and post-study radiographs for the third metacarpal bone (MCIII), nuclear scintigraphy, blood sampling and analysis of plasma bone biomarkers (CTX-1 and osteoclastin), post-study bone

fluorescent labeling and biopsy. Bone biopsy specimens were further processed for micro-computed tomography, bone histomorphometry and immunostaining of CatK. Blood cellular components were also evaluated. Repetitive dosing of this CatK inhibitor transiently inhibited plasma CTX-1 (reflecting inhibition of bone collagen resorption) and increased bone plasma osteoclastin levels, but did not prevent normal adaptive bone remodeling to exercise which was evident by a radiographic narrowing of the MCIII cortex occurred in both groups. Bone morphology, and density were not different between groups by all intensive measures. This CatK inhibitor may be a moderator of abnormal bone turnover in animals with bone diseases with minimal negative influence on normal young exercising animals.

## **INTRODUCTION:**

Our investigations evaluated the effect of VEL-0230, alternatively named NC-2300, a highly specific irreversible inhibitor of bone Cathepsin K (CatK) (1). Cathepsin K is a lysosomal protease highly expressed in osteoclasts and is the primary enzyme involved in bone matrix resorption and cartilage degradation (2). Moreover, CatK sits at the nexus of both musculoskeletal system and the immune system as it contributes to both bone resorption and the production of pro-inflammatory cytokines (3).

The CatK inhibitor (CatKI) used in this study suppressed osteoclast-mediated bone resorption *in vitro* and *in vivo* in rat models through binding to intracellular and extracellular CatK (3). In addition, effective oral dose and dosing interval of this CatKI,



VEL-0230, have been identified in healthy young horses and were correlated to a significant decrease in a plasma bone resorption biomarker (4). This CatKI induced a rapid, short-acting inhibitory effect of bone resorption and inflammation as proven by efficacy studies in humans, non-human primates, horses, dogs and rats (1, 3, 4,). Unlike bisphosphonate anti-resorptive drugs, CatK inhibition did not lead to over-suppression of bone turnover (5). These findings suggested that this CatKI, with its dual anti-resorptive and anti-inflammatory properties, maybe a potential specific therapeutic for osteo-inflammatory disorders in human and animals.

The effect of CatK inhibition on bone resorption has been studied in horses (6). In addition, it has been concluded that CatK plays a role in the pathogenesis of equine osteoarthritis by degrading collagen type II (7). There are several reports on using anti-resorptive drugs in equine bone resorption disorders (8, 9). Moreover, some anti-resorptives have been proposed for the treatment of dorsal metacarpal disease (bucked shins) and other bone resorption disorders in horses (10, 11).

The purpose of this study therefore was to evaluate bone structure and turnover in healthy young exercising horses receiving repeated oral dosing of a CatKI in a randomized, controlled, double-blinded, prospective, sufficiently powered clinical trial. The first specific objective was to determine whether repeated dosing of a CatKI produced a desired inhibition of the bone resorption biomarker carboxy-terminal cross-linking telopeptide of type I collagen (CTX-1). The second objective was to determine the effect of repeated dosing of this CatKI on bone homeostasis as evaluated by quantitative imaging, bone histomorphometric measurements, immuno-staining of bone CatK and the quantitative analysis of bone turnover related gene expression. We

also evaluated the effect of CatKI administration on blood cellular components for evidence of influence on the immune pathway.

## **MATERIALS AND METHODS:**

### ***Experimental design:***

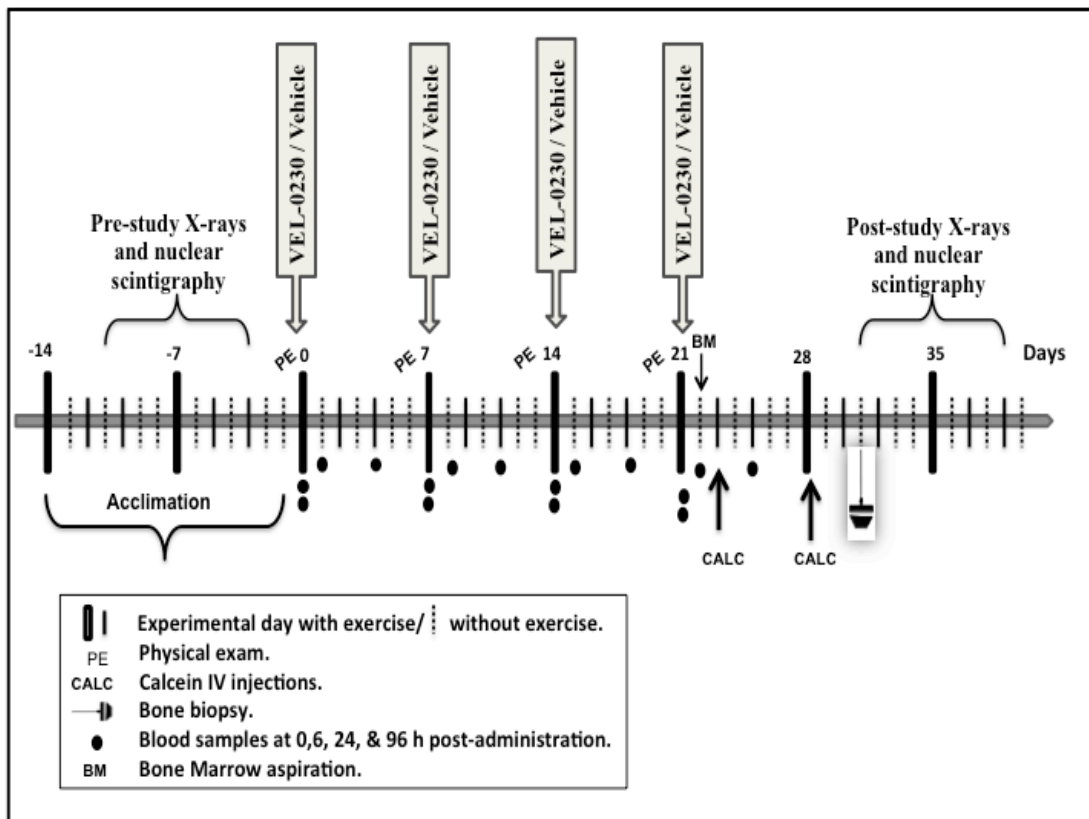
Twelve young horses (2-5 years), sufficiently sound to be exercised without medication on a treadmill, were aligned in a randomized, controlled, double-blinded prospective clinical trial. The horses were acclimated to a treadmill at the Veterinary Medical Center, The Ohio State University, and baseline data were obtained. The horses were exercised 3 times a week to sustain a constant level of conditioning and to mimic a training schedule. Horses were randomly assigned to either a treatment (n=6) or a control (n=6) group in a blinded manner. Starting at day 0, a CatKI or a vehicle (Freestride Therapeutics, Inc. MI, USA) was administered orally as previously described (4) at a dose of 4 mg/kg body weight (b.w.) once a week for 4 successive weeks in the treatment and control group, respectively. All experimental procedures were done and data was obtained according to the schedule shown in the experimental design (Fig. 9). The study was approved by The Ohio State University Institutional Animal Care and Use Committee.

### ***Blood sampling and analysis:***

Blood samples were obtained via jugular venipuncture from each animal at 4 time points around each dose administration (0 hour [h], 6h, 24h, and 96h). Plasma was

separated and stored at  $-20^{\circ}\text{C}$  for further analysis of biomarkers of bone turnover.

Additional blood



**Figure 9:** Experimental design.

samples were obtained for complete blood count (CBC) pre-first dose and post-last dose administration. Samples were processed at the same day of collection for total red blood cell count (RBCs), total white blood cell (WBCs) count, total platelet count, differential WBCs by automated hematology analyzer and manual cytology.

***Biochemical markers of bone turnover:***

Using pre-validated specific ELISA kits, plasma biomarkers of bone formation (osteocalcin; MyBioSource, Inc., CA, USA) and resorption (CTX-1; Serum Crosslaps<sup>®</sup>, Crosslaps, Osteometer A/S, Ballerup, Denmark) were assessed at all sampling time points in duplicates. The optical density was obtained using a standard colorimetric method and averaged concentrations were calculated for each duplicate. The CTX-1 % changes from baseline post each drug or vehicle administration were calculated.

***Radiographic analysis:***

Pre and post-study lateral digital radiographs for the third metacarpal bone (MCIII) of right and left forelimbs were taken and the width of dorsal cortex at mid-diaphysis as well as the widest cortex were measured for each limb (Fig. 10) (Sedecal<sup>™</sup>, Sedecal USA, Arlington Heights, IL or Prestige II, GE Medical Systems, Milwaukee, WI; digital radiography system EDR-6, Sound-Eklin, Carlsbad, CA; viewing software E-film, Merge Healthcare, Milwaukee, WI). Measurement of the MCIII dorsal cortex estimates adaptive bone remodeling in young galloping racehorses with bone resorption

(narrowing of the cortex) preceding bone deposition (widening of the dorsal cortex) (12).

***Nuclear scintigraphy:***

Nuclear scintigraphy was performed for distal forelimbs to assess bone metabolic activity pre and post-study. All horses received 4.625 GBq (125 mCi) of technetium TC 99m medronate (SynCor, Oklahoma City, OK), injected via an IV catheter into the left or right jugular vein 2 hours prior to imaging. Horses were then sedated with 0.01mg/kg b.w. detomidine hydrochloride IV for image acquisition. All images were obtained using a rectangular gamma camera (Omega 500, Technicare, Solon, OH) with low energy general-purpose collimator and 90 seconds static images were processed using an image acquisition software (Gamma 600, Strichman Medical Equipment, Inc., Richmond, VA). Three regions of interest (ROIs) were evaluated relative to a standard flood source syringe; the dorsal cortex of MCIII (ROI1), the distal epiphysis of MCIII (ROI2), and the dorsoproximal region of the first phalanx (ROI3) (Fig. 11). Radioactivity (counts/ROI) was measured and decay rate was corrected. The counts/ROI were converted to a percent-injected dose/ROI (% injected dose) using the following set of formulas:

Counts/ROI measured for each ROI, including the flood source syringe and decay corrected

- ***Decay Corrected Counts*** = 
$$\frac{\text{Counts}}{e^{-0.693(\frac{t}{363})}}$$

- t = time from injection of radiopharmaceutical to the start of imaging

(minutes)

Radioactivity of flood source syringe measured (uCi) prior to imaging and decay corrected

- ***Decay Corrected uCi*** =  $\frac{uCi}{e^{-0.693(\frac{t}{363})}}$
- t = time from when the flood source syringe was measured to the start of imaging (minutes)

uCi:DCC ratio determined for flood source syringe

- ***uCi: DCC<sub>0</sub>*** =  $\frac{\text{Decay Corrected uCi}}{\text{Decay Corrected Counts for syringe}}$

Radioactivity for each ROI was calculated as uCi/ROI

- ***uCi/ROI*** = ***Decay Corrected Counts/ROI x (uCi: DCC<sub>0</sub>)***

Radioactivity of pre-injection dose of radiopharmaceutical was measured (mCi) prior to injecting into horse and then decay corrected

- ***Decay Corrected mCi<sub>pre</sub>*** = ***mCi x e<sup>-0.693( $\frac{t}{363}$ )</sup>***
- t = time from when dose was measured to when it was injected (minutes)

After injecting radiopharmaceutical into horse, the radioactivity of the dose remaining in the syringe was measured (mCi) and decay corrected

- ***Decay Corrected mCi<sub>post</sub>*** =  $\frac{mCi}{e^{-0.693(\frac{t}{363})}}$

- $t$  = time from when dose was injected to when it was measured (minutes)

The total amount of radiopharmaceutical was determined and then converted from mCi to uCi

- ***Injected dose mCi*** =  $\frac{\text{Decay Corrected mCi}_{pre}}{\text{Decay Corrected mCi}_{post}}$
- ***Injected dose uCi*** = ***Injected dose mCi***  $\times \frac{1000 \text{ uCi}}{\text{mCi}}$

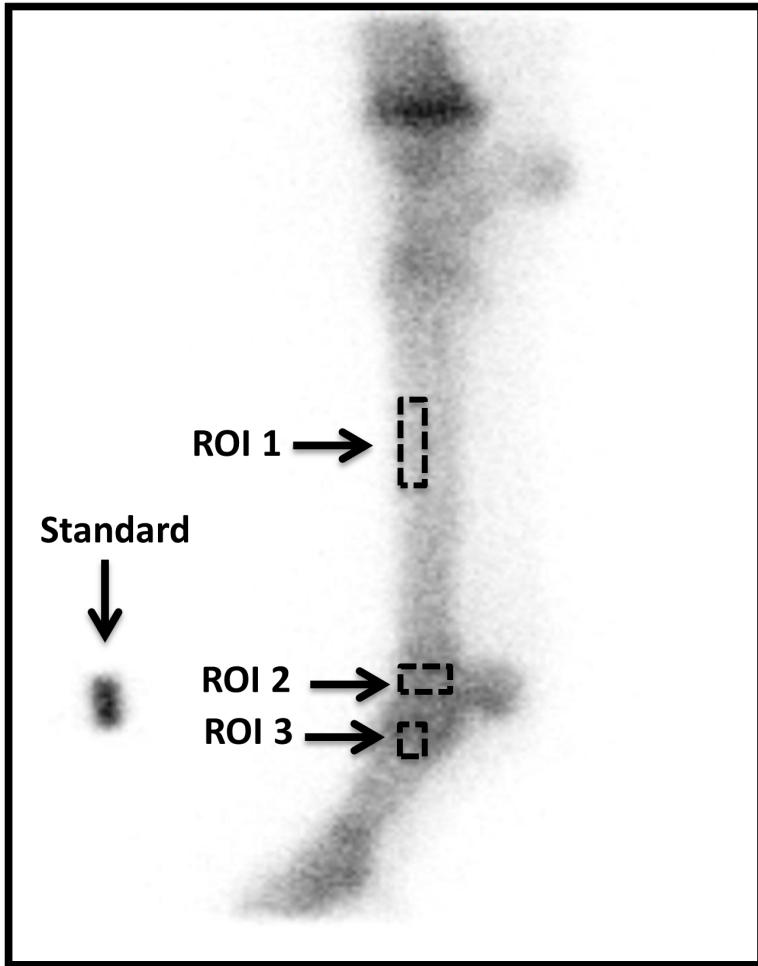
The % injected dose/ROI detected by the gamma camera was calculated

- ***% Injected dose / ROI*** =  $\frac{\text{uCi}/ROI}{\text{Injected dose uCi}}$





**Figure 10:** Mid-point of the diaphysis (black oval) established half-way between the distal aspect of the metacarpal tuberosity (top white arrow) and the dorsoproximal aspect of the mid-sagittal ridge (bottom white arrow) and widest point of the dorsal cortex (white oval).



**Figure 11:** The three ROIs in distal forelimb delineated using square/rectangular boundaries of equal size per horse; Standard = flood source syringe, ROI 1= dorsal cortex of MCIII, ROI 2 = distal epiphysis of MCIII, ROI 3= dorsoproximal P1.

***Bone fluorescent labeling and biopsy:***

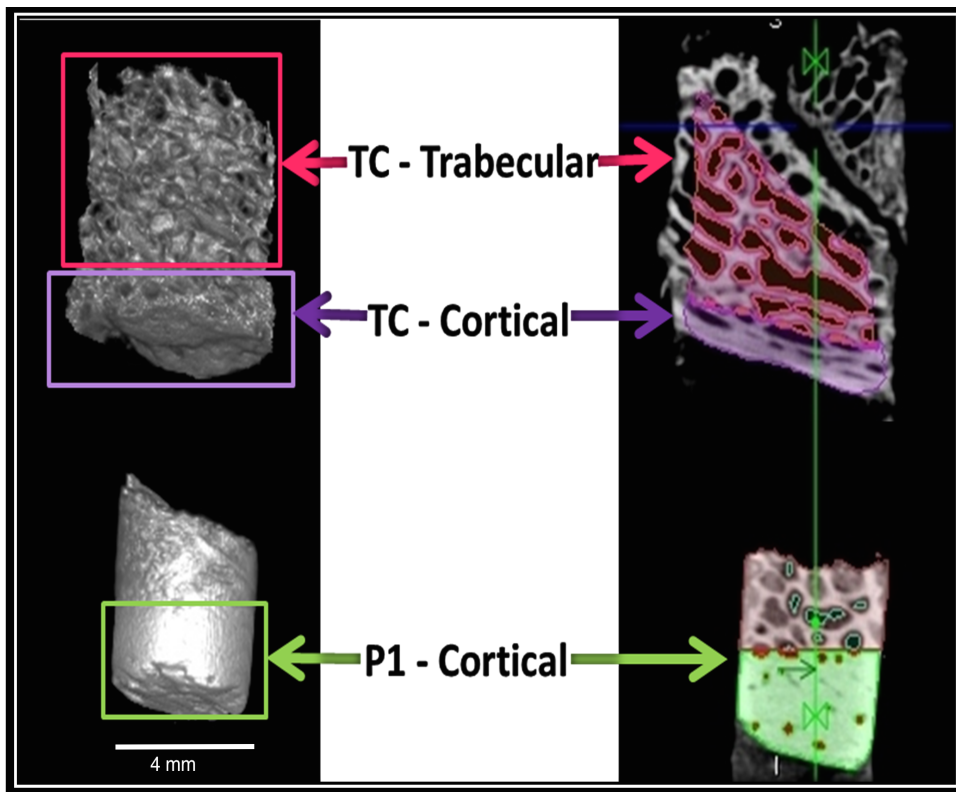
Bone fluorescent labeling and post-study bone biopsies were performed in all horses. For fluorescent labeling of bone mineralization activity, calcein (Sigma-Aldrich) dissolved in 2% sodium bicarbonate solution (Abbott Laboratories, North Chicago, IL, USA) was administered IV at a dose of 20 mg/kg b.w. on d 23 and d 28. Post-study bone biopsies were obtained 3 days after second calcein dose administration from TC, a non-weight bearing bone, and P1, a weight bearing bone, according to the sampling time points. Horses were randomly assigned to left or right TC and forelimb P1. Horses were then sedated with Detomidine (0.01 mg/kg, IV) and biopsy collection sites were prepared aseptically and locally infused with 2 ml (P1) or 20 ml (TC) of 2% Mepivacaine hydrochloride. Over the prepared biopsy sites, a 2-cm skin and subcutaneous tissue incision was performed using a #10 scalpel blade. Cortical bone biopsies were collected from P1 first with a 4mm Michele trephine, then the same site was overlapped with a 6mm Michele trephine to create two biopsy specimens. Cortical and trabecular bone were collected from the TC from two separate locations through a similar incision using a 6mm Michele trephine. Skin incisions were closed using 2-0 polypropylene sutures in an interrupted cruciate pattern. Horses received Phenylbutazone (Butazolidin; Schering, Kenilworth, NJ) 2 g orally for 3 days including the day of biopsy and a single injection of Penicillin G procaine (Crystacillin; Solvay, Marietta, GA) 22,000

units/kg Intramuscular at the time of biopsy. Biopsies were obtained from the Tuber coxae (TC), a non-weight bearing bone, and the proximal phalanx (P1), a weight bearing bone, according to the sampling time points. Core biopsies of P1 were obtained proximally at a known site of increased bone remodeling in young racehorses (13). Horses were stall rested and limbs were bandaged for 7 days.

***Micro-computed tomography of bone:***

Micro-computed tomography ( $\mu$ CT) was performed for P1 and TC biopsy specimens. A 4 mm diameter biopsy specimen of P1 and a 6mm diameter specimen of TC from each horse were placed together in a 5 mL cryo tube with 0.1 mL 0.9% NaCl. The specimens were separated in the tube by a small piece of styrofoam sheeting. Tubes were kept on ice for up to 2 hours before being transferred to -80°C. The specimens were maintained at -80°C for 3 to 7 days and transferred to the micro-computed tomography ( $\mu$ CT) laboratory at The Ohio State University, on dry ice. Three ROIs were established in the following bone biopsies; Tuber coxae trabecular bone (ROI1), tuber coxae cortical bone (ROI2), P1 cortical bone (ROI3). A calibrated Lucite phantom was scanned with the same acquisition protocol as the samples to establish the correlate absolute density for each  $\mu$ CT scan. Regions of interest were analyzed in Siemens Inveon Research Workplace (Siemens PreClinical, Knoxville, TN) taking the raw CT values of the bone and determining a set point that eliminated as much background noise as possible from the evident bone. The convenient set point for cortical bone was -700 raw CT units that corresponded to 1057 mg/cc in

absolute density. Care was taken to exclude any crushed bone or obviously foreign debris from the measurement volumes. The hydroxyapatite-doped rods correspond to 1750, 1250, 1050, and 1000 mg/cc. The acquisition was a full rotation of one projection per degree. The source was run for 175 ms at 80 kVp and 500 mA for each projection. The resultant pixel size was 38.8  $\mu\text{m}$  using a modified Feldkamp algorithm for reconstruction and a binning of 4. Three ROIs were established in the bone biopsies as following; TC trabecular bone (ROI 1), TC cortical bone (ROI 2), P1 cortical bone (ROI 3) (Fig. 12). The surface area (SA) to volume (V) ratio (SA:V), percent-bone volume (%BV), and bone mineral density (BMD) were calculated for each ROI.



**Figure 12:** The three different ROIs analyzed for surface area to volume ratio (SA:V), percent bone volume (%BV) and bone mineral density (BMD); 1. Tuber coxae (TC) trabecular bone. 2. TC cortical bone. 3. P1 cortical bone.

***Bone histomorphometric measurements:***

Bone biopsies obtained from TC and P1 3 days after the second calcein dose administration (as described) were carefully fractionated and processed for both decalcified and non-decalcified bone histomorphometric evaluation. For preparing the non-decalcified sections, calcified bone specimens were dehydrated in alcohol, embedded in methacrylate, cut into 10- $\mu$ m sections and mounted unstained using non-fluorescent medium. The non-decalcified bone specimens were quantitatively evaluated for dynamic bone measurements in 2 ROIs within the P1 cortical bone and 3 ROIs within the TC trabecular bone using a microscope interfaced with a semiautomatic analysis system (Bioquant OSTEO 7.20.10; Bioquant Image Analysis Co.). Mineral apposition rate (MAR) of the osteonal and trabecular bone was estimated as the distance between the midpoints of the two calcein labels (Out:Rd and In:Rd) divided by the time between the midpoints of the interval within the 1-mm<sup>2</sup> cortical bone area under 200 magnification. Additionally, the number of single-labeled osteon (sL.On) and double-labeled osteon (dL.On) were counted in the two representative ROIs.

$$\text{MAR } (\mu\text{m per day}) = (\text{Out:Rd} - \text{In:Rd}) \div 5$$

Bone formation rate (BFR) per bone surface (BS), which represented the volume of mineralized bone (MS) formed per unit time and per unit bone surface (BS), was estimated for TC trabecular bone.

$$\text{BFR}=\text{MAR} * (\text{MS}/\text{BS})$$

For preparing the decalcified sections, the TC and P1 samples of each animal were fixed in 10% neutral buffered formalin for 48 hours and then decalcified in neutral 10% EDTA for 20 days. Samples were then embedded in glycol methacrylate resin (GMA; Historesin, Nussloch, Leica). Decalcified bone sections (1.5  $\mu\text{m}$  thick) were prepared using a Reihert Jung microtome 2050 Supercut (Cambridge Instrument Gmbh, Heiderberger, Switzerland). All sections were stained with tartrate-resistant acid phosphatase (TRAP) and counterstained with 0.05% toluidine blue (pH 7.2). They were observed under a light microscope (Axiophoto; Jena, Carl Zeiss, Germany) at 450 $\times$  and 4 ROIs within the TC trabecular bone were quantified for static bone measurements as the ratio of bone volume (BV) to tissue volume (TV); BV/TV, eroded surface (ES) regardless of osteoclast (OC) presence to BS; ES/BS, and osteoclast number per ES; N.OC /ES were calculated using Bioquant OSTEO software (Bioquant OSTEO 7.20.10; Bioquant Image Analysis Co.). In addition, 2 ROIs within the P1 trabecular bone were analyzed for the same three parameters. The non-decalcified bone sections were then quantitatively evaluated for dynamic bone measurements, bone formation rate (BFR) per bone surface (BS), in 2 ROIs within the P1 cortical bone and 3 ROIs within the TC trabecular bone. Formalin-fixed decalcified sections were stained with tartrate-resistant acid phosphatase (TRAP) and 4 ROIs within the TC trabecular bone were quantified for static bone measurements as the ratio of bone volume (BV) to tissue volume (TV); BV/TV, eroded surface (ES) regardless of osteoclast (OC) presence to BS; ES/BS, and osteoclast number per ES; N.OC /ES were



calculated using Bioquant OSTEO software. In addition, 2 ROIs within the P1 trabecular bone were analyzed for the same three parameters.

***Immunostaining of bone CatK:***

Cathepsin K was immunostained in representative sections of formalin-fixed decalcified bone samples using a standard Biotin/streptavidin horseradish peroxidase method. The paraffin sections were de-waxed through Xylene (Sigma-Aldrich, St. Louis, MO) and descending gradient of ethanol treatments. To reduce nonspecific binding, the tissue samples were treated with the avidin/biotin blocking kit per manufacturer's instructions (SP-2001, Vector Laboratories, Burlingame, CA), followed by three 5 minutes washes with Phosphate buffered Saline (PBS). Bone CatK was then immunostained using a pre-validated Rabbit polyclonal cathepsin K antibody (1:50) as a primary antibody (Biorbyt Ltd, Cambridgeshire, UK) with which samples were incubated at 4°C overnight followed by incubation for 30 minutes with biotinylated Goat anti-Rabbit immunoglobulins (Vector Laboratories, UK) as a secondary antibody. Bone samples were developed with a peroxidase substrate solution (NovaRED, Vector Laboratories, Burlingame, CA) for 10 minutes to stain CatK-positive osteoclasts. Slides were washed quickly in PBS to terminate the reaction, taped dry, counterstained with hematoxylin, taped dry, cover-slipped, and allowed to dry in the dark for 2 days. Two ROIs under 20x magnification were analyzed for determination of total (number of osteoclasts), CatK-positive osteoclasts and BS using Bioquant OSTEO software. The percentage of CatK-positive osteoclasts was calculated as  $(\text{CatK-positive OC}/\text{total OC}) \times 100$ . In addition CatK-positive OC were quantified per bone surface (CatK- positive OC/BS).

***Quantitative real-time PCR:***

Relative gene expression analysis for Osteopontin (OPT) and CatK genes in relation to a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was performed using specific custom-designed primers. Frozen, unfixed bone samples were pulverized in liquid nitrogen using a mortar and pestle and homogenized for 30 seconds at 8000 rpm. Total RNA was isolated using Guanidinium thiocyanate-phenol-chloroform extraction method (TRIzol® and Chlorophorm; Invitrogen). RNA concentrations were then quantified by duplicate absorbance determinations at 260 nm using UV spectrophotometer (NanoDrop ND-1000). Complimentary (c) DNA was generated from 1 µg total RNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions using MJ Research PTC-150 Thermal Cycler (MJ Research, USA). Relative gene expression analysis for Osteopontin (OPT) and CatK genes in relation to a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was performed using specific custom-designed primers using Primer3 software (Steve Rozen, Helen J. Skaletsky, 1998). Real-time PCR was performed in a total volume of 50 µl using 96-well microwell plates and an ABI PRISM 7000 sequence detector (Applied BioSystems). Five microliters of purified DNA were added to 25 µl SYBR® Green PCR Master Mix (Applied BioSystems) with a 200 nM concentration of forward and reverse primers.

To reach a total volume of 50  $\mu$ l per well, DNase-RNase-free distilled water (Sigma) was added. The reaction was run for one cycle at 80°C for 3 minutes, and then 40–45 cycles of 94°C for 10 s, 69°C for 1 minute, and a final extension period at 72°C for 7 minutes. Real-time PCR was performed and cycle threshold (Ct) values were obtained for all analyzed genes during log phase of the cycle. The levels of OPT and CatK were normalized to GAPDH for each sample and  $\Delta$ Ct were calculated ( $\Delta$ Ct = Ct gene of interest – Ct GAPDH). All PCRs were performed in triplicate. Table (5) shows the forward and reverse primer nucleotides sequences for each gene of interest.

<b>Gene</b>	<b>Forward primer sequence (5'-3')</b>	<b>Reverse primer sequence (5'-3')</b>
<b>Equine OPT</b>	CGCAGATCTGAAGACCAGTA	GGAATGCTCACTGGTCTCAT
<b>Equine CatK</b>	CAGAGATTGCCATTCCGTTT	ATAGAATCAGCCCCAGGACA
<b>Equine GAPDH</b>	CAACGAATTTGGCTACAGCA	CTGTGAGGAGGGGAGATTCA

**Table 5:** Nucleotides sequences for forward and reverse designed primers for quantitative real time PCR analysis.

***Data analysis and statistics:***

Using a sample size of (6) in each group, the study had at least an 80% power to detect percent-difference as low as those reported in Table 6. Statistical analyses for all data were performed using SAS for Windows (SAS Institute Inc., Cary, NC, USA) and data was graphed as mean with standard error of the mean (SEM). Baseline and post-study values of all data were statistically analyzed using repeated measures analysis of variance (ANOVA) with GLM of SAS. For plasma osteocalcin values, osteocalcin % change from baseline at 6 hours post-dose administration was compared between groups. For the nuclear scintigraphy, individual values of each ROI as well as the sum of the three ROIs for left and right MCIII were compared between groups. Since there was no significant difference between left and right MCIII measurements, right and left limb data for each animal were combined. Objective data obtained at the termination of the study were compared between the two groups using *t* test for normally distributed data and Mann–Whitney *U* test for skewed data.

Parameter	Study reference	Calculated population's SD	Calculated population's mean	The least detectable % difference	Estimated power
Radiographic dorsal cortical width of MCIII mm	McCarthy and Jeffcott, 1992 (25)	1.79	38.975	6	0.89
Osteoclasts number per 50 field		0.61	1.5	50	0.85
Histomorphometric Bone area %		1.13	96.15	2	0.99
Histomorphometric MAR $\mu\text{m}/\text{day}$	McCarthy and Jeffcott, 1992 (25)	0.15	1.4205	13	0.84
Plasma CTX-1 ng/ml	Hussein <i>et al.</i> , 2014 (10)	0.206666667	0.626666667	40	0.86
Plasma Osteocalcin ng/ml	Cooper <i>et al.</i> , 2000 (26)	4.5	27.65333333	20	0.85
$\mu\text{CT}$ BV%	Kulmala <i>et al.</i> , 2012 (27)	9.680612245	72.95	16	0.84
$\mu\text{CT}$ Bone SA:V ratio 1/mm		1.919897959	7.255	31	0.82
$\mu\text{CT}$ BMD $\text{g}/\text{cm}^3$		0.027040816	0.78	4.5	0.9

**Table 6:** Sample size and power calculations for some evaluated parameters.

## **RESULTS:**

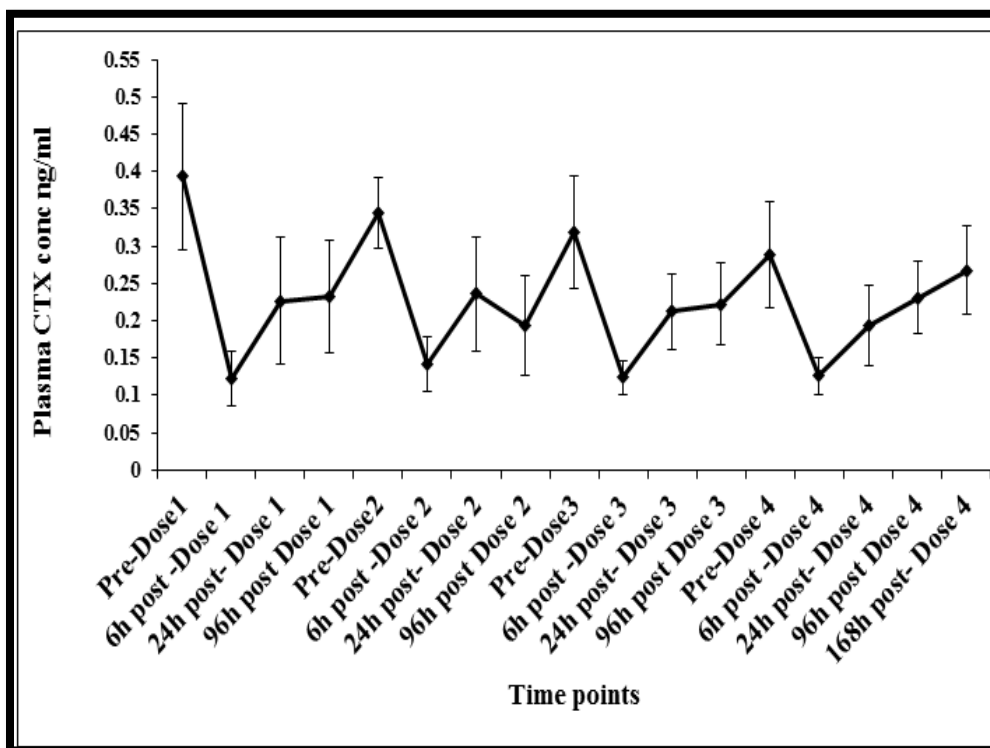
All horses completed the study and received full doses of the drug or vehicle as per the assigned times.

### ***Complete blood picture and differential cytology:***

All values of CBC and differential count were within reference ranges (Clinical Hematology laboratory, Veterinary Medical Center, The Ohio State University). There was no cell morphological change detected in blood smears.

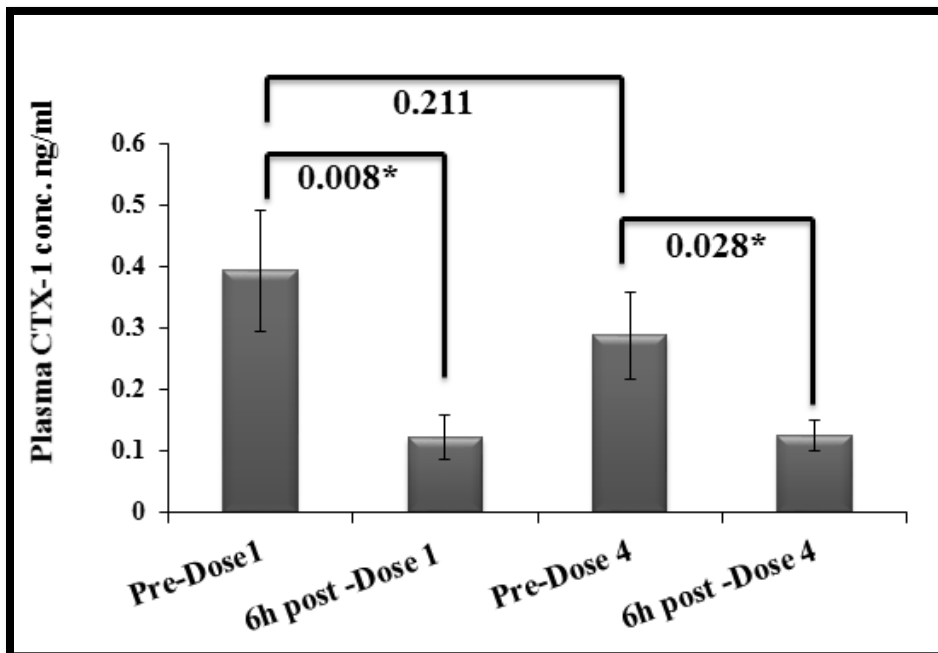
### ***Biochemical markers of bone turnover:***

There was an immediate CTX-1 inhibition in the CatKI-treated group that returned to be not statistically different from baseline values before next dosing (Fig. 13). Plasma CTX-1 concentrations 6 hours after CatKI dose administration were significantly lower than pre-dose concentrations while no significant reduction in CTX-1 concentrations was observed after vehicle administration in the control group. Results are shown for the first and fourth doses administrations in the CatKI group (Fig. 14). There was no statistical difference between pre-dose 4 and pre-dose 1 CTX-1 concentrations in CatKI group. Plasma osteocalcin concentrations were significantly greater at CatKI group. Figure 15 shows the mean osteocalcin % change from pre each dose administration in both CaTKI and control groups.

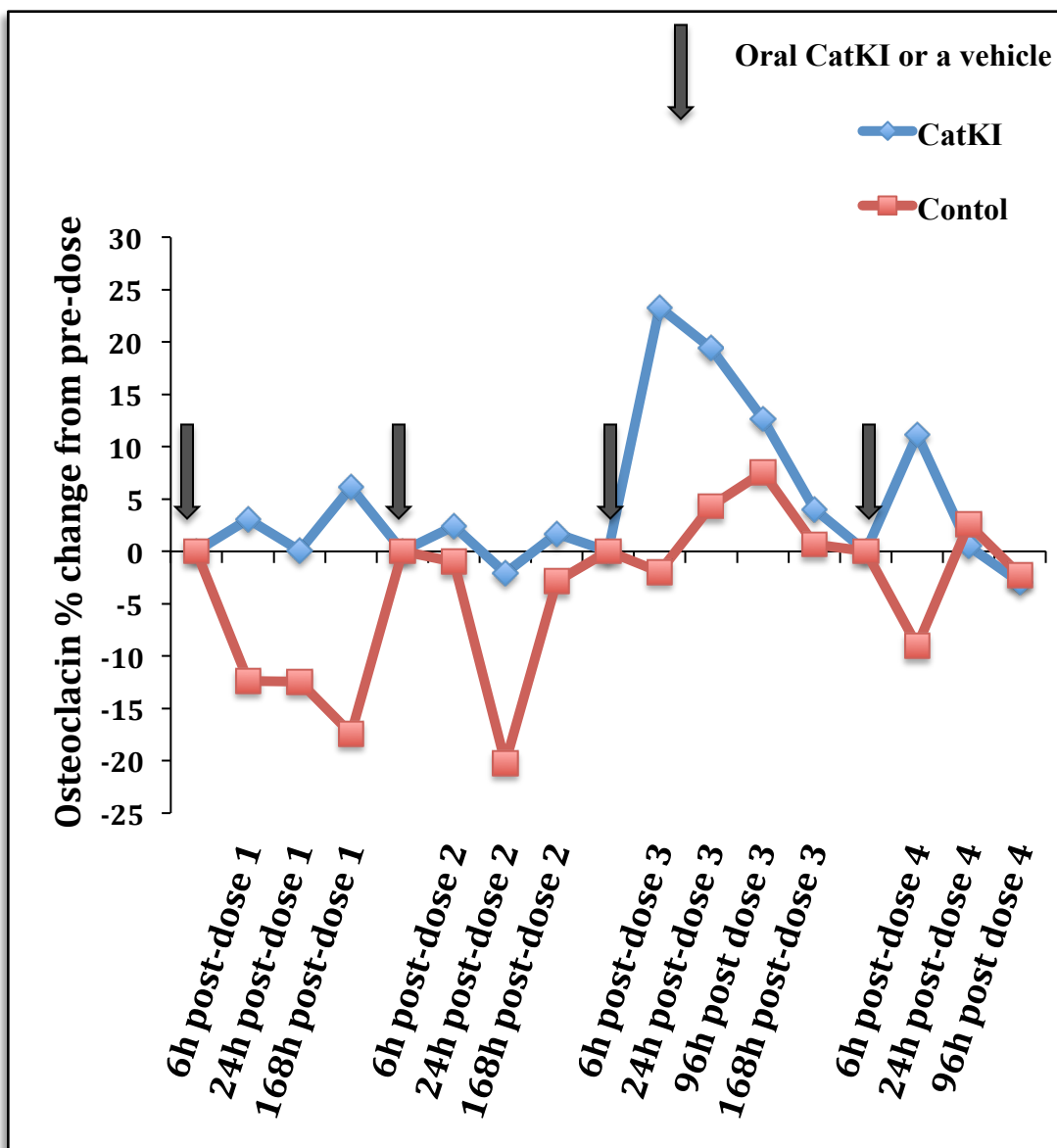


**Figure 13:** Mean±SEM plasma CTX-1 % inhibition from baseline value in CatKI group showing an immediate CTX-1 inhibition that returns to be not statistically different from baseline values before next dosing.





**Figure 14:** Mean  $\pm$  SEM plasma CTX-1 concentrations ng/ml before and 6 hours after first and fourth dose administrations in the CatKI group showing significant reduction \* ( $P < 0.05$ ) in concentrations after drug administration and no significant difference between pre-dose 1 and pre-dose 4 time points.



**Figure 15:** Mean plasma osteocalcin % change from pre-dose. Cathepsin K inhibitor group showed significantly greater osteocalcin % changes from pre-dose following all doses administration.

***Radiographic analysis:***

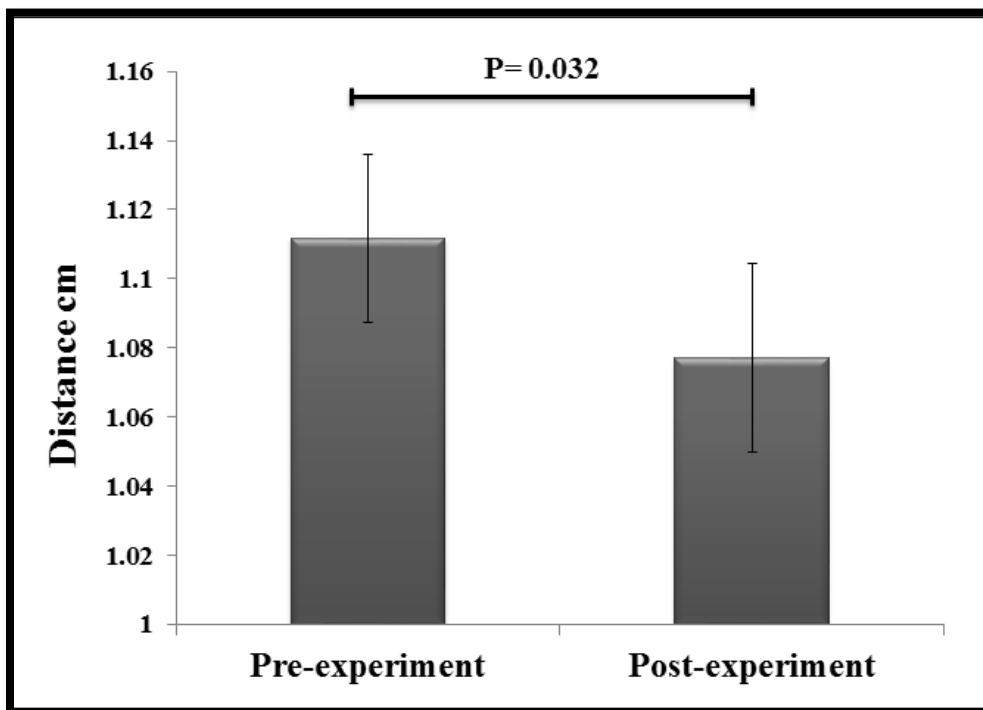
Pre and post-study radiographs for MCIII of right and left limbs indicated that there were no significant differences between groups in the MCIII dorsal midpoint cortical width and width of the widest cortex. However, a significant decrease ( $P= 0.032$ ) in the width of the dorsal cortex was observed in both groups with time in exercising horses (Fig. 16). There was no significant difference between left and right MCIII values.

***Nuclear scintigraphy:***

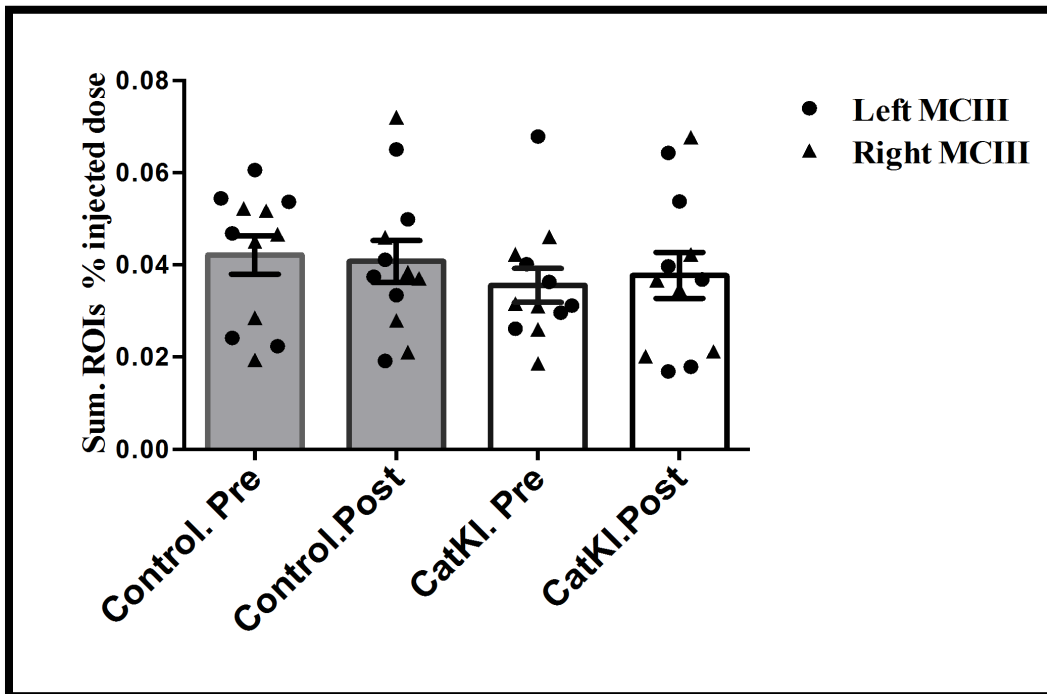
There was no significant difference between groups for the three ROIs or summed ROIs for MCIII % injected dose (Fig. 17) or in the ROI % injected dose change from baseline. There was no significant difference between left and right MCIII values.

***Micro-computed tomography of bone:***

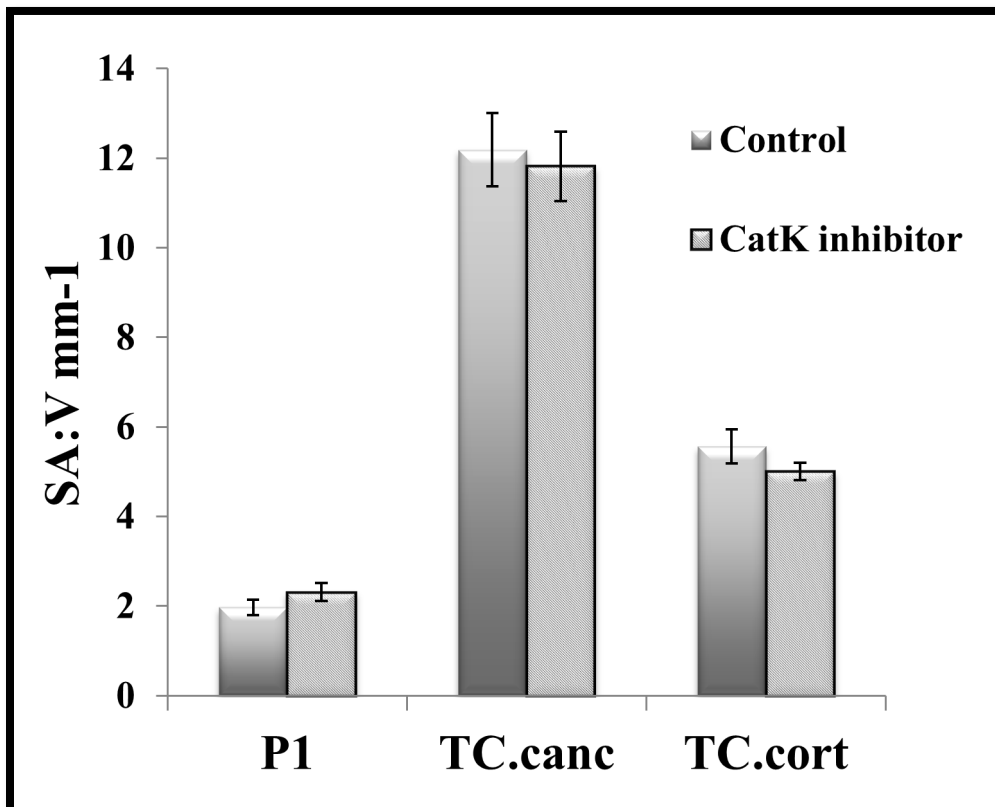
There was no statistical significant difference between the two groups for all measured parameters including the SA:V, %BV, and BMD of P1 cortical bone, TC trabecular bone and TC cortical bone (Fig. 18-20).



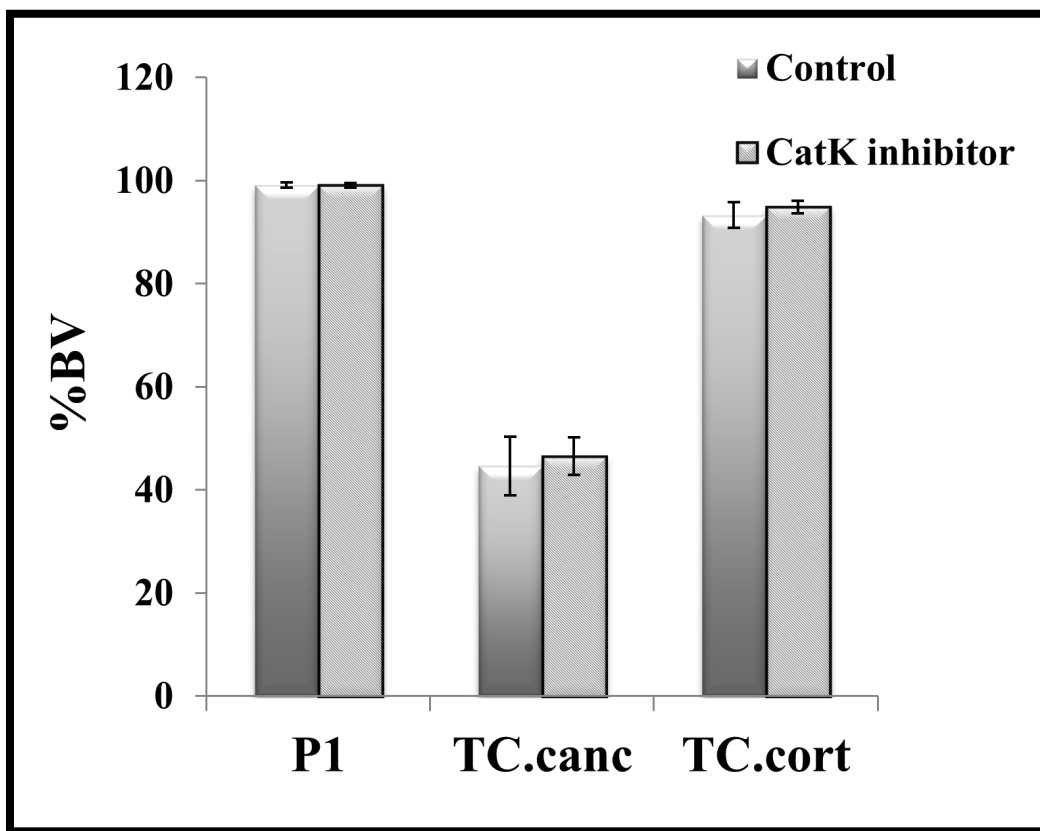
**Figure 16:** Mean  $\pm$  SEM dorsal cortical width of MCIII (black oval) measured on radiographs obtained pre- and post-study. Pre- to post-study values differed in both control and CatKI-treated groups ( $P=0.032$ ); values for both group and limbs were grouped together to emphasize the effect of time (exercise).



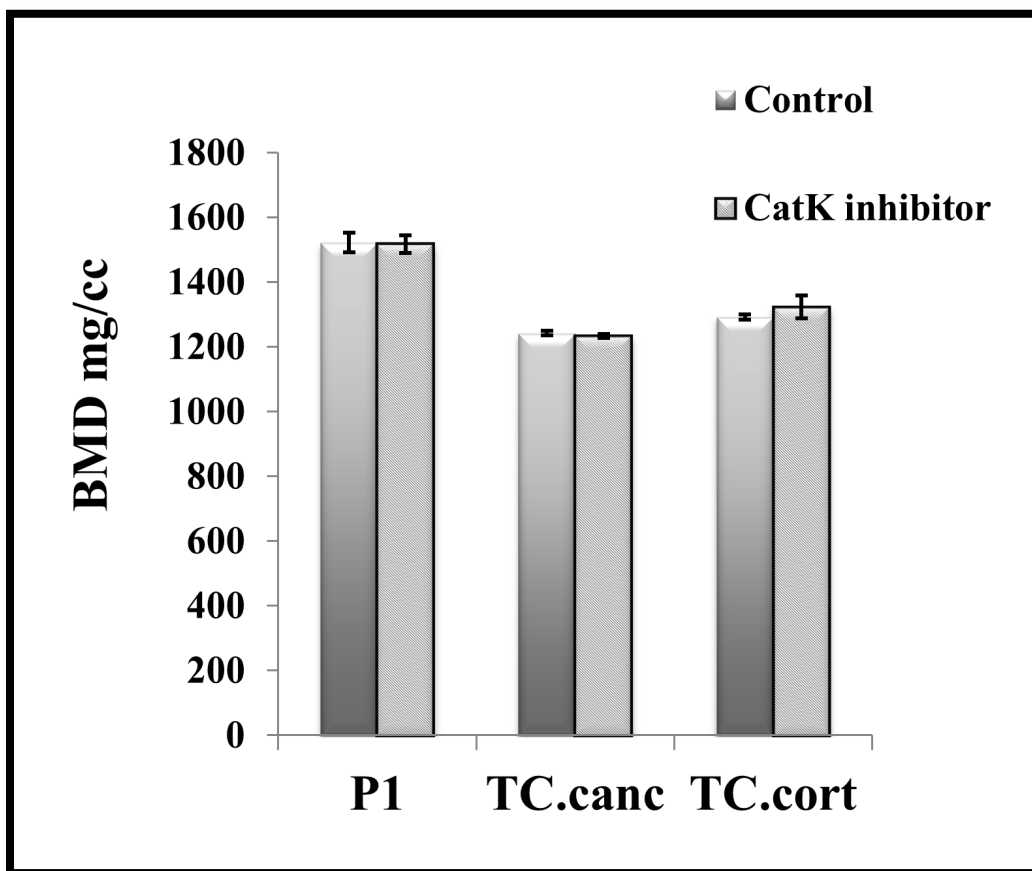
**Figure 17:** Mean  $\pm$  SEM of % injected dose summed for the three ROIs for each limb.



**Figure 18:** Calculated SA:V ratio using ex-vivo Micro-computed tomography for P1, TC trabecular bone and TC cortical bone.



**Figure 19:** Calculated BV using ex-vivo Micro-computed tomography for P1, TC trabecular bone and TC cortical bone.



**Figure 20:** Calculated BMD using ex-vivo Micro-computed tomography for P1, TC trabecular bone and TC cortical bone.



***Bone histomorphometric measurements:***

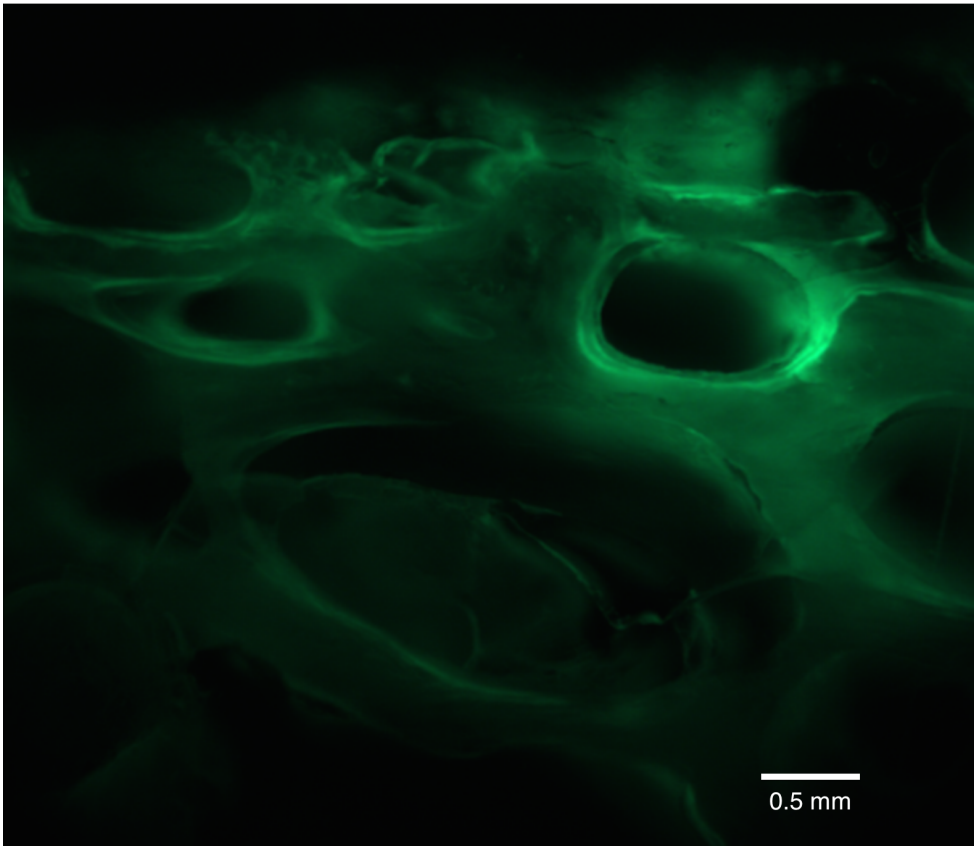
There was no significant difference between the two groups in the dynamic bone histomorphometric measurements; BFR and the static measurements; BV/TV, N.OC/ES, and ES/BS (Fig. 21-26).

***Immunostaining of bone CatK:***

Regardless of group, all osteoclasts in all examined microscopic fields were stained positive for CatK (Fig. 27) and there was no statistical difference between the two groups in number or calculated indices of CatK-positive OC/BS. A moderate number of active bone lining osteoblasts were CatK-positive (Fig. 28).

***Quantitative real-time PCR:***

Based on raw Ct values, CatK had significantly greater values (lower gene expression) compared to OPT and GAPDH regardless of group. There were no statistical differences between groups in  $\Delta$ Ct values for either OPT or CatK genes (Fig. 29).



**Figure 21:** Fluorescent microscopic image showing calcein double bone labeling for TC cancellous bone.

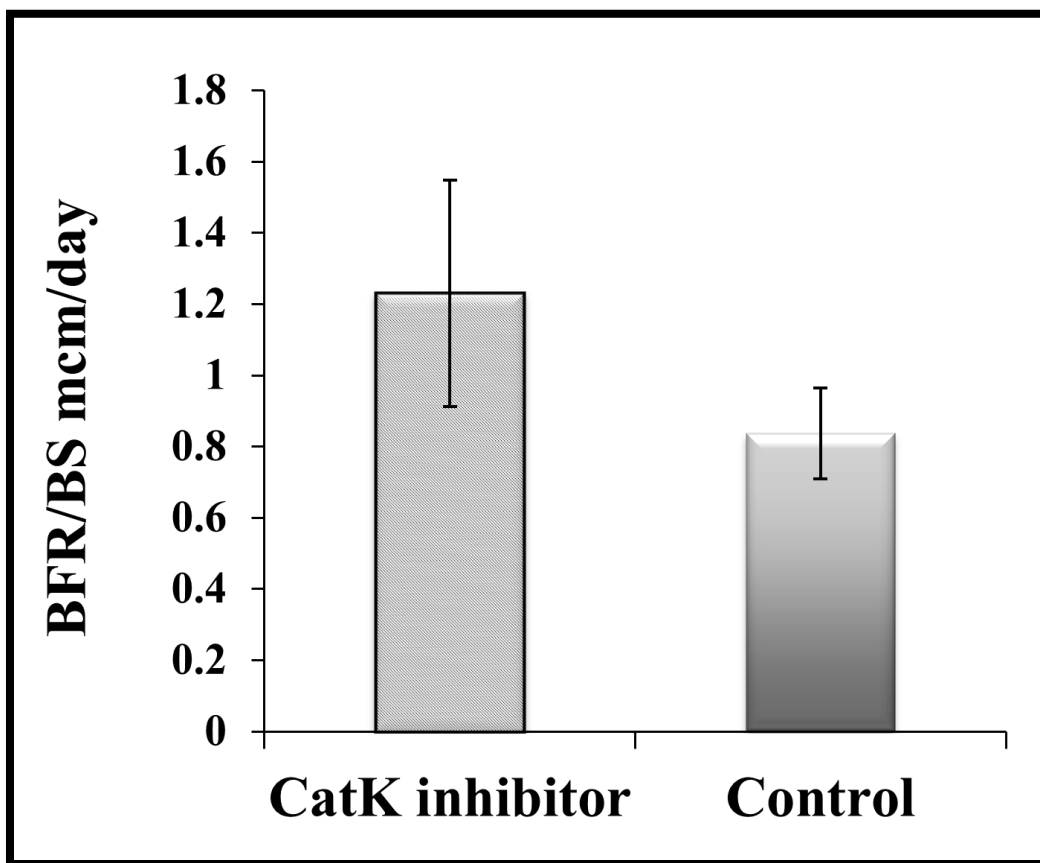
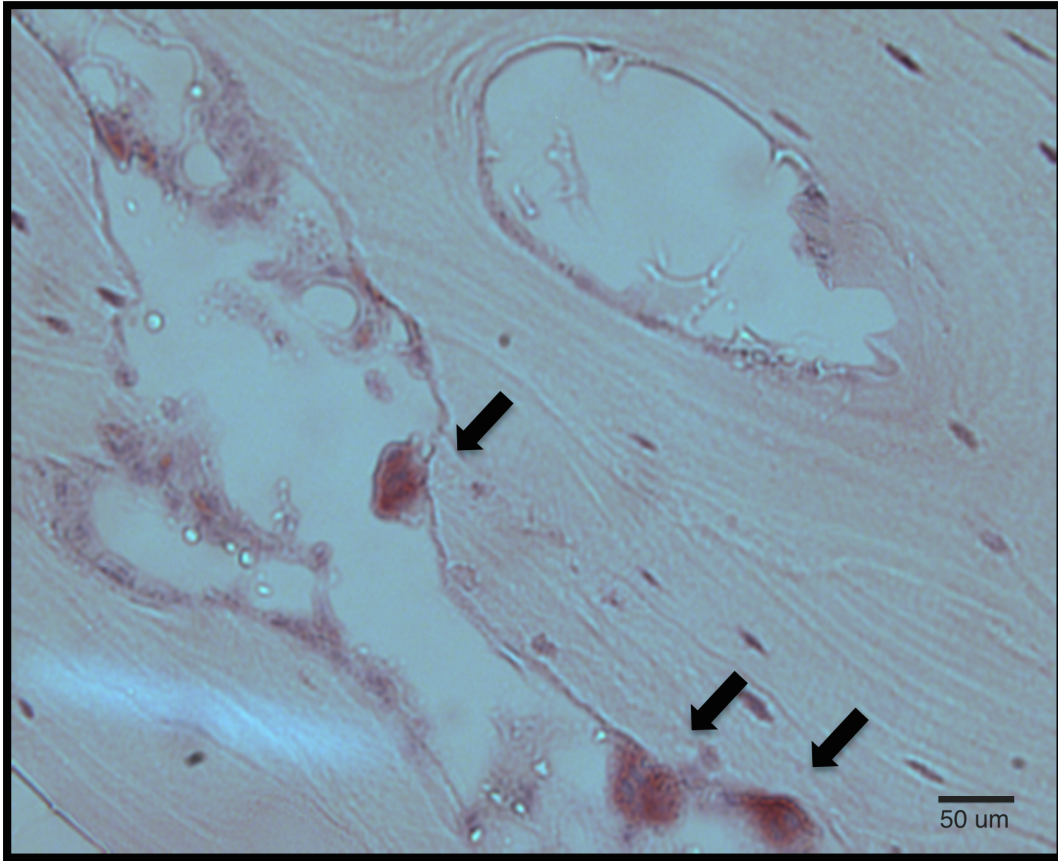
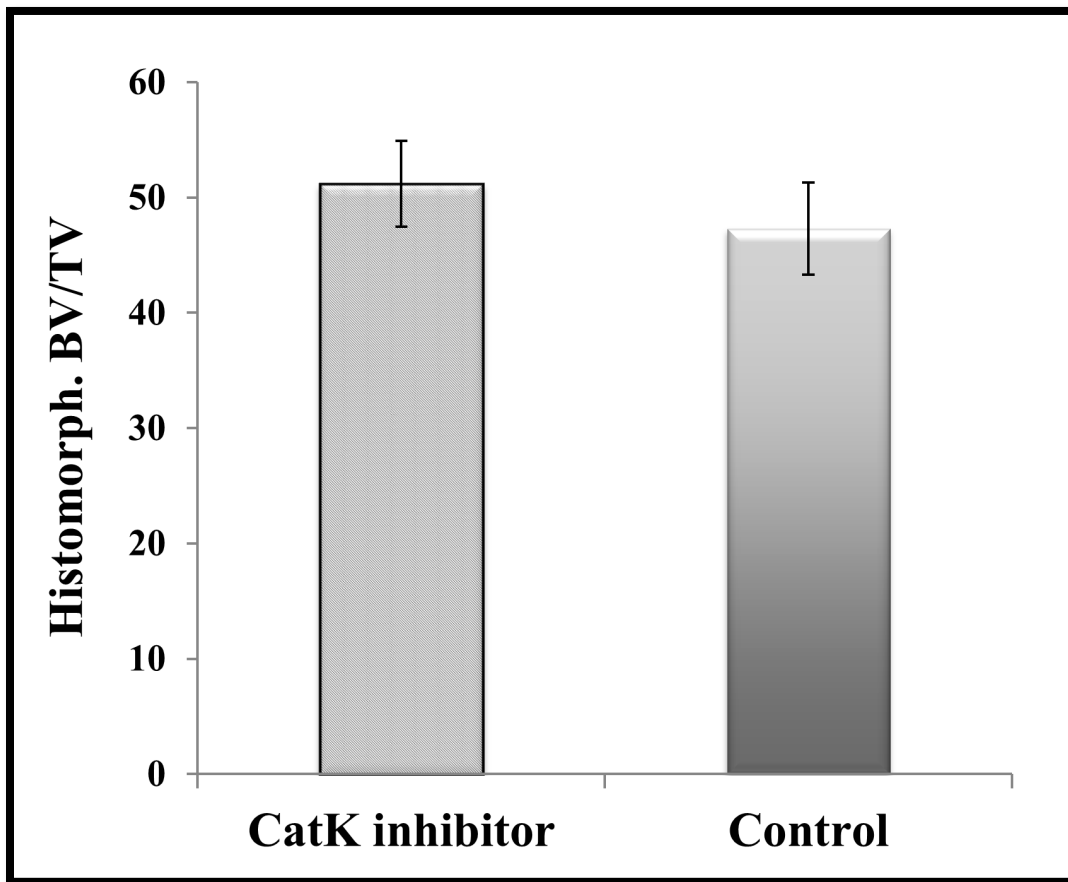


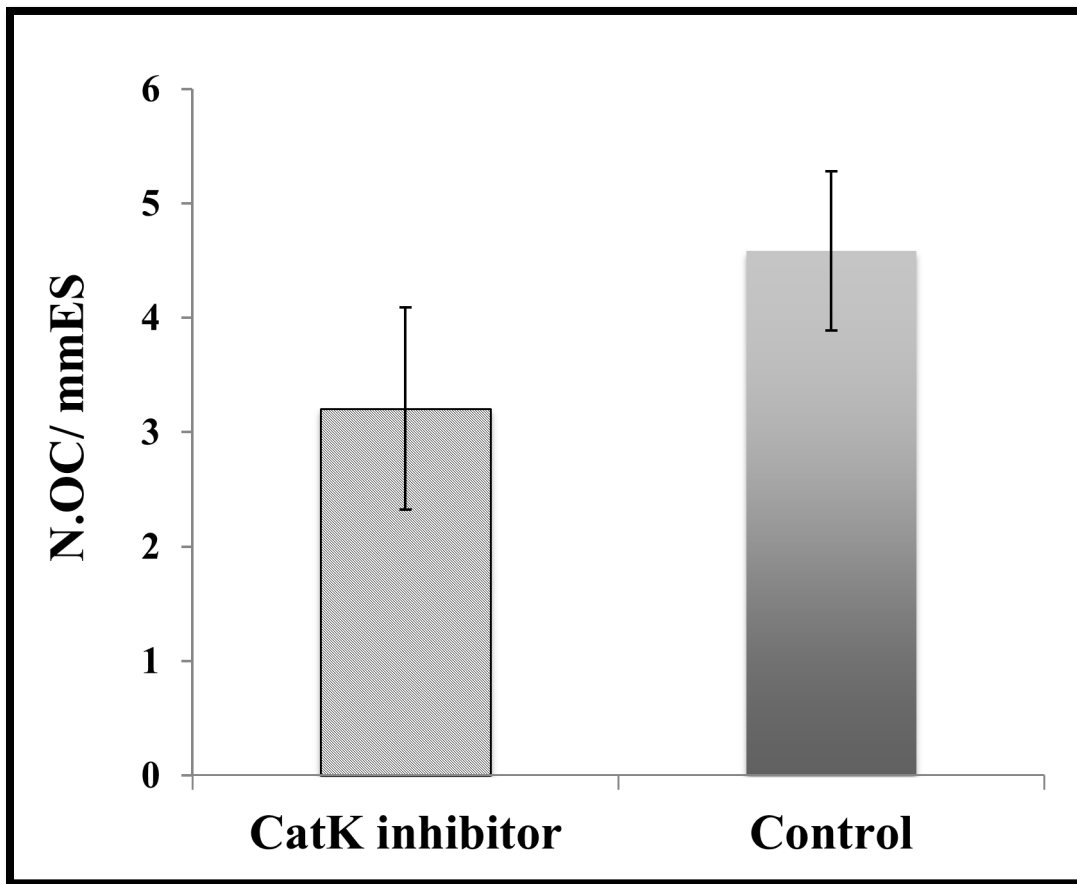
Figure 22: Mean  $\pm$  SEM BFR.



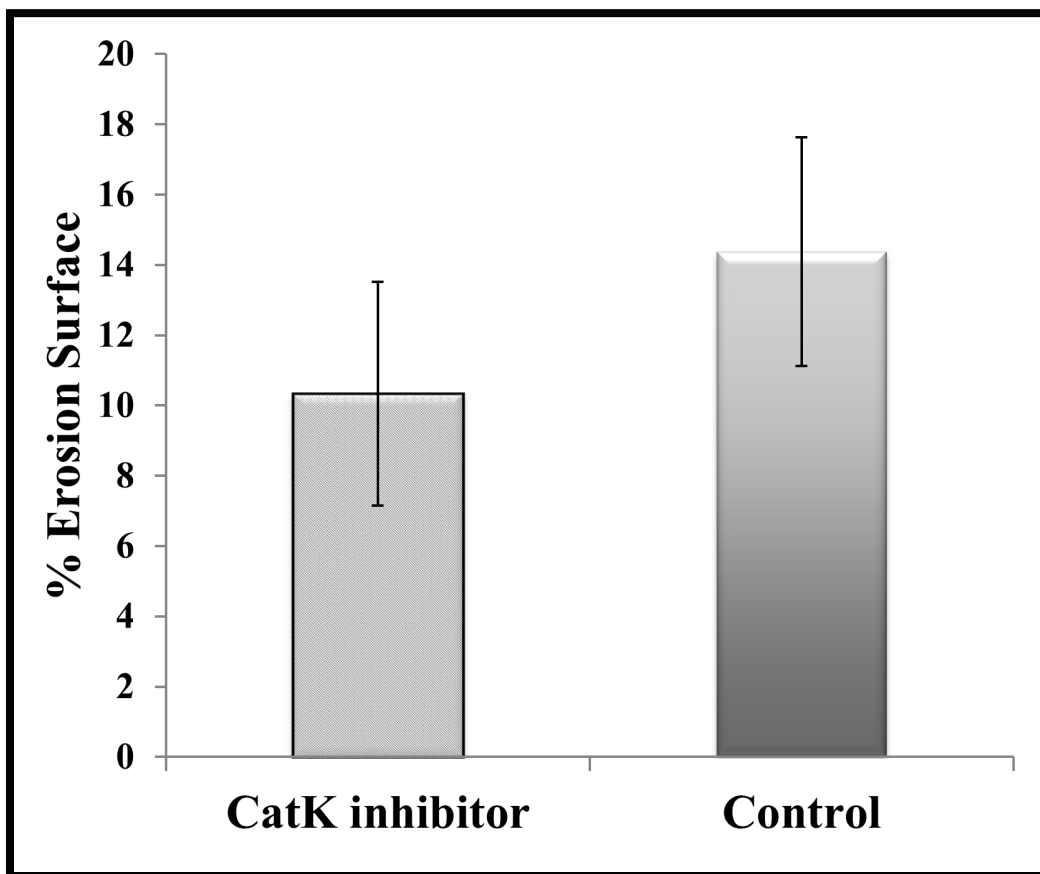
**Figure 23:** Microscopic image of TRAP positive (red) osteoclasts within the resorption pits (black arrows).



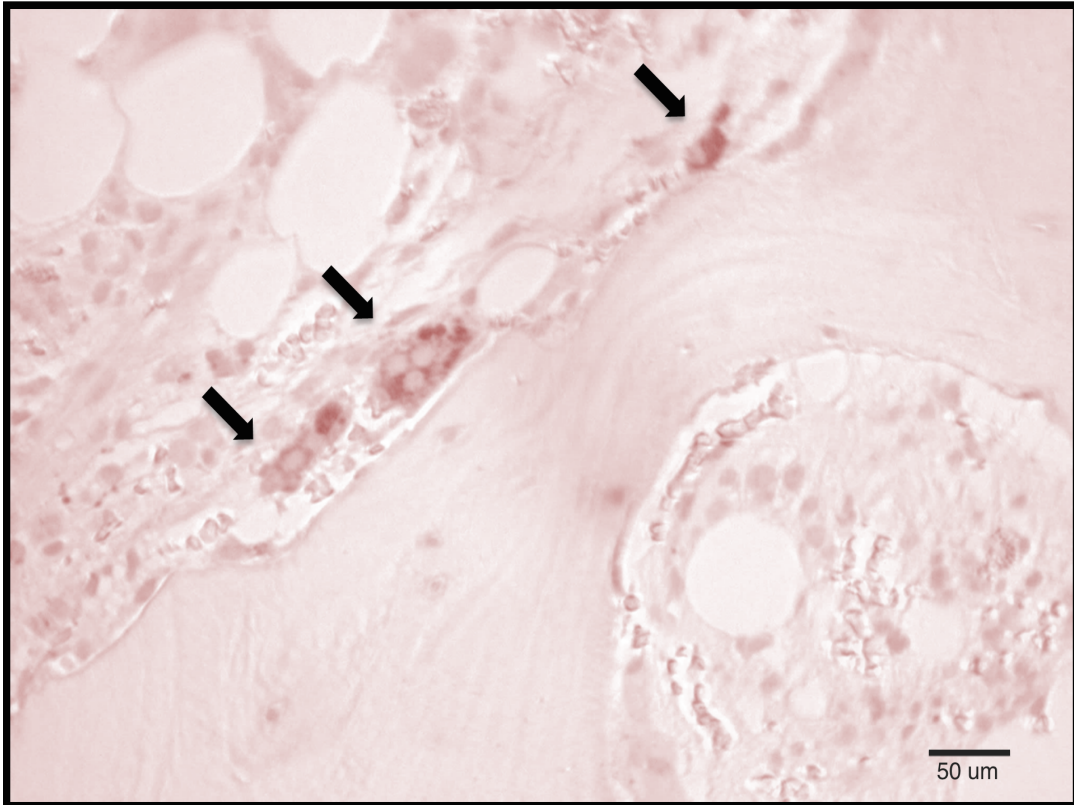
**Figure 24:** Mean  $\pm$  SEM histomorphometric BV/TV. No statistical differences were found between groups.



**Figure 25:** Mean  $\pm$  SEM histomorphometric N.O.C/ES. No statistical differences were found between groups.

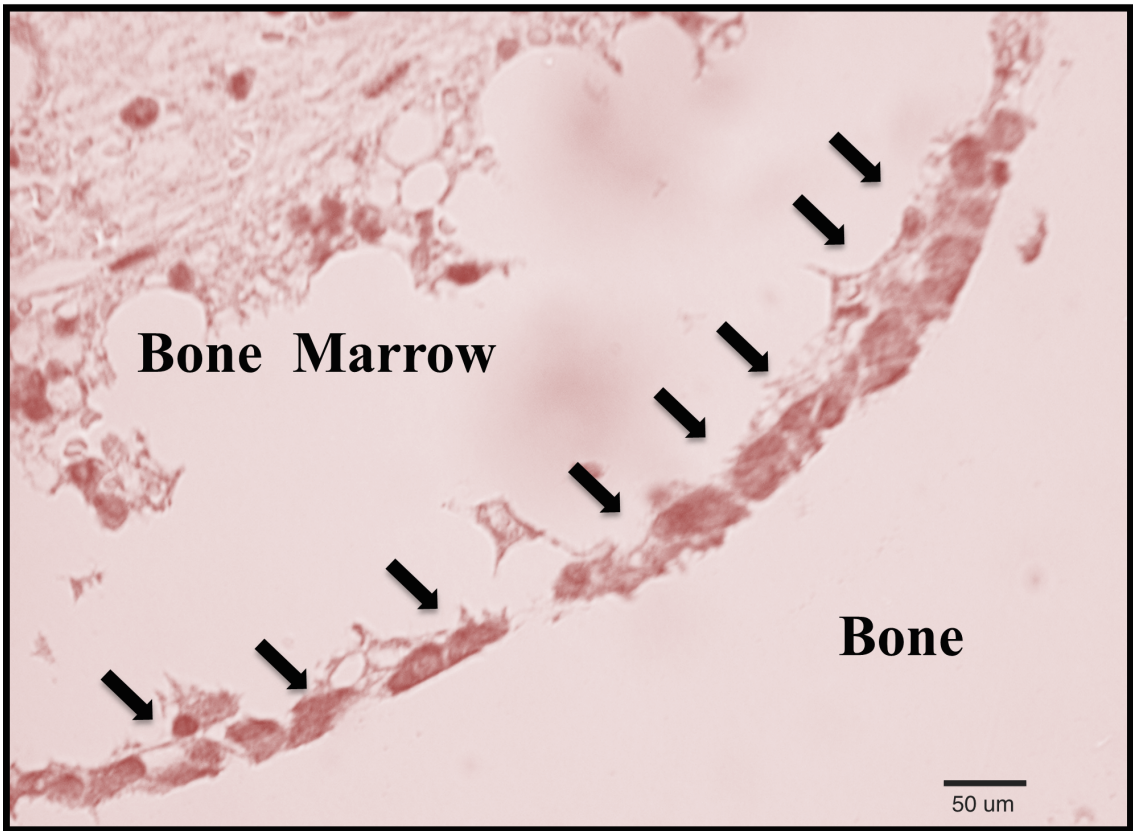


**Figure 26:** Mean $\pm$ SEM histomorphometric ES/BS. No statistical differences were found between groups.

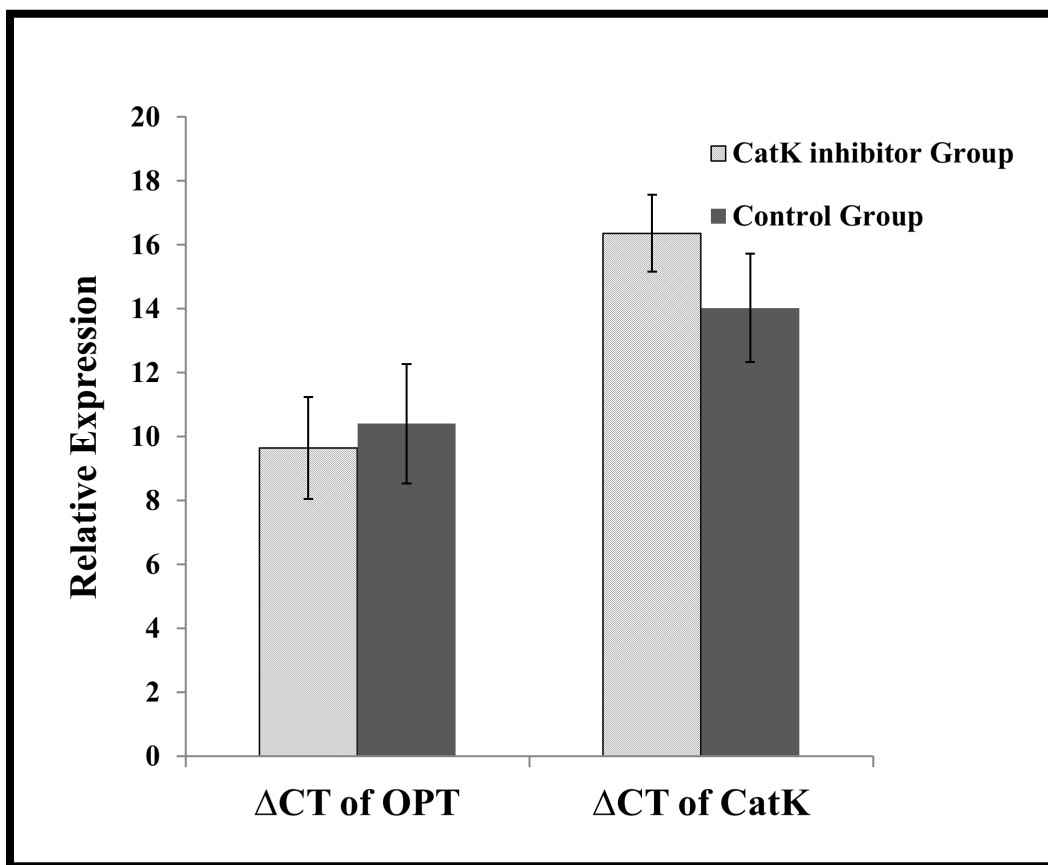


**Figure 27:** Microscopic image of Cathepsin K-positive, multinucleated osteoclasts (black arrows) within the resorption pits.





**Figure 28:** Microscopic images of Cathepsin K-positive osteoblasts (black arrows).



**Figure 29:** Relative gene expression measured by quantitative real time PCR and expressed as  $\Delta C_t$  values of osteopontin (OPT) and cathepsin K (CatK) calculated using a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene.

## **DISCUSSION:**

Our paper is the first to report the safe, repetitive oral dosing of a CatK inhibitor (VEL-0230) in young exercising animals, specifically horses. No abnormal systemic effects were detected with repeated administration of this CatKI. Our study additionally confirmed that VEL-0230 administration caused a significant and sustained (5–6 days) decrease in the plasma bone resorption biomarker CTX-1 following each weekly dose. Plasma CTX-1 returned to pre-dosing values before each subsequent dose administration (7 days later) with no evidence of drug accumulation. These data also confirmed and extended the results of a previous study (4) showing that young exercising horses with prior active race training showed markedly elevated baseline plasma CTX-1 values and exhibited the greatest percent-inhibition of CTX-1. Similar results were seen in a previous study that reported elevated levels of baseline CTX-1 in racehorses (17).

Our study provided evidence of bone resorption inhibition with each dose without negative influence on adaptive bone remodeling (the accelerated normal process of coupled bone resorption preceding bone deposition) induced by exercise in these young animals. Multiple studies demonstrated repeated evidence of bone resorption inhibition without negative influence on bone remodeling using other CatKIs (18, 19) but not with bisphosphonates treatment (20, 21). Exercised horses in both CatKI-treated and control

groups had remodeled the dorsal metacarpal cortex during the 5 weeks of exercise with detectable alteration in bone formation between the groups as measured by bone formation biomarker, osteocalcin. The reduction in cortical width of MCIII in exercising young horses is an anticipated finding due to exercise-induced homeostatic changes in endosteal and periosteal bone resorption. This reduction suggested an effect of exercise; what is known as exercise-induced bone resorption (22). Bone resorption preceding formation is a well-established fact in healthy and diseased bone (23, 24). This is a recognized feature in young racehorses in training (25, 26). The increase in bone remodeling with bone resorption preceding formation has been noted in other exercise-based investigations and has been associated with micro-damage to the bone (27). Moreover, the dorsal cortex is considered the major site of exercise-induced bone remodeling (10). We interpreted this finding as evidence that repeated CatKI administration did not interfere with active bone remodeling in healthy exercising young horses. Our study was not designed to test for the effect of exercise, thus bone biopsies were only obtained at the end of the study because galloping horses would fracture at the bone biopsy site. However, the data from the vehicle control group demonstrated that CatK inhibition did not influence basal levels of remodeling. As expected, exercising horses demonstrated a greater percentage of active trabecular osteons and mineralizing surface than resting equine dorsal cortex previous data and likely reflects the stimulation of exercise (data not shown) (28, 29).

Cathepsin K is the primary enzyme responsible for osteoclast-mediated degradation of organic bone matrix during bone resorption (6, 30). Unlike other CatKIs, VEL-0230 is unique in that it is also a powerful anti-inflammatory (3, 31). Inflammation-induced

bone resorption is often associated with disease processes of injured periosteum, bone microfracture or fracture, particularly in racehorses (32), human athletes (33) and soldiers (34). This osteo-inflammatory process represents a different mechanism than adaptive physiologic, load-induced bone resorption and exercise-induced bone resorption. Previous work showed that VEL-0230 inhibition of pro-inflammatory cytokines reduced the susceptibility of Cathepsin K deficient mice to autoimmune encephalitis; it also prevented and treated adjuvant-induced arthritis in rodent disease models (3). This implies a functional role for CatK in the innate immune and chronic inflammatory responses that may be important in inflammatory bone diseases such as excessive exercise-induced stress remodeling.

The CatKI-treated horses were not different from control horses in the amount or focal intensity of bone uptake of the radiopharmaceutical indicating similar bone turnover and activity. Nuclear bone scans are functional tests that measure an aspect of bone metabolism or bone remodeling (35) and thus allow evaluation of exercise-induced bone remodeling differences. Bone biopsies, taken from both weight bearing (P1) and non-weight bearing (TC) bone and analyzed by *ex-vivo*  $\mu$ CT, showed no significant effect of CatKI administration on SA:V, %BV, and BMD of either bone type. Histomorphometric bone formation rate was greater, but not significantly different, in CatKI group than control group. However, bone formation biomarker was greater in CatKI group compared to the control one. In concert, these data supported the conclusion that VEL-0230 had no significant effect on bone remodeling in young, exercising healthy horses.

Bone biopsy studies demonstrated that horses receiving CatKI did not differ from control horses in both numbers of TRAP-positive osteoclasts, number of osteoclasts per eroded surface, or CatK-positive osteoclasts. The lack of change in blood monocytes also supported this observation considering that osteoclasts are known to be driven from monocytic-macrophage cell origin (36).

No significant effect on bone formation was detected in the measures of bone quality and mineral density using various bone imaging modalities. Interestingly, some bone lining active osteoblasts did stain positive for CatK. Similar findings were reported in human osteoblasts (37) and may represent a recent novel finding. Interestingly, CatKI appeared to be activating osteoblast function in bone formation and not reducing the number of these CatK-positive osteoblasts than the control group (data not shown).

There is a time lag in exercise-induced remodeling with bone resorption preceding the signaling to induce bone formation, usually taking 5 weeks (38). Consistent with this, a significant increase in bone mineral densities in other species (non-human primates and rats) following daily VEL-0230 treatment were not seen until 2 -3 months of treatment (1). Thus, measurable increase in equine bone mineral densities might be detected if VEL-0230 administration had continued beyond 4 weeks. Investigations of other CatKIs-mediated suppression of bone resorption in humans and animals have failed either to detect a change on bone formation biomarkers or actually reported a significant decrease in bone formation over 21-27 weeks of treatment (39, 40).

Other ongoing *in vitro* studies demonstrated that VEL-0230 inhibited immune response and pro-inflammatory cytokine secretion of horses' bone marrow cells in response to lipopolysaccharide and unmethylated CpG motifs (Authors, unpublished data). The

anticipated outcome of CatKI therapy is reduced bone resorption during inflammatory bone diseases such as excessive exercise-induced bone trauma. However, this would not be expected in normal bone of young animals remodeling in response to training exercise (e.g., racehorse or other performance athletes), as homeostatic control mechanisms differ. In healthy animals, turnover is regulated predominately by the RANK/RANK-Ligand system and matrix-released growth factors such as TGF $\beta$ ; pro-inflammatory cytokines do not typically play a role in normal bone homeostasis (41-45).

Overall, the data from our investigations support the concept that bone CatK inhibition is unique as it produced marked inhibition of bone resorption and increase in bone formation without reducing osteoclast numbers or affecting bone homeostasis in healthy horses. This is unlike other studies using anti-resorptive drugs (e.g. bisphosphonates and Anti-RANKL). This critical difference from other drugs that permanently suppress osteoclast activity and numbers (e.g. bisphosphonates) is important. Valid concerns exist about using drugs on young athletes (animal and human) that interfere with the necessary normal adaptive bone remodeling to exercise (46, 47).

In summary, administration of CatKI to equine athletes in training for 1 month did not influence the ability of bone to normally adapt to exercise. Bone morphology and density were not different to controls by all intensive measures. Bone formation was greater due to repeated CatKI administration. Our findings support the potential use of CatK inhibition by VEL-0230 in young racehorses that may have bone inflammatory disorders and combined with changes in training protocols, would be expected to

accelerate convalescence and maintenance of fitness. Continued investigation of this compound for stress related bone diseases is warranted.



## **Chapter 4**

# **Effect Of Repeated Oral Administration Of A Cathepsin K Inhibitor On Equine Stem And Progenitor Cells Differentiation *In Vitro*.**

### **SUMMARY:**

This study aimed to determine the effect of repeated oral administration of a Cathepsin K inhibitor (CatKI), VEL-0230, in a randomized controlled clinical trial for 4 weekly doses on equine bone marrow-derived stem and progenitor cells differentiation *in vitro*. Bone marrow was harvested from the sternum of six horses (3 assigned to receive CatKI and 3 assigned to receive control vehicle) prior the start of the study and from all twelve horses 24 hours after the fourth weekly dose of CatKI (n=6) or vehicle (n=6). Bone marrow was cultured in monolayer to select/deselect bone marrow derived-mesenchymal stromal cells (BMD-MSCs) and cell differentiation assays, characterization by flow cytometry and differentiation capacities were studied. We hypothesized that neither exercise nor repeated oral administration of VEL-0230, a potent and selective CatKI, would affect the

characterization or differentiation capacity of equine bone marrow derived-stem and progenitor cells, specifically chondrogenic, osteogenic or osteoclastogenic tri-lineage differentiation or expression of stem cell markers. Bone marrow cells from both groups were multi-potent and showed strong capacity to differentiate into chondrogenic, osteogenic and osteoclastic pathways with no difference in staining scores between the CatKI and control groups. There was no significant difference in expression of CD90, CD34, CD11b, MHCI, and MHCII molecules from before exercise to after exercise in either the control or CatKI groups. There was no difference in viability or differentiation capacity between control and CatKI groups as determined by 7AAD, Toluidine blue, von Kossa, Alizarin red, and Tartrate-resistant acid phosphatase (TRAP) staining. Cathepsin K inhibition for 4 weeks did not alter the regenerative ability of bone marrow, specifically for bone formation (osteoblastogenesis) or remodeling (osteoclastogenesis).

## **INTRODUCTION:**

Cathepsin K (CatK) is a lysosomal cysteine protease that is highly expressed in osteoclasts, the bone resorbing cells and has a high collagenase activity, specifically to Type I collagen, a major component of bone extracellular matrix (1). The emerging evidence that CatK is the primary enzyme involved in osteoclastic bone resorption has made it an important target for the treatment of bone resorption disorders (2). An un-expected role for CatK in the immune response and inflammation has been also revealed (3). We have evaluated the use of a potent CatKI, VEL-0230, in healthy exercising horses for future therapeutic application in

equine patients with osteo-inflammatory conditions (4, Hussein et al., un-published data). Cathepsin K expression has been detected in cells other than osteoclast, and its inhibition was found to affect specific signaling pathways such as TLR9 and TLR4 down-stream signals (3, 5, Hussein et al., un-published data). Equine bone marrow serves as a source of mononuclear cells, growth factors and stem and progenitor cells. It has been used clinically to promote repair of musculoskeletal tissues in attempt to accelerate fracture healing and articular cartilage regeneration (6). The purposes of this study therefore, is to determine the effects of sustained CatK inhibition on equine bone marrow derived-stem and progenitor cells in monolayer culture *in vitro*.

We hypothesized that neither exercise nor repeated oral administration of VEL-0230 for a month would affect the characterization or differentiation capacity of equine bone marrow derived-stem and progenitor cells, specifically chondrogenic, osteogenic or osteoclastogenic tri-lineage differentiation or expression of stem cell markers *in vitro*.

#### **MATERIAL AND METHODS:**

Sternal bone marrow was aspirated from 18 horses; 12 horses administered repeated oral doses (4mg/kg) of CatKI (n=6) or a vehicle (n=6) at the termination of the 4-week protocol (Fig. 9), from 6 normal horses prior to the experiment.

***Oral administration of VEL-0230 and vehicle:***

A CatKI, VEL-0230, and a vehicle (Freestride Therapeutics, Inc. MI, USA) were administered orally as previously described (4) at a dose of 4mg/kg b.w. once a week for 4 successive weeks in the treatment and control group, respectively.

***Bone marrow aspiration, processing, and analysis:***

All horses were sedated with 0.01mg/kg body weight (b.w.) detomidine hydrochloride (Domitor, Pfizer Animal Health, Exton, PA) and 0.02 mg/kg b.w. butorphanol (Torbugesic, Fort Dodge Animal Health, Fort Dodge, Iowa) intravenously. An area of 5 x 20 cm over the sternum was clipped, scrubbed, aseptically prepared and the intersternbral spaces were identified by palpation or ultrasonography. Local infiltration of 2 ml anesthetic solution, mepivacaine hydrochloride 2% (Carbocaine, Cooke-Waite Laboratories, Inc., NY), was performed subcutaneously over the midpoint in the sagittal plane of two adjacent sternbrae. A stab incision using a No. 11 scalpel blade was then made through the skin and a Jamshidi biopsy needle (11 gauge, 10 cm, Ranfac Corp, Avon, Mass) pre-rinsed with heparin (Multiparin 5000 iu/ml) was introduced for approximately 4-6 cm until it contacted the sternbra. The needle was then further pushed 3 or 4 cm into the sternbra and 60cc of bone marrow was aspirated into two 30 ml

syringes, preloaded with 3 ml heparin each. The two 30 cc syringes were then loaded into one Marrowstim<sup>®</sup> concentration system (BIOMET Biologics, Warsaw, IN, USA). Bone marrow nucleated cells were immediately isolated according to the manufacturer's instructions and the method described by Ishihara *et al.* [14], and then transferred into sterile 10 ml syringes. The syringes were then placed on ice for immediate transfer to the laboratory

The concentrated bone marrow was washed, centrifuged at 500 g and re-suspended in 10 ml phosphate buffered saline for further analysis. Processed bone marrow was analyzed for automated total and differential WBCs count, differential WBCs manual cytology, bone marrow smears (Clinical Hematology lab, College of Veterinary Medicine, The Ohio State University), flow cytometry, isolation of non-adherent cells, and cultured to select the adherent mesenchymal fraction as described below.

***Flow cytometry: Immunostaining and analysis of stem cell markers and MHC molecules:***

An aliquot of the processed fresh concentrated bone marrow ( $5 \times 10^5$  cells) were stained for 30 minutes in the dark at 4°C with one of the following fluorochrome-labeled antibodies (Abs): anti-CD90, anti-CD11b, anti-CD34 (BD Biosciences and eBiosciences, San Diego, CA). For MHC I and MHC II immuno-staining, cells were stained with either anti-equine MHC I monoclonal Ab (Clone CVS22, AbD Serotec, Raleigh, NC) followed by anti-mouse secondary antibody (FITC rat anti-mouse IgG1, clone A85-1, BD Biosciences, San Jose, Calif), or anti-equine MHC II

monoclonal Ab (Clone CVS20, AbD Serotec, Raleigh, NC) followed by anti-mouse secondary antibody. After incubation, cells were washed twice in FACS buffer and re-suspended in 200  $\mu$ l FACS buffer for flow cytometry analysis (Accuri Cytometers, San Jose, CA). A gating procedure was generated by generating a cytogram of SSC versus forward scatter light and gated to exclude cell debris by including only cells with relatively high SSC and forward scatter light values. Quadrant cursors were set by using an appropriate negative control; either unstained control cell preparation or fluorochrome-labeled secondary antibody control preparation. Quadrants were set so that in negative controls, 99% of the cells were localized in the left lower quadrant. The percentage and absolute number of cells labeled by each antibody was determined for each experimental condition per each animal.

***Osteoclastogenesis assay:***

An aliquot of processed fresh concentrated bone marrow were cultured to select non-adherent cells, which represented hematopoietic and monocyte/ macrophage progenitor cells (precursors of osteoclasts), as well as other white blood cells. These selected cells were driven to differentiate into equine osteoclast-like cells (OCLs) in culture using the following protocol; Cultures were kept sterile at 37° C in 5 % CO<sub>2</sub>. The cell suspension was pre-cultured at approximately  $1 \times 10^7$  cells  $\text{cm}^{-2}$  in a 75  $\text{cm}^2$  culture flask for 6 hours at 37°C, % CO<sub>2</sub>. The non-adherent cells, which were enriched for mono- nuclear cells were collected and centrifuged at 500 g, 4° C for 10 minutes and re-suspended in a MEM, pH 7.2, supplemented with 10 % (v/v)

FBS, 25ngml<sup>-1</sup> rhM-CSF (R&D Systems), 10ngml<sup>-1</sup> rhRANKL (R&D Systems), 10<sup>-8</sup> M 1,25 dihydroxyvitamin D3 (vitamin D3, Affiniti Research Products, UK). Cells were plated at a density of 2.5X10<sup>6</sup> cells cm<sup>-2</sup> in collagen-coated 6 well plates. Half the medium was replaced twice weekly. After culture of cells for 7 days, non-adherent cells were removed by trypsinisation for 2 minutes. After 15 minutes of further trypsinisation the remaining cells, which were enriched for OCLs, were collected by gentle scraping using a cell scraper and cytopins prepared on poly-L-lysine coated slides (Sigma, St Louis, MO). The cytopin preparations of OCLs were processed for TRAP staining using a commercial acid phosphatase leucocyte kit (Sigma, St Louis, MO) according to the manufacturer's protocol. The number of TRAP positive multinucleated cells ( $\geq 3$  nucleus) in 5 representative fields under 20X magnification was counted and averaged number of OCLs for each experimental condition was obtained.

### ***Chondrogenesis:***

Mesenchymal stromal cells (MSCs), selected by adherence in monolayer culture of processed fresh concentrated bone marrow for up to 3 days, were isolated and differentiated to chondrocytes using Lonza® Poietics™ human mesenchymal stem cell media and modified protocol. Fifteen ml centrifuge tubes were used for chondrogenic induction, with 1 X 10<sup>6</sup> MSCs/tube to form a pellet. After induction for 14 to 28 days, pellets were removed and fixed by incubation in formalin for 24 hours. Formalin-fixed chondrocyte pellets were sectioned and stained with Toluidine blue (Fisher scientific, USA) for analysis by microscopy. Degree of

cartilaginous extracellular matrix staining (purple color) was scored by a blinded evaluator (HH) as: 0 (no staining), 1 (mild staining), 2 (moderate staining), 3 (marked staining).

***Osteogenesis assay:***

The MSCs were also differentiated to osteoblasts using Lonza® Poietics™ human mesenchymal stem cell media and modified protocol. A 6-well plate was used for osteogenic induction with  $1 \times 10^6$  MSCs/well. Basal medium was used for the first 24 hours to allow adherence. Induction medium was changed every 3 days thereafter. The cells were analyzed after 2 to 3 weeks for calcium, in accordance with a von Kossa and alizarin red S staining protocols. In brief, the wells were washed twice with PBS solution and then fixed in phosphate-buffered 10% formalin for 10 minutes. The monolayers were then processed for von Kossa and alizarin red S staining. For von Kossa staining, cells were serially dehydrated in solutions of ethanol (70%, 95%, and 100%; 2 times for each concentration) and then allowed to air dry. Cells were then serially rehydrated in solutions of ethanol (100%, 95%, and 80%; 2 times for each concentration). Finally, cells were rehydrated in water twice. Silver nitrate solution (2%) was added, and the cells were exposed to sunlight for 20 to 40 minutes. Cells were then rinsed 2 times with water, and 5% sodium thiosulfate was added; cells were allowed to equilibrate for 3 minutes. Cells then were rinsed with water, and acid fuchsin counterstain (5 mL of 1% acid fuchsin with 95 mL of picric acid and 0.25 mL of 12N HCl) was added. After incubation for 5 minutes, the cells were washed with water (2 times), 95%



ethanol, and 100% ethanol before image analysis by use of a viewing microscope where mineralized nodules were stained dark brown or black. For Alizarin red S staining, 2% alizarin red S (Sigma) was prepared in distilled water and the pH was adjusted to 4.1–4.3 using 0.5% ammonium hydroxide. Formalin-fixed osteogenic monolayers were washed, and stained with alizarin red S for 10–15 minutes. After removal of unincorporated excess dye with distilled water, the mineralized nodules were labeled as red spots. The degree of mineralized nodules staining for both stains was scored as following: 0 (no staining), 1 (mild staining), 2 (moderate staining), 3 (marked staining).

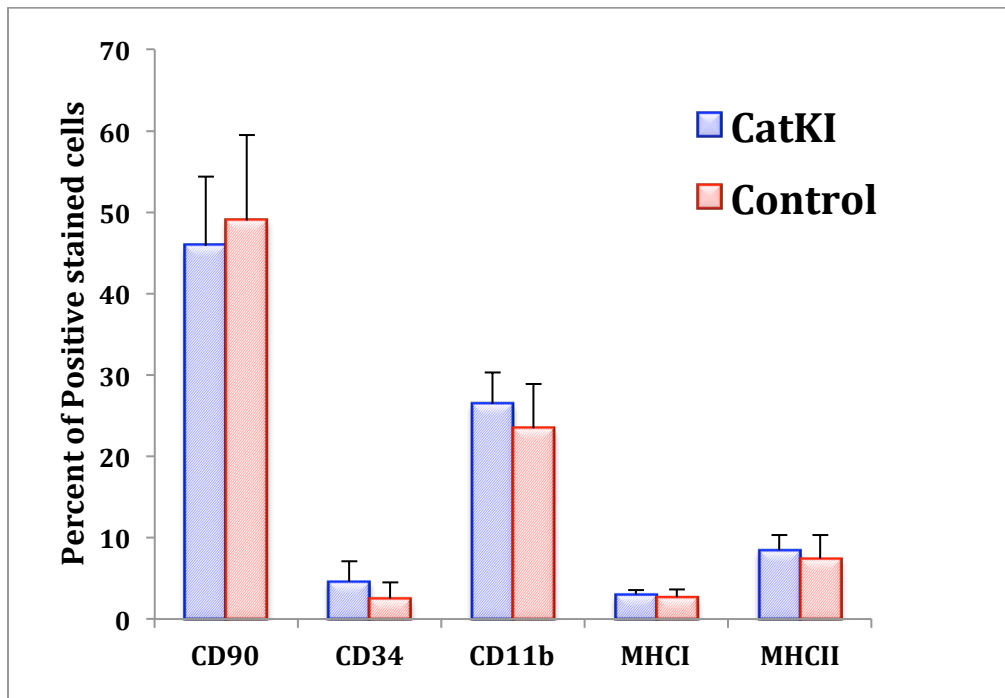
***Statistical analysis:***

Data for all outcomes were statistically analyzed using SPSS v.18.0 (IBM Corp., Armonk, NY, USA). The data was then graphed as mean with standard deviation (SD). The normal distribution within the different variables was investigated using Shapiro-Wilk test. The values in the CatKI and the control groups obtained post-study were compared using student t-test for normally distributed data and Mann-Whitney U rank test for skewed data. The effect of exercise (time) and treatment (CatKI) were evaluated in the values of the CatKI and the control groups obtained pre-study and post-study using repeated measures analysis of variance. Statistical significance was determined when  $P < 0.05$  and trend differences were discussed when  $0.10 > P > 0.050$ .

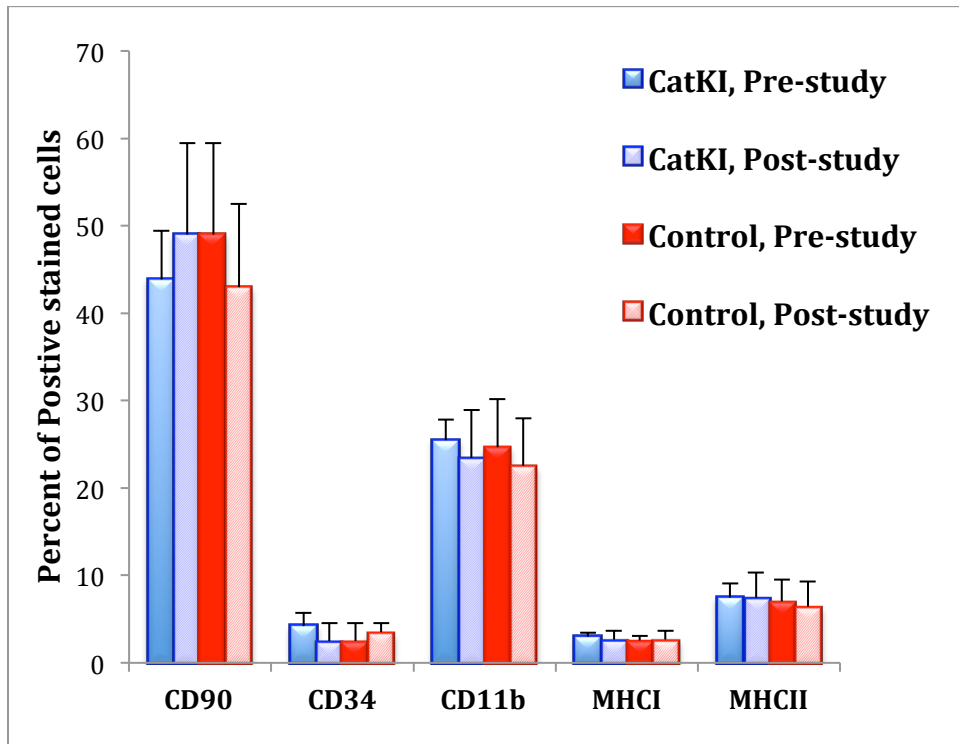
## **RESULTS:**

### ***Repeated oral administration of CatKI did not alter the stem cell markers or immune related molecules expression:***

Bone marrow automated and manual differential count as well as flow cytometric characterization of different cell surface markers did not reveal differences between CatKI treatment and vehicle or before and after exercise for the 1-month protocol. Figure (30) shows mean $\pm$ SD of the percent of cells in the fresh concentrated bone marrow that stained positive for CD90, CD34, CD11b, MHCI, and MHCII in CatKI and control group post-study. Figure (31) shows the same parameters compared between CatKI and control group at pre to post-study.



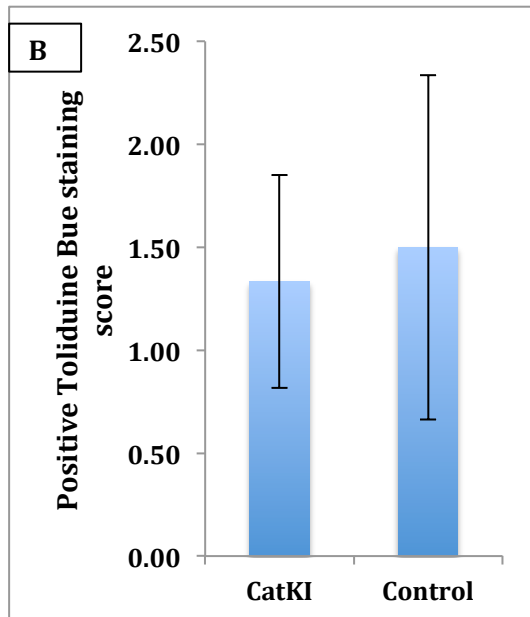
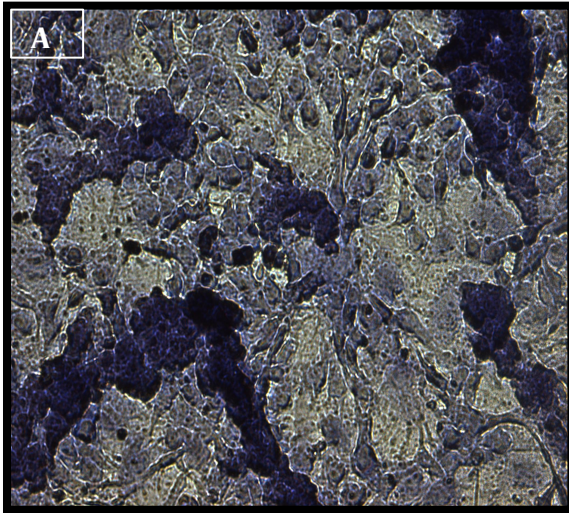
**Figure 30:** Mean±SD of the percent of fresh concentrated processed bone marrow cells stained positive for CD90, CD34, CD11b, MHC I, and MHC II in CatKI and control group post-study. No significantly statistical difference was found between groups.



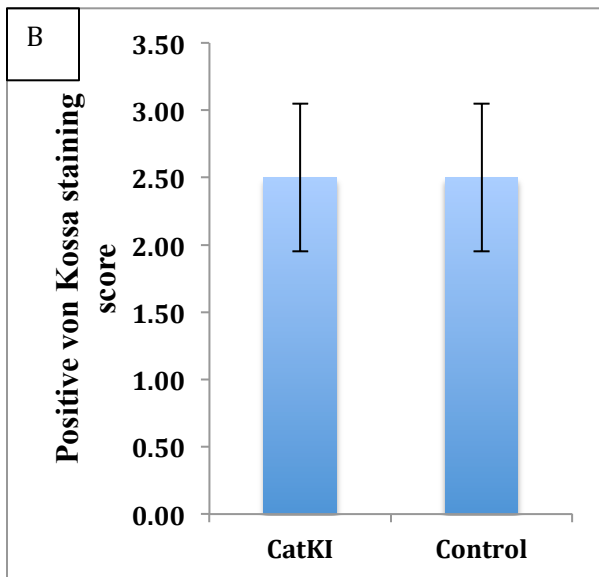
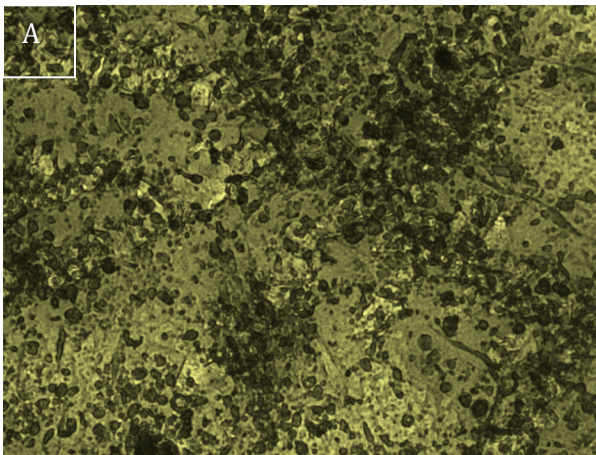
**Figure 31:** Mean±SD of the percent of fresh concentrated processed bone marrow cells stained positive for CD90, CD34, CD11b, MHCI, and MHCII in CatKI and control group post-study. No significant statistical effect for exercise (time pre to post-study) or for the CatKI treatment.

***Repeated oral administration of CatKI did not alter the chondrogenic, osteogenic, and osteoclastogenic differentiation capacities of equine stem and progenitor cells in vitro:***

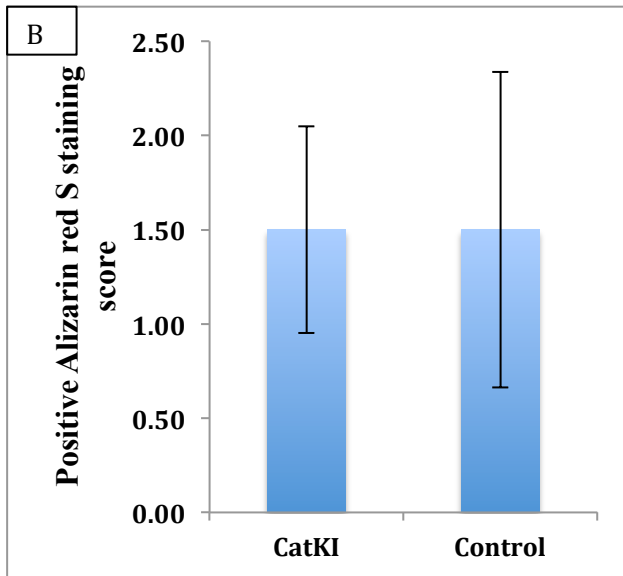
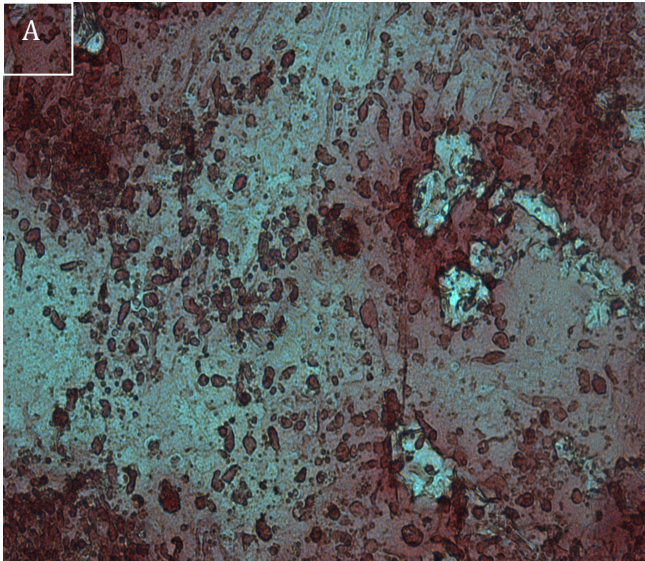
Bone marrow semi-quantitative cellular staining microscopic analyses did not reveal a difference in differentiation capacities of equine bone marrow-derived stem and progenitor cells. Chondrogenic assay data are shown in Fig. 32; Fig. 32A shows Toluidine blue stained chondrocyte extracellular matrix, and Fig. 32B shows mean $\pm$ SD Toluidine blue scoring for both CatKI and control groups. Osteogenic assay data are shown in Fig. 33 & 34; Fig. 33A shows von Kossa stained mineralized matrix of osteoblast like cells and Fig. 33B shows mean $\pm$ SD von Kossa scoring for both CatKI and control groups. Alizarin red S stained mineralized matrix is shown in Fig. 34A and Fig. 34B shows mean $\pm$ SD Alizarin red S scoring for both CatKI and control groups. Osteoclastogenesis assay data are shown in Fig. 35; Fig. 35A shows TRAP stained multinucleated OCLs and Fig. 35B shows mean $\pm$ SD of averaged number of TRAP positive multinucleated cell for both CatKI and control groups.



**Figure 32:** Toluidine blue staining and scoring. A: Bone marrow derived-mesenchymal stromal cells isolated following repeated oral administration of a CatKI or a vehicle and cultured in chondrogenic media showing marked Toluidine blue positive staining. B: Mean±SD Toluidine blue scoring for bone marrow derived-mesenchymal stromal cells isolated following repeated oral administration of a CatKI or a vehicle and cultured in chondrogenic media.

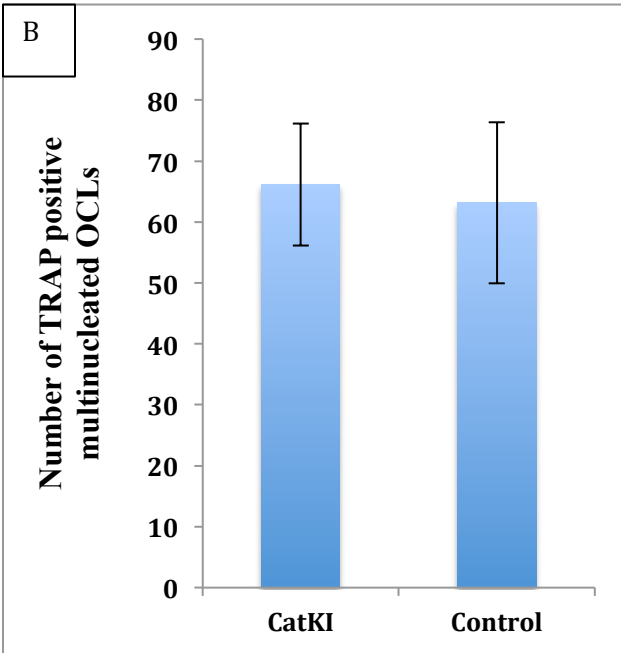
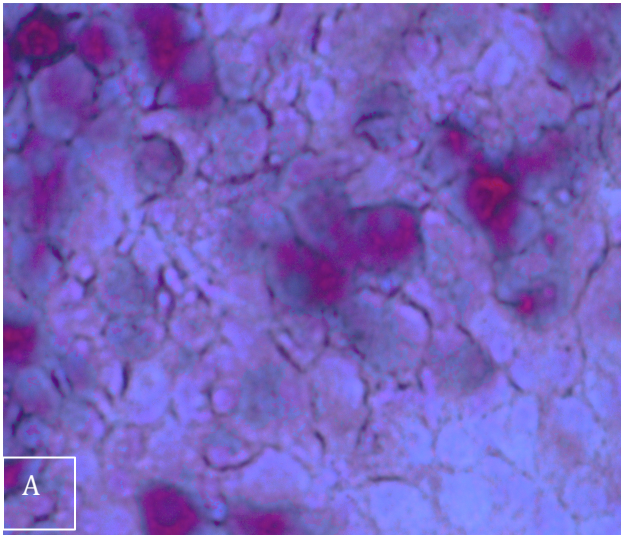


**Figure 33:** von Kossa staining and scoring. A: Bone marrow derived-mesenchymal stromal cells isolated following repeated oral administration of a CatKI or a vehicle and cultured in osteogenic media showing marked von Kossa positive staining. B: Mean±SD von Kossa stain scoring for bone marrow derived-mesenchymal stromal cells isolated following repeated oral administration of a CatKI or a vehicle and cultured in osteogenic media.



**Figure 34:** Alizarin red S staining and scoring. A: Bone marrow derived-mesenchymal stromal cells isolated following repeated oral administration of a CatKI or a vehicle and cultured in osteogenic media showing marked alizarin red S positive staining. B: Mean±SD alizarin red S stain scoring for bone marrow derived-mesenchymal stromal cells isolated following repeated oral administration of a CatKI or a vehicle and cultured in osteogenic media.





**Figure 35:** TRAP staining and analysis. A: Bone marrow derived-mononuclear fraction isolated following repeated oral administration of a CatKI or a vehicle and cultured in osteoclastogenic media showing marked TRAP positive multinucleated OCLs. B: Mean±SD TRAP positive multinucleated OCLs, per 20X high power field, of bone marrow derived-mononuclear non-adherent fraction isolated following repeated oral administration of a CatKI or a vehicle and cultured in osteoclastogenic media.

## **DISCUSSION:**

In the present study, we have evaluated the effect of repeated oral administration of a CatKI, VEL-0230, on bone marrow derived- stem and progenitor cell differentiation *in vitro*. Repeated oral administration of VEL-0230 for 4 weekly doses did not affect the ability of bone marrow stem cells and osteoclast progenitor cells to differentiate into chondrocytes, osteoblasts or osteoclasts-like cells when subjected *in vitro to culture in various differentiating media*. This is, in fact, a valuable finding as it provided additional data for the CatKIs safety profile and further elucidated the mechanism of action of this class of anti-resorptive agents. The CatKI reportedly acts by transiently inhibiting the resorptive activity of osteoclasts (8) without affecting their viability unlike the bisphosphonates, which reside in the bone compartment for long periods causing significant loss of osteoclast numbers (9). We anticipated that stem and progenitor cells isolated from horses which had been administered CatKI for a month would be able to restore their CatK activity and differentiate *in vitro* over the 7-28 days of the different assays.

Although the coupling between bone resorption and formation has been established and could account, in part, for the lack of, or decrease in, osteoblastic activity due to anti-resorptive administration (10), it has been recently concluded that osteoclast-

specific CatK deletion stimulates S1P-dependent bone formation (11). Another report has also reported an increased level of osteoclastogenesis in CatK-deficient mice possibly as a positive feedback of impaired bone resorption (12). Our work did not measure the feedback influence of arresting CatK activity on the producing cells. It is possible that a positive feedback loop could initiate osteoclastogenesis or up-regulation of osteoclast activity to compensate for the loss of CatK activity and the pause in bone resorption. Our work *in vivo* did not detect a difference in number of osteoclasts and osteoblasts per bone surface between CatKI and control groups using bone histomorphometric analysis (Chapter 3). Despite the lack of structural changes in the number of cells in the bone, the biomarkers of bone formation (osteocalcin) and bone resorption (CTX-1) did demonstrate an increase in the function of the cells present in bone of horses treated repeatedly with CatKI compared to controls.

In conclusion, our data suggested that horses who received CatKI in repeated dosages that induced repeated CatK inhibition, did not demonstrate a loss of bone marrow capability to replace osteoblastic cells, osteoclastic cells, or multi-potent stem cells. This is relevant in that several indications, such as systemic, local or regional application of CatKI to modulate bone resorption and inflammation in equine osteo-inflammatory conditions, it would not be anticipated to interfere with the stem and progenitor cell's regenerative and differentiation capacities *in vivo*.

## **Chapter 5**

### **Cathepsin K inhibition renders equine bone marrow nucleated cells hypo-responsive to LPS and unmethylated CpG stimulation *in vitro***

#### **SUMMARY:**

Cathepsin K (CatK) is an important enzyme regulating bone degradation and has been shown to contribute to the immune response. We have studied two inflammatory models in equine bone marrow nucleated cells (BMNCs); the Lipopolysaccharides (LPS) and the unmethylated CpG stimulation with the following objectives: 1. To determine whether CatK inhibition will alter the cytokine secretion by stimulated BMNCs; specifically IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , and 2. To determine the changes in BMNCs surface markers' expression and major histocompatibility complex (MHC) molecules under CatK inhibition. Cathepsin K inhibition promoted BMNCs viability and reduced cell apoptosis. Moreover, CatK

inhibition significantly decreased cytokine secretion of either naïve or stimulated BMNCs, and altered their MHC molecules expression. In conclusion, CatK inhibition in horses did affect BMNCs other than mature osteoclasts rendering them hypo-responsive to both TLR4- and TLR9-induced inflammation, predicting a proteolytic activity for CatK within the MyD88 pathway and/or the following proteolytic events required for the cytokines secretion.

## **INTRODUCTION:**

Cathepsin K (CatK) is a cysteine protease that is an important enzyme regulating bone and cartilage degradation and has been shown to contribute to the immune response (1). It is the most abundant cysteine protease expressed in the osteoclast and is believed to be instrumental in collagen and other extra-cellular matrix degradation that is necessary for bone and cartilage turnover (2). Low expression of CatK has been detected in normal heart, lung, small intestine, colon, testes, ovaries, placenta, skeletal muscles, thyroid epithelial cells, macrophages, and bone marrow-derived dendritic cells (3-5). While elevated CatK expression has been reported in psoriatic skin lesions, and in synovial fluid and lining tissue of joints with rheumatoid arthritis (6). The involvement of CatK in Toll like receptors (TLRs) signaling has recently been established as another function for CatK in addition to its known role in extracellular matrix degradation (3).

The TLR are a class of pattern recognition molecules with key functions in the innate and the acquired immune systems. They are type I trans-membrane proteins

with ectodomains containing leucine-rich repeats that mediate the recognition of pathogen-associated molecular patterns, trans-membrane domains, and intracellular Toll–interleukin (IL) 1 receptor (TIR) domains required for downstream signal transduction. The cellular localization of these receptors can vary with some localized at the cell surface (e.g. TLR1, TLR2, TLR4, TLR5 and TLR6) and others localized within endolysosomal compartments (TLR3, TLR7, TLR8 and TLR9). The TLRs interact with a variety of extracellular and intracellular ligands and influence the activity of a wide range of tissues and cell processes (7). Moreover, they have been found to be major regulators of multiple immune-mediated diseases such as pulmonary asthma, acute respiratory distress syndrome, inflammatory bowel disease, transplant–allograft rejection, vascular collapse, and rheumatoid arthritis (8). The proteases responsible for the cleavage of the TLRs have not been definitively identified, in part owing to disagreement among several reports. Most notably, inhibitors of CatK have revealed a crucial role for CatK in the function of TLR4 and/or TLR9 (3, 9). However, other reports denied this role in either TLR4 or TLR9 signaling pathway (8).

Multiple synthetic inhibitors of CatK have been developed over recent years with few of them proceeded to clinical trials in animal models and human (10-12). Our study aimed to investigate the effect of CatK inhibition on TLR4 and TLR9 signaling pathway in equine bone marrow nucleated cells (BMNCs). Infectious and inflammatory diseases are major equine health problems, particularly due to the inherent sensitivity of horses to microbial molecules, especially Lipopolysaccharides (LPS). Unlike rodents, horses are extremely sensitive to LPS

showing the most comparable sensitivity to that of people (13). Among currently available CatK inhibitors, VEL- 0230 (alternatively named NC-2300) is a highly selective and potent inhibitor of CatK that has shown efficacy in inhibiting bone resorption biomarkers in exercising horses and has been proposed as therapeutic for equine osteo-inflammatory conditions (10).

We hypothesized that CatK inhibition in horses will affect BMNCs other than mature osteoclasts rendering them hypo-responsive to induced inflammation. Thus, careful considerations to the side effects of CatK inhibitors on the immune system during its therapeutic application in bone resorption disorders should be taken, whereas they may have dual benefits in the treatment of osteo-inflammatory disorders. We have studied two different inflammatory models in horses' BMNCs; The LPS (TLR4 ligand) and the unmethylated CpG (TLR9) stimulation with the objectives of: 1.To determine whether CatK inhibition will alter the cytokine secretion by stimulated BMNCs; specifically IL-1 $\beta$ , IL-6, and tumor necrosis factor alpha (TNF- $\alpha$ ), and 2.To determine the changes in BMNCs surface markers' expression and the major histocompatibility complex (MHC) molecules under CatK inhibition; Cluster of differentiation (CD) 11b, CD2, CD4, CD5 (Iy-1), and CD90 (Thy-1), MHCI, and MHCII.

#### **MATERIAL AND METHODS:**

All experimental procedures were approved by the "Institutional Animal Care Committee" at The Ohio State University.

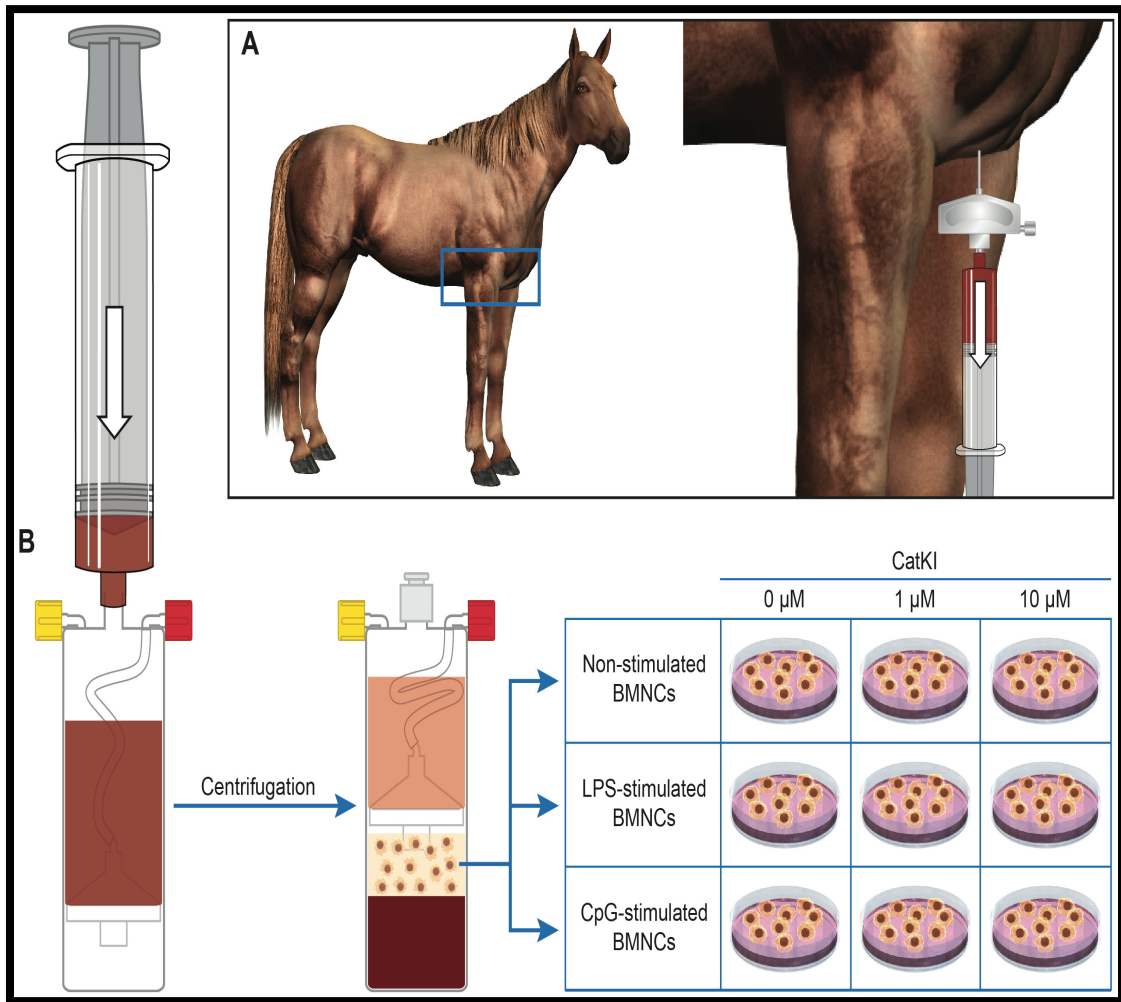
***Bone marrow aspiration and nucleated cell isolation:***

Six horses were sedated with 0.01mg/kg body weight (b.w.) detomidine hydrochloride (Domitor, Pfizer Animal Health, Exton, PA) and 0.02 mg/kg b.w. butorphanol (Torbugesic, Fort Dodge Animal Health, Fort Dodge, Iowa) intravenously. An area of 5 x 20 cm over the sternum was clipped, scrubbed, aseptically prepared and the intersternbral spaces were identified by palpation or ultrasonography. Local infiltration of 2 ml anesthetic solution, mepivacaine hydrochloride 2% (Carbocaine, Cooke-Waite Laboratories, Inc., NY), was performed subcutaneously over the midpoint in the sagittal plane of two adjacent sternbrae. A stab incision using a No. 11 scalpel blade was then made through the skin and a Jamshidi biopsy needle (11 gauge, 10 cm, Ranfac Corp, Avon, Mass) pre-rinsed with heparin (Multiparin 5000 iu/ml) was introduced for approximately 4-6 cm until it contacted the sternbra. The needle was then further pushed 3 or 4 cm into the sternbra and 60 ml of bone marrow was aspirated into two 30 ml syringes, preloaded with 3 ml heparin each (Fig. 36A). The two 30 ml syringes were then loaded into one Marrowstim<sup>®</sup> concentration system (BIOMET Biologics, Warsaw, IN, USA). Bone marrow nucleated cells were immediately isolated according to the manufacturer's instructions and the method described by Ishihara *et al.* (14), and then transferred into sterile 10 ml syringes. The syringes were then placed on ice for immediate transfer to the laboratory.



### ***Cell culture and experimental treatments:***

Horse BMNCs were collected and pelleted (all centrifugation steps were performed at 300 g at 6°C for 5 min). The cells were washed twice with 1X DPBS (Invitrogen, Carlsbad, CA) and cultured in Dulbecco's Modified Eagle's medium (DMEM; GIBCO) supplemented with 10% heat inactivated fetal bovine serum (GIBCO). For application of the different experimental conditions, cells were diluted to  $10^6$  cell/ml and dispensed to 12-well plates. Cells were then seeded as per well alone or with either one of two CatKI (VEL-0230) concentrations; 1 $\mu$ M or 10 $\mu$ M. The cells were seeded non-stimulated or stimulated with either LPS (Sigma-Aldrich, MO, USA) at concentration of 1 $\mu$ g/ml or unmethylated CpG motive (InvivoGen, CA, USA) at concentration of 5  $\mu$ g/ml with each CatKI concentration in the media. The plates were incubated for 48 hours in 5% CO<sub>2</sub> at 37°C. The CatKI concentrations were chosen based on the effective concentration on previous research while LPS concentration and CpG sequence and concentrations were chosen on the basis of a pilot experiment using horses peripheral blood mononuclear cell (Comparative Orthopedic Research Laboratory, The Ohio State University, Unpublished data). Fig. 36B illustrates the steps of BMNCs isolation and different cell treatments.



**Figure 36: Sternal bone marrow isolation, BMNCs isolation and treatments. (A) Sternal bone marrow isolation from deeply sedated horse under sterile conditions. (B) Isolation of BMNCs using specific centrifugation kit and application of different treatments *in vitro*; BMNCs were seeded either non-stimulated, LPS-stimulated, or CpG-stimulated in the presence of 0, 1, or 10  $\mu\text{M}$  of a CatKI (VEL-0230).**

***Cell count and measurements of cell viability:***

Cell total count and viability were performed using a hemocytometer and trypan blue staining (InvivoGen). Further assessment of apoptosis and viability was performed using Annexin V/PE Apoptosis detection kit (BD Biosciences, Bedford, MA) and subsequent flow cytometric analysis.

***Cytokines assay:***

The concentrations of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in culture media were measured using equine-specific ELISA kits (Genorise Scientific, INC. Glen Mills, PA, USA) according to the manufacturers' protocols. All measurements were performed in duplicates, optical density was obtained using a standard colorimetric method and averaged concentrations were calculated for each duplicate.

***Quantification of IL-6 and TNF- $\alpha$  mRNA by real-time polymerase chain reaction:***

Quantification of IL-6 and TNF- $\alpha$  mRNA by real-time polymerase chain reaction was performed as a confirmatory step since their protein levels were closely above

the lower limit of ELISA detection range. Total RNA was isolated using Guanidinium thiocyanate-phenol-chloroform extraction method (TRIzol<sup>®</sup> and Chlorophorm; Invitrogen). Complimentary (c) DNA was generated from 1 µg total RNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions using MJ Research PTC-150 Thermal Cycler (MJ Research, USA). Relative gene expression analysis for equine IL-6 and TNF- $\alpha$  in relation to a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was performed using specific custom-designed primers using Primer3 software (Steve Rozen, Helen J. Skaletsky, 1998). Real-time PCR was performed in a total volume of 50 µl using 96-well microwell plates, an ABI PRISM 7000 sequence detector and SYBR<sup>®</sup> Green PCR Master Mix (Applied BioSystems). Cycle threshold (Ct) values were obtained for all analyzed genes during log phase of the cycle. The levels of IL-6 and TNF- $\alpha$  were normalized to GAPDH for each sample and  $\Delta$ Ct were calculated ( $\Delta$ Ct of IL-6/TNF- $\alpha$  = Ct of IL-6/TNF- $\alpha$  – Ct GAPDH). All PCRs were performed in triplicate.

***Immunostaining and analysis of BMNCs lineages and MHC molecules:***

Single-cell preparations ( $5 \times 10^5$  cells) were stained for 30 minutes in the dark at 4°C with one of the following fluorochrome-labeled antibodies (Abs): anti-CD90, anti-CD11b, anti-CD2, anti-CD4, and anti-CD5 (BD Biosciences and eBiosciences, San Diego, CA). For MHCI and MHCII immuno-staining, cells were stained with either anti-equine MHCI monoclonal Ab (Clone CVS22, AbD Serotec, Raleigh, NC)

followed by anti-mouse secondary antibody (FITC rat anti-mouse IgG1, clone A85-1, BD Biosciences, San Jose, Calif), or anti-equine MHCII monoclonal Ab (Clone CVS20, AbD Serotec, Raleigh, NC) followed by anti-mouse secondary antibody. After incubation, cell washed twice in FACS buffer and re-suspended in 200  $\mu$ l FACS buffer for flow cytometry analysis (Accuri Cytometers, San Jose, CA). A gating procedure was generated by generating a cytogram of SSC versus forward scatter light and gated to exclude cell debris by including only cells with relatively high SSC and forward scatter light values. Quadrant cursors were set by using an appropriate negative control; either unstained control cell preparation or fluorochrome-labeled secondary antibody control preparation. Quadrants were set so that in negative controls, 99% of the cells were localized in the left lower quadrant. The percentage and absolute number of cells labeled by each antibody as well as the mean fluorescence intensity (MFI) were determined for each experimental condition per each animal.

***Data and statistical analyses:***

Data for all outcomes were statistically analyzed as raw values and percent change from control values (normalized to non-stimulated cell, LPS-stimulated cells, or CpG- stimulated cell with 0  $\mu$ M CatKI). Normalized data was then graphed as mean with standard error of the mean (SEM). Statistical analysis was performed using SPSS v.18.0 (IBM Corp., Armonk, NY, USA). The normal distribution within the different variables was investigated using Shapiro-Wilk test. Comparison between different experimental conditions was performed using the one-way analysis of

variance with repeated measures for normally distributed data followed by Duncan's Multiple Range Test, and Friedman test for skewed data. Significance was determined when  $P < 0.05$  and trend differences were discussed when  $0.10 > P > 0.050$ .

## **RESULTS:**

### ***Cathepsin K inhibition promoted BMNCs viability and proliferation, and reduced cell apoptosis:***

Treatment of non-stimulated, LPS-stimulated, and CpG-stimulated cells with the CatKI (VEL-0230) significantly increased total cell count and cell viability by more than 50% as measured by trypan blue staining (Data not shown). This was further confirmed by Annexin-7AAD fluorescent staining which revealed a significant reduction in the percent of cells undergoing apoptosis with CatKI treatment in all experimental conditions (Fig. 37). The decrease in percent apoptotic cells was CatKI-concentration dependent.

### ***Cathepsin K inhibition significantly decreased cytokine production of either naïve or stimulated BMNCs:***

Different cytokine protein analyses revealed significant reduction in IL-1  $\beta$ , IL-6, and TNF $\alpha$  concentrations in culture supernatants of non-stimulated cells, or cells

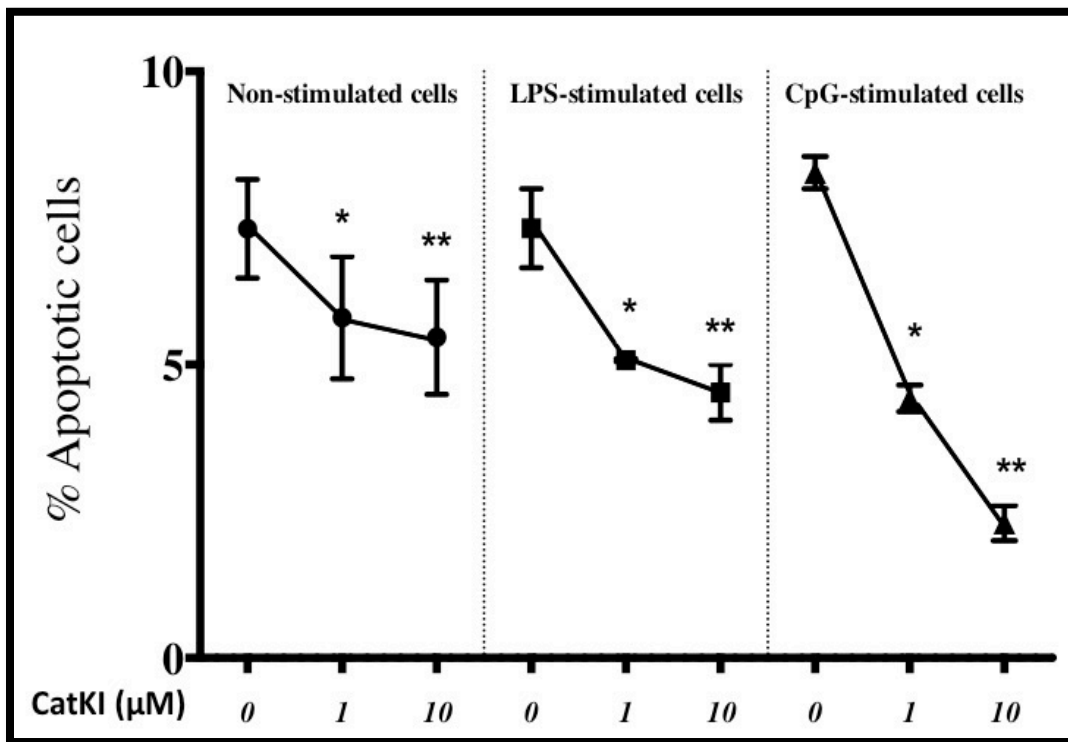
stimulated with LPS and CpG. This reduction in cytokine secretion was evident with either one or two CatKI molar concentrations. The cytokine secretions of the non-stimulated cells showed either a trend or significant decrease with the greater CatKI concentration (10  $\mu$ M). Horse was a significant different variable in all analyzed parameters of all experimental conditions ( $P < 0.001$ ). Table (7) is showing mean $\pm$ SEM cytokine concentrations (pg/ml of culture supernatant) of BMNCs in different experimental conditions. Table (7) shows the mean $\pm$ SEM cytokine concentrations (pg/ml of culture supernatant) of BMNCs in different experimental conditions.

The IL-1  $\beta$ , IL-6, and TNF $\alpha$  normalized data are shown in Fig. (38-40). The non-stimulated cells (Fig. 38) showed a significant decrease in IL-1 $\beta$  secretion with both CatKI molar concentrations, a significant decrease in IL-6 and a trend reduction in TNF $\alpha$  secretions with the greater CatKI concentration only. The LPS-stimulated cells (Fig. 39) showed a significant decrease in all cytokine secretions with both CatKI concentrations with the exception of TNF $\alpha$  secretion showing a trend reduction at the greater CatKI concentration only. The CpG-stimulated cells (Fig. 40) showed a significant reduction in the secretion of all analyzed cytokines with both CatKI concentrations.

Further gene expression analysis of IL-6 and TNF $\alpha$  confirmed a CatKI concentration-dependent decrease in mRNA levels for both IL-6 and TNF $\alpha$  in all experimental conditions (non-stimulated, LPS-stimulated and CPG-stimulated cells). Comparison of IL-6  $\Delta$ Ct values between different CatKI concentrations revealed significance effect for CatKI concentration with P values equals of 0.04, 0.023, and 0.035 for non-

stimulated, LPS-stimulated and CPG-stimulated cells respectively. Similarly, TNF $\alpha$   $\Delta$ Ct values revealed significance effect for CatKI concentration with P values 0.034, 0.043, and 0.025 for non-stimulated, LPS-stimulated and CPG-stimulated cells respectively



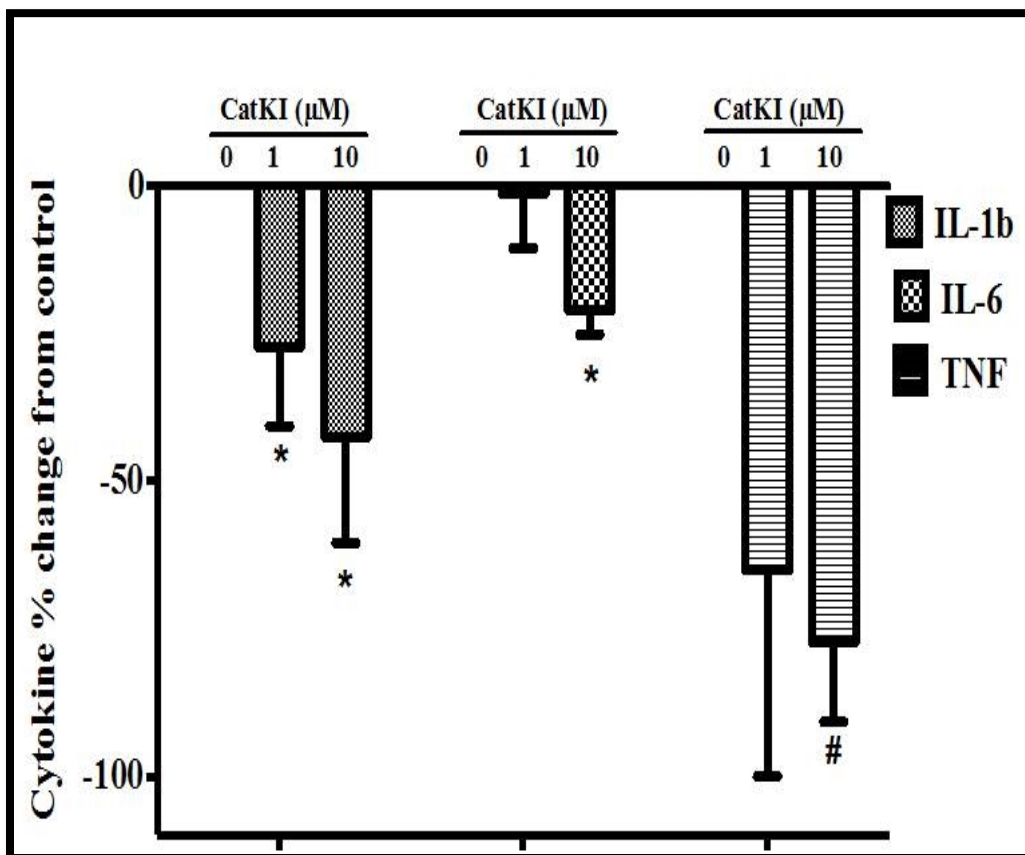


**Figure 37:** Cathepsin K inhibition significantly decrease the percent of apoptotic cells in horse BMNCs. The BMNCs were stimulated with either LPS or unmethylated CpG, treated with 2 different CatKI concentrations for 48 hours, and stained with Annexin and 7AAD to measure the percentage of apoptotic cells by flow cytometry. The error bars represent the standard error of the mean. Statistical comparison results are denoted; (\*) when group is significant compared to the CatKI (0μM),  $P < 0.05$ . (\*\*) when group is highly significant compared to the CatKI (0μM),  $P < 0.01$ .

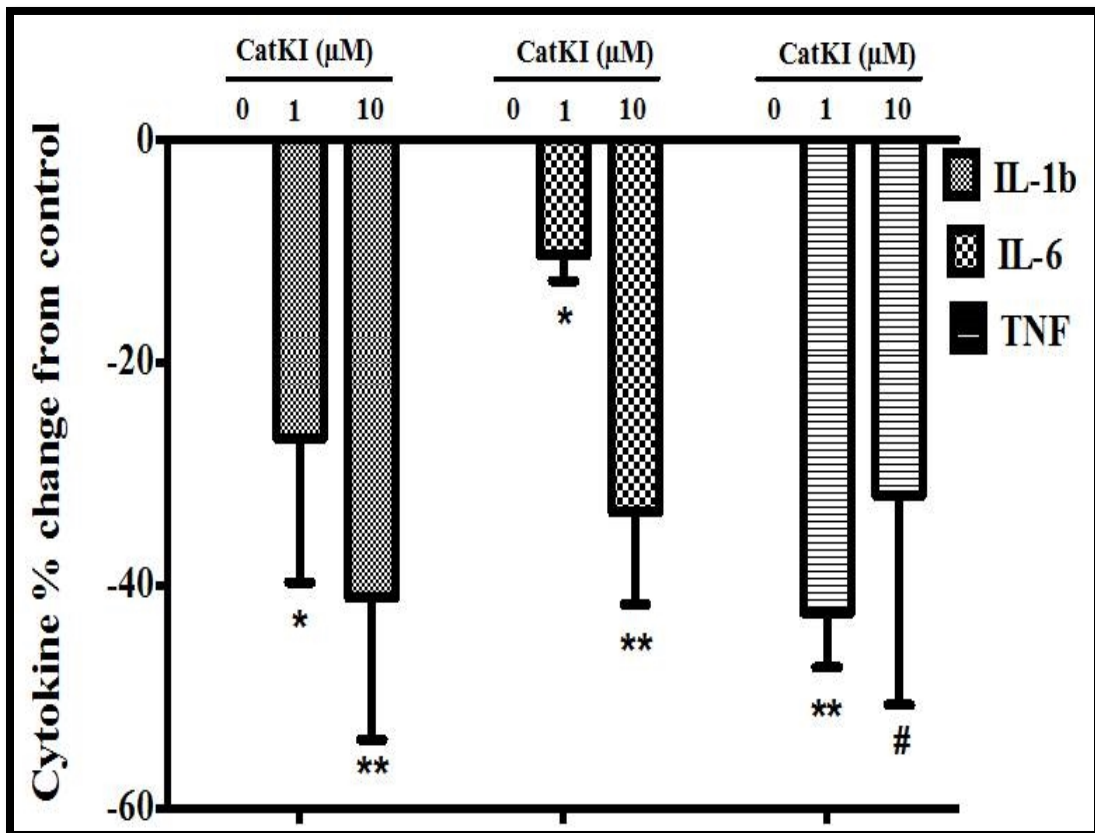
Cytokine	Un-stimulated cells			LPS-stimulated cells			CpG-stimulated cells		
	CatKI (0 $\mu$ M)	CatKI (1 $\mu$ M)	CatKI (10 $\mu$ M)	CatKI (0 $\mu$ M)	CatKI (1 $\mu$ M)	CatKI (10 $\mu$ M)	CatKI (0 $\mu$ M)	CatKI (1 $\mu$ M)	CatKI (10 $\mu$ M)
<b>IL-1<math>\beta</math></b> (pg/ml)	2014.8 $\pm$ 611.6	1731.3 $\pm$ 603.8	1682.3 $\pm$ 597.2 <sup>#</sup>	3677.5 $\pm$ 1185.1	3177.0 $\pm$ 1162.6 <sup>#</sup>	2736.3 $\pm$ 1062.6 <sup>**</sup>	2612. $\pm$ 3 785.5	2067. $\pm$ 3 573.5	1632.0 $\pm$ 459.8 <sup>*</sup>
<b>IL-6</b> (pg/ml)	38.8 $\pm$ 3.3	38.8 $\pm$ 5.3	30.8 $\pm$ 3.2 <sup>**</sup>	82.0 $\pm$ 6.9	73.3 $\pm$ 6.1 <sup>*</sup>	55.3 $\pm$ 8.3 <sup>*</sup>	88.3 $\pm$ 11.3	79.5 $\pm$ 11.8 <sup>*</sup>	64.5 $\pm$ 10.1 <sup>*</sup>
<b>TNF<math>\alpha</math></b> (pg/ml)	5.3 $\pm$ 2.1	2.8 $\pm$ 2.8	1.0 $\pm$ 0.7 <sup>#</sup>	100.4 $\pm$ 21.2	58.0 $\pm$ 13.2 <sup>*</sup>	68.8 $\pm$ 24.6	23.4 $\pm$ 6.8	12.9 $\pm$ 5.4 <sup>*</sup>	9.6 $\pm$ 3.5 <sup>**</sup>

**Table 7:** Mean $\pm$ SEM cytokine concentrations (pg/ml of culture supernatant) of BMNCs in different experimental conditions.

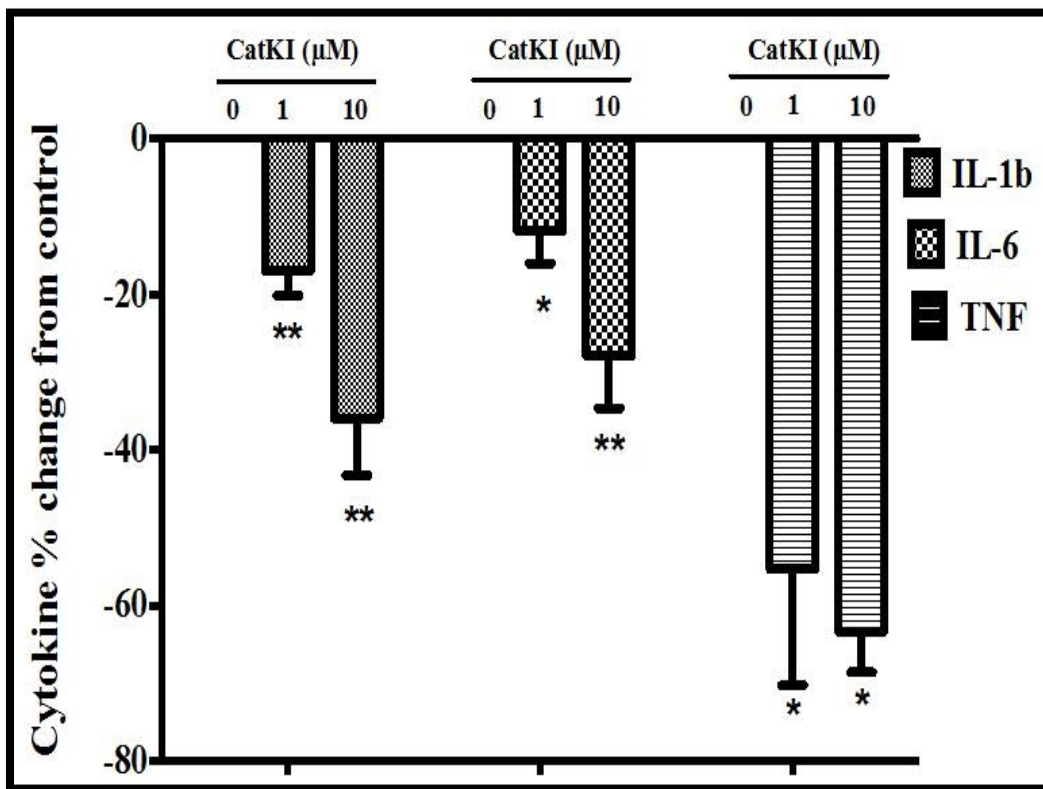
(<sup>#</sup>) 0.10 > P > 0.05 compared to CatKI (0  $\mu$ M) within the same experimental group. (<sup>\*</sup>) P < 0.05 compared to CatKI (0  $\mu$ M) within the same experimental group. (<sup>\*\*</sup>) P < 0.01 compared to CatKI (0  $\mu$ M) within the same experimental group.



**Figure 38:** Cathepsin K inhibition significantly decrease cytokine production of non-stimulated BMNCs. The mean±SEM % change from control in IL-1β, IL-6, and TNFα production is shown for BMNCs treated with 0, 1, or 10 0μM of a CatKI. Statistical comparison results are denoted; (\*) when group is significant compared to the CatKI (0μM), P<0.05. (\*\*) when group is highly significant compared to the CatKI (0μM), P<0.01. (#) when group has a trend change compared to the CatKI (0μM), 0.10>P>0.05.



**Figure 39:** Cathepsin K inhibition significantly decrease cytokine production of LPS-stimulated BMNCs. The mean±SEM % change from control in IL-1 β, IL-6, and TNFα production is shown for BMNCs treated with 0, 1, or 10 0μM of a CatKI. Statistical comparison results are denoted; (\*) when group is significant compared to the CatKI (0μM), P<0.05. (\*\*) when group is highly significant compared to the CatKI (0μM), P<0.01. (#) when group has a trend change compared to the CatKI (0μM), 0.10>P>0.05.



**Figure 40:** Cathepsin K inhibition significantly decreases cytokine production of CpG-stimulated BMNCs. The mean±SEM % change from control in IL-1 β, IL-6, and TNFα production is shown for BMNCs treated with 0, 1, or 10 0μM of a CatKI. Statistical comparison results are denoted; (\*) when group is significant compared to the CatKI (0μM), P<0.05. (\*\*) when group is highly significant compared to the CatKI (0μM), P<0.01. (#) when group has a trend change compared to the CatKI (0μM), 0.10>P>0.05.

***Cathepsin K inhibition altered different equine BMNCs surface markers and MHC molecules expression:***

One or two concentrations of the CatKI (VEL-0230) treatments significantly altered cell surface markers and MHC molecules expression as shown in table (8). The horse was a significant different variable in all analyzed parameters of all experimental conditions ( $P < 0.001$ ). The normalized data for the non-stimulated cells are shown in Fig. (41) for % positive cells and in Fig. (42) for MFI. There were significant reductions in the % of cells positive for CD90, CD11b, and MHC I with one or both CatKI concentrations. A trend decrease in the % of CD4 positive cells was found at the greater CatKI concentration. Meanwhile, significant reductions in the MFI of CD2 and MHC I with one or both CatKI concentrations were observed. A significant increase in the MFI of MHC II was detected at the greater CatKI concentration only.

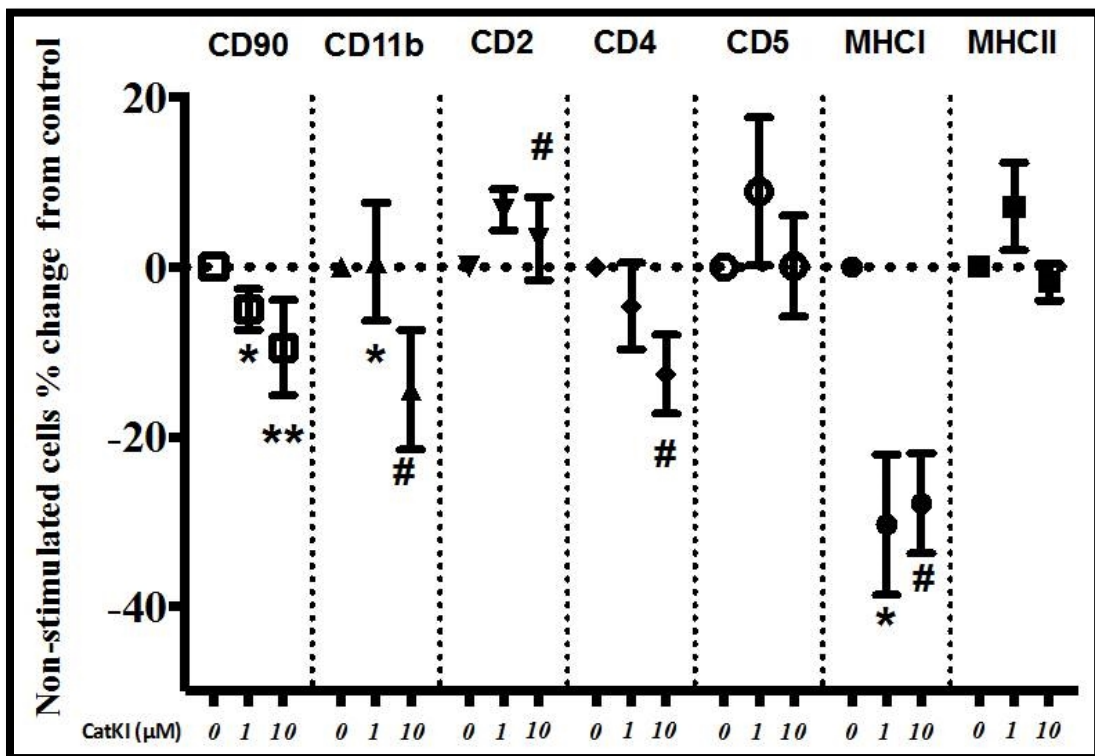
The normalized data for the LPS-stimulated cells is shown in Fig. (43) for % positive cells and in Fig. (44) for MFI. There were significant reductions in the % of cells positive for CD90, CD11b, CD4, MHC I and MHC II with one or both CatKI concentrations. Meanwhile, significant reductions in the MFI of CD90, CD11b, CD2, CD4, CD5, and MHC I with one or both CatKI concentrations were observed. A significant increase in the MFI of MHC II was detected at the lower CatKI concentration.

The normalized data for the CpG-stimulated cells is shown in Fig. (45) for % positive cells and in Fig. (46) for MFI. There were significant reductions in the % of cells positive for CD2, CD4, CD5 and MHCII with one or both CatKI concentrations. A biphasic change in the % of CD11b positive cells were found with a significant increase observed at the lower CatKI concentration and a significant decrease observed at the greater CatKI concentration. Meanwhile, significant reductions in the MFI of CD11b, CD2, CD4, MHCI and MHCII with one or both CatKI concentrations were observed. A significant increase in the MFI of CD5 was detected at the greater CatKI concentration.

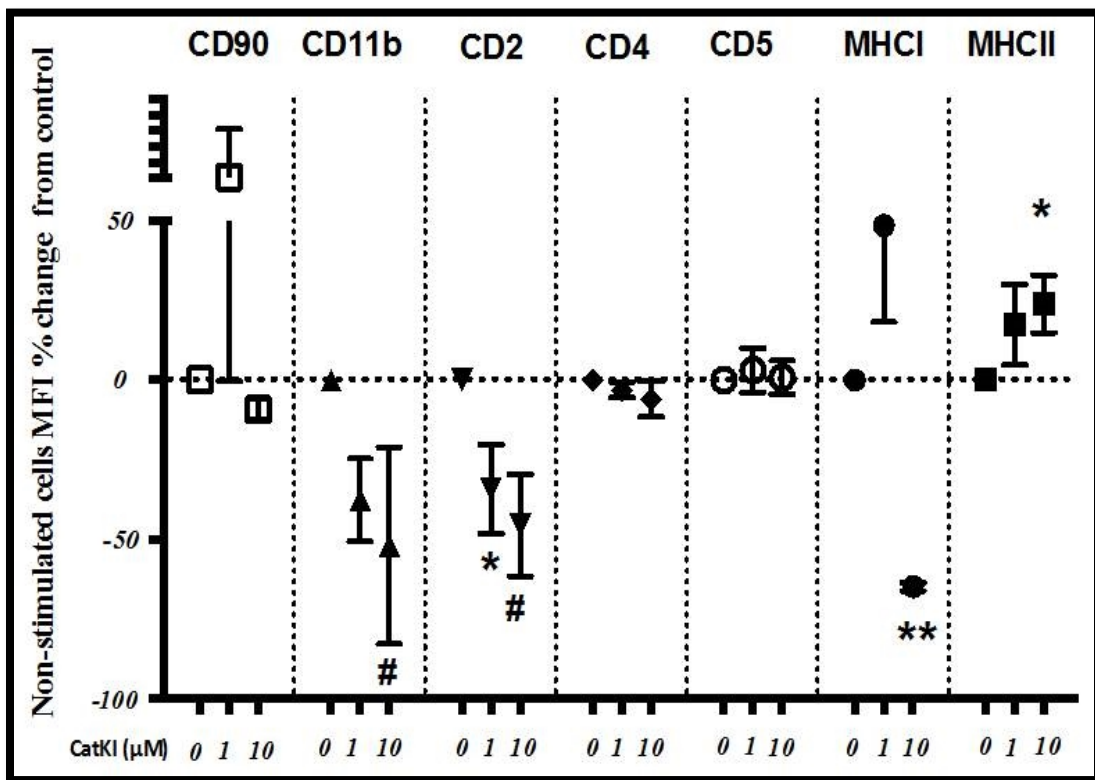
Parameter		Non-stimulated cells			LPS-stimulated cells			CpG-stimulated cells		
		CatKI (0 $\mu$ M)	CatKI (1 $\mu$ M)	CatKI (10 $\mu$ M)	CatKI (0 $\mu$ M)	CatKI (1 $\mu$ M)	CatKI (10 $\mu$ M)	CatKI (0 $\mu$ M)	CatKI (1 $\mu$ M)	CatKI (10 $\mu$ M)
CD 90	% positive cells	59.4 $\pm$ 1.1	56.3 $\pm$ 0.8	53.5 $\pm$ 2.5	55.7 $\pm$ 0.5	47.5 $\pm$ 0.4 <sup>**</sup>	44.6 $\pm$ 0.8 <sup>#</sup>	45.3 $\pm$ 0.3	46.6 $\pm$ 2.5	46.4 $\pm$ 0.1 <sup>#</sup>
	MFI	190,744 $\pm$ 103,026	954,716 $\pm$ 850,553	160,812 $\pm$ 83,947	205,421 $\pm$ 106,277	177,199 $\pm$ 90,512 <sup>*</sup>	131,074 $\pm$ 102,114 <sup>#</sup>	97,356 $\pm$ 71,963	97,786 $\pm$ 70,958	98,823 $\pm$ 72,906
CD 11b	% positive cells	29.6 $\pm$ 2.6	29.3 $\pm$ 2.4	24.6 $\pm$ 1.4	24.3 $\pm$ 3.8	17.7 $\pm$ 4.7 <sup>*</sup>	16.2 $\pm$ 3.9 <sup>**</sup>	12.8 $\pm$ 4.9	16.0 $\pm$ 4.7	9.0 $\pm$ 3.2
	MFI	1,406,935 $\pm$ 876,636	718,522 $\pm$ 485,808	425,997 $\pm$ 130,825	504,013 $\pm$ 336,537	362,151 $\pm$ 243,318	285,039 $\pm$ 183,059 <sup>**</sup>	453,724 $\pm$ 221,244	340,531 $\pm$ 168,176	175,750 $\pm$ 91,701
CD 2	% positive cells	7.9 $\pm$ 3.1	8.1 $\pm$ 3.0	8.9 $\pm$ 3.8	13.7 $\pm$ 5.5	14.3 $\pm$ 6.1	12.6 $\pm$ 3.9	15.3 $\pm$ 6.0	14.3 $\pm$ 6.0	12.8 $\pm$ 5.4 <sup>*</sup>
	MFI	248,559 $\pm$ 67,821	125,669 $\pm$ 33,620 <sup>#</sup>	81,966 $\pm$ 14,450 <sup>*</sup>	109,422 $\pm$ 27,341	75,857 $\pm$ 30,524 <sup>#</sup>	49,695 $\pm$ 18,703 <sup>*</sup>	66,077 $\pm$ 34,128	42,184 $\pm$ 17,732	30,491 $\pm$ 10,905
CD 4	% positive cells	14.0 $\pm$ 2.7	13.6 $\pm$ 2.9	12.7 $\pm$ 2.9	22.7 $\pm$ 4.7	19.9 $\pm$ 4.5 <sup>**</sup>	16.7 $\pm$ 4.5 <sup>**</sup>	18.3 $\pm$ 5.0	17.9 $\pm$ 4.9	16.5 $\pm$ 4.8 <sup>**</sup>
	MFI	34,386 $\pm$ 3,002	33,373 $\pm$ 3,168	32,811 $\pm$ 4,515	39,477 $\pm$ 4,284	37,453 $\pm$ 4,028	31,336 $\pm$ 2,992 <sup>**</sup>	73,770 $\pm$ 46,449	30,201 $\pm$ 4,752	24,513 $\pm$ 2,657
CD 5	% positive cells	8.0 $\pm$ 2.2	8.4 $\pm$ 2.3	8.3 $\pm$ 2.5	15.9 $\pm$ 6.0	16.2 $\pm$ 5.3	14.2 $\pm$ 5.0	13.4 $\pm$ 5.3	12.7 $\pm$ 5.1 <sup>#</sup>	12.2 $\pm$ 5.5
	MFI	13,719 $\pm$ 1,980	14,133 $\pm$ 2,432	13,886 $\pm$ 2,444	14,965 $\pm$ 1,439	13,251 $\pm$ 1,678 <sup>*</sup>	12,966 $\pm$ 1,636 <sup>**</sup>	12,397 $\pm$ 1,267	12,695 $\pm$ 2,500	13,847 $\pm$ 1,474 <sup>*</sup>
MH CI	% positive cells	0.5 $\pm$ 0.09	0.4 $\pm$ 0.06 <sup>*</sup>	0.3 $\pm$ 0.06 <sup>#</sup>	1.5 $\pm$ 0.5	1.3 $\pm$ 0.5 <sup>#</sup>	0.8 $\pm$ 0.4 <sup>*</sup>	1.3 $\pm$ 0.6	1.1 $\pm$ 0.5	1.0 $\pm$ 0.5
	MFI	133,896 $\pm$ 25,255	222,121 $\pm$ 69,753	47,050 $\pm$ 9,107 <sup>*</sup>	193,294 $\pm$ 38,063	101,641 $\pm$ 25,687 <sup>*</sup>	67,766 $\pm$ 22,717 <sup>*</sup>	116,403 $\pm$ 27,860	59,763 $\pm$ 13,902 <sup>*</sup>	23,073 $\pm$ 2,968 <sup>*</sup>
MH CII	% positive cells	9.7 $\pm$ 3.0	10.1 $\pm$ 2.9	9.7 $\pm$ 3.2	12.7 $\pm$ 4.6	11.6 $\pm$ 4.1	10.1 $\pm$ 3.2	19.4 $\pm$ 7.0	17.0 $\pm$ 7.4 <sup>*</sup>	15.4 $\pm$ 6.5 <sup>*</sup>

**Table 8:** Mean $\pm$ SEM % positive cells and MFI for different analyzed cell surface markers (CD90, CD11b, CD2, CD4, CD5, MHCII, and MHCI).

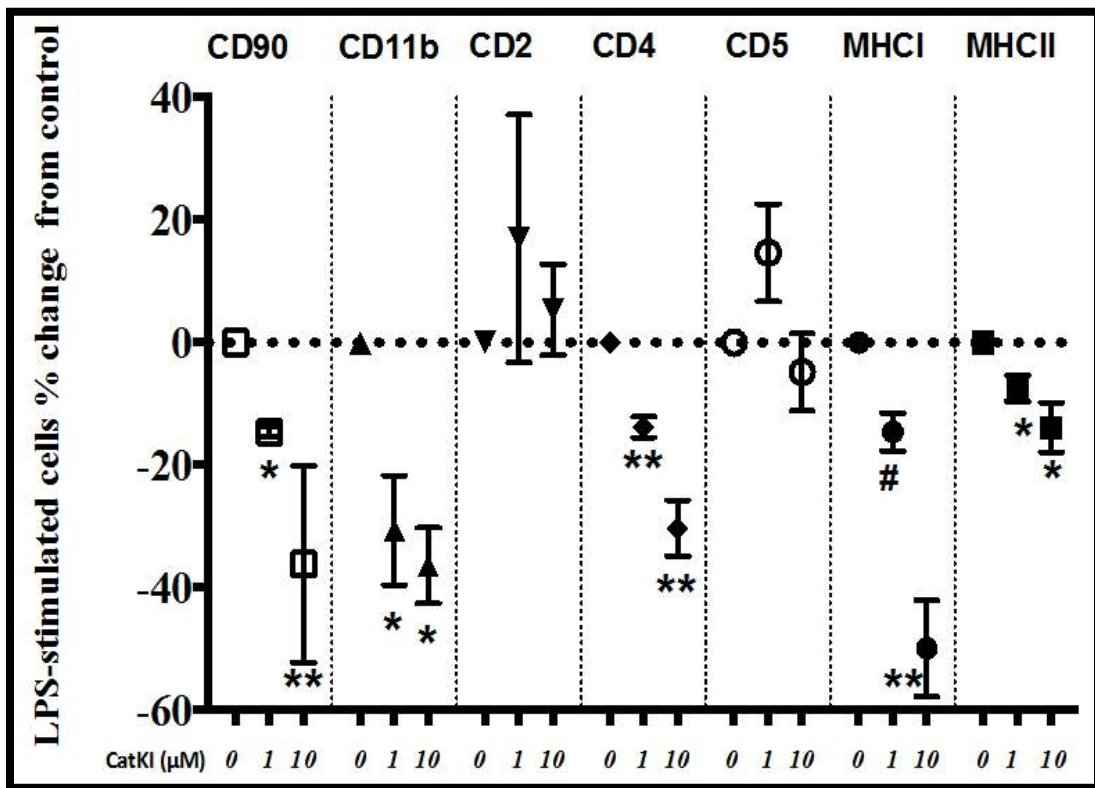




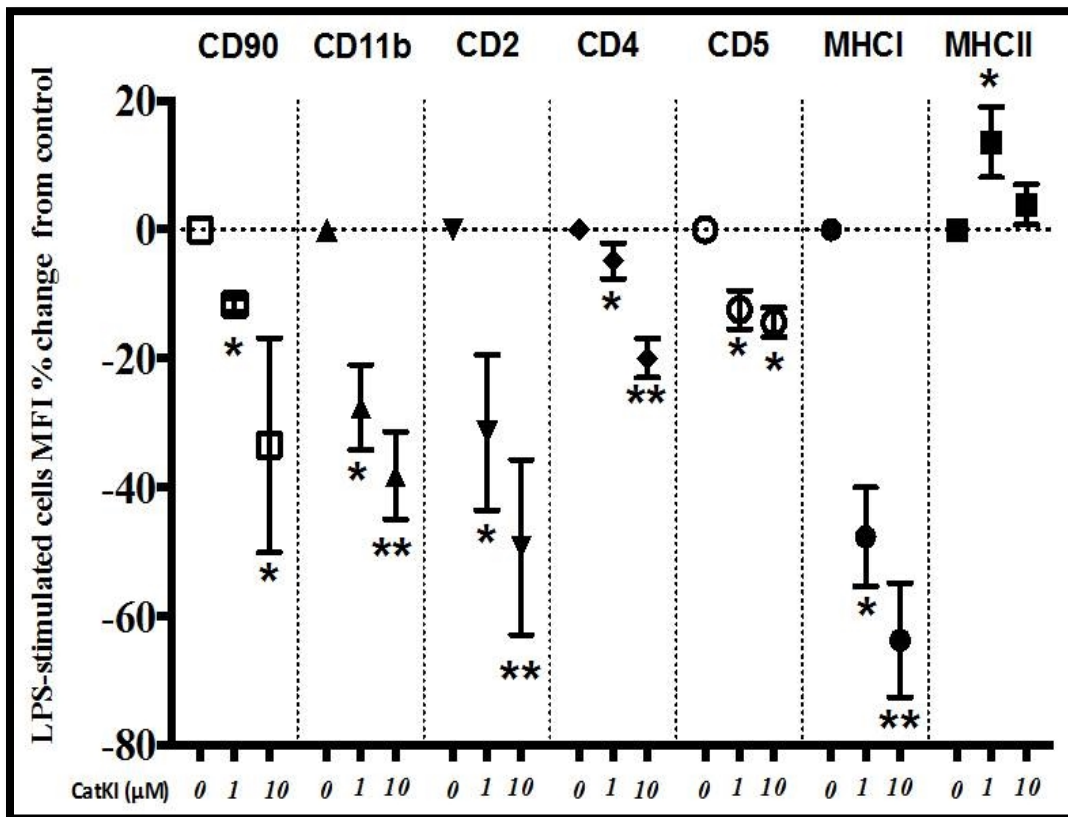
**Figure 41:** Cathepsin K inhibition alters different lineages and MHC molecules expression of non-stimulated BMNCs in horses. Mean±SEM % changes from control in percent positive cells are shown for CD90, CD11b, CD2, CD4, CD5, MHC I, and MHC II. Statistical comparison results are denoted; (\*) when group is significant compared to the CatKI (0μM),  $P < 0.05$ . (\*\*) when group is highly significant compared to the CatKI (0μM),  $P < 0.01$ . (#) when group has a trend change compared to the CatKI (0μM),  $0.10 > P > 0.05$ .



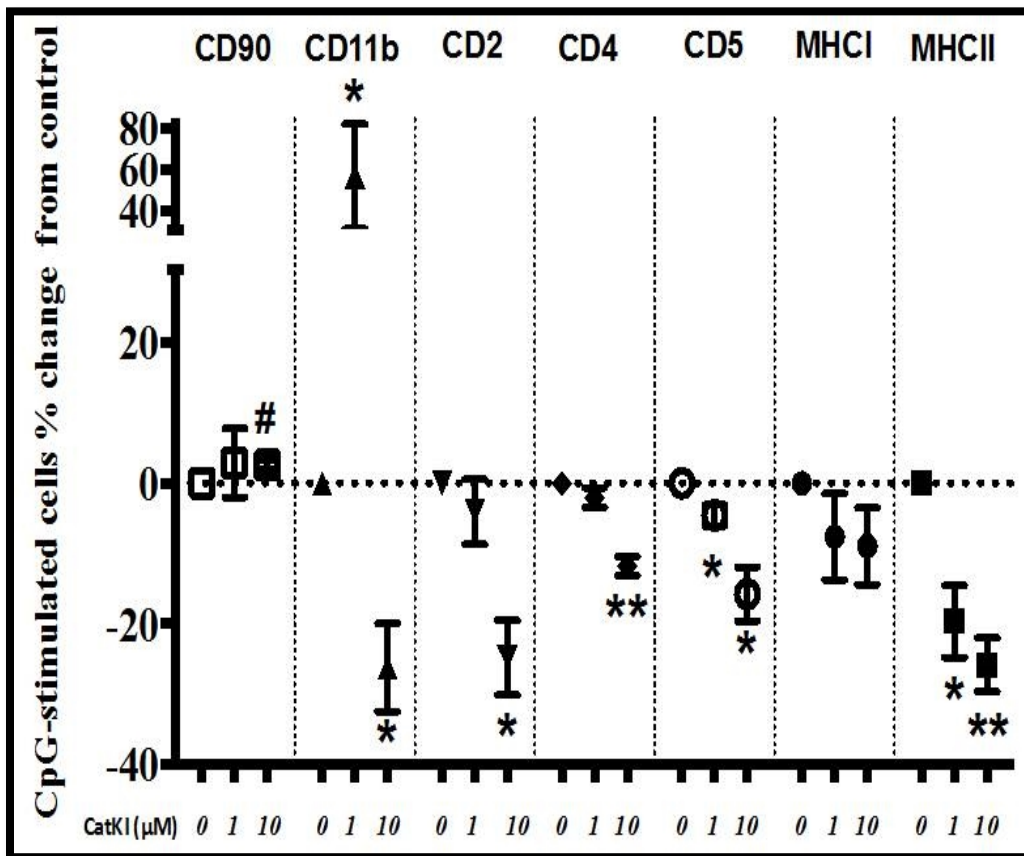
**Figure 42:** Cathepsin K inhibition alters different lineages and MHC molecules expression of non-stimulated BMNCs in horses. Mean±SEM % changes from control in mean fluorescence intensity (MFI) are shown for CD90, CD11b, CD2, CD4, CD5, MHC I, and MHC II. Statistical comparison results are denoted; (\*) when group is significant compared to the CatKI (0μM),  $P < 0.05$ . (\*\*) when group is highly significant compared to the CatKI (0μM),  $P < 0.01$ . (#) when group has a trend change compared to the CatKI (0μM),  $0.10 > P > 0.05$ .



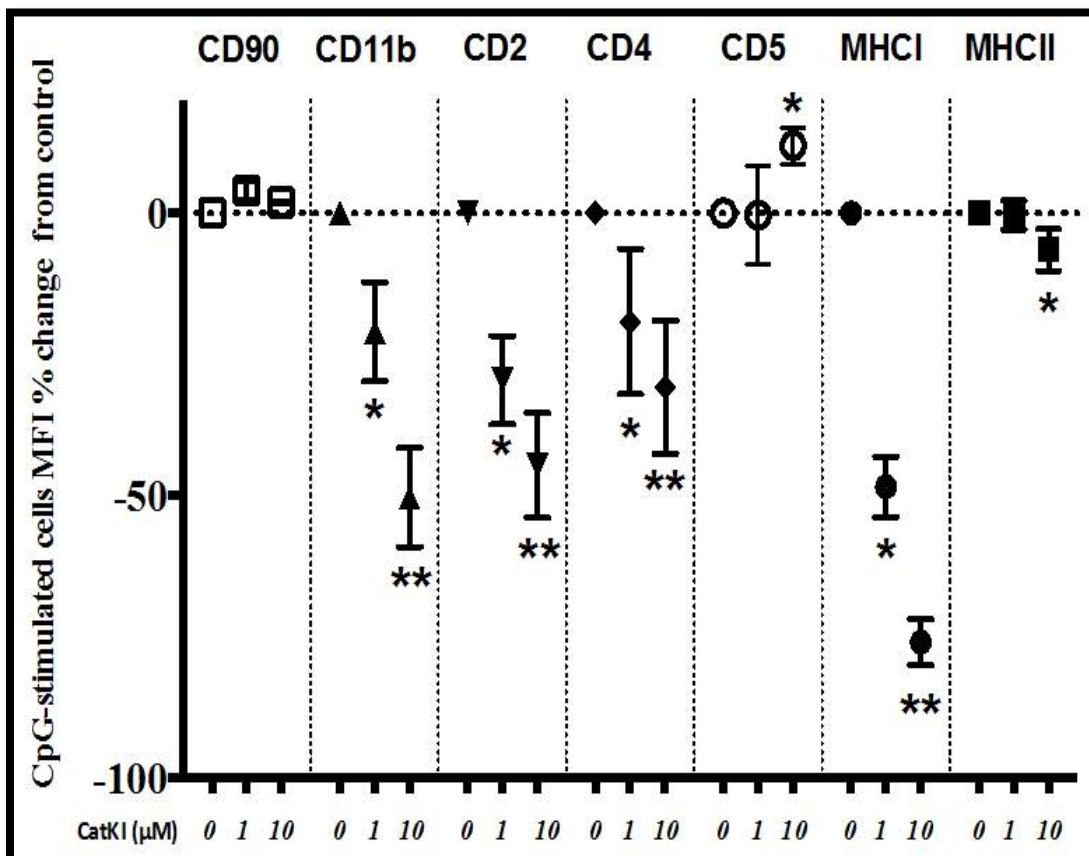
**Figure 43:** Cathepsin K inhibition alters different lineages and MHC molecules expression of LPS-stimulated BMNCs in horses. Mean±SEM % changes from control in percent positive cells are shown for CD90, CD11b, CD2, CD4, CD5, MHCI, and MHCII. Statistical comparison results are denoted; (\*) when group is significant compared to the CatKI (0μM), P<0.05. (\*\*) when group is highly significant compared to the CatKI (0μM), P<0.01. (#) when group has a trend change compared to the CatKI (0μM), 0.10>P>0.05.



**Figure 44:** Cathepsin K inhibition alters different lineages and MHC molecules expression of LPS-stimulated BMNCs in horses. Mean±SEM % changes from control in mean fluorescence intensity (MFI) are shown for CD90, CD11b, CD2, CD4, CD5, MHC I, and MHC II. Statistical comparison results are denoted; (\*) when group is significant compared to the CatKI (0μM), P<0.05. (\*\*) when group is highly significant compared to the CatKI (0μM), P<0.01. (#) when group has a trend change compared to the CatKI (0μM), 0.10>P>0.05.



**Figure 45:** Cathepsin K inhibition alters different lineages and MHC molecules expression of CpG-stimulated BMNCs in horses. Mean±SEM % changes from control in percent positive cells are shown for CD90, CD11b, CD2, CD4, CD5, MHCI, and MHCII. Statistical comparison results are denoted; (\*) when group is significant compared to the CatKI (0μM), P<0.05. (\*\*) when group is highly significant compared to the CatKI (0μM), P<0.01. (#) when group has a trend change compared to the CatKI (0μM), 0.10>P>0.05



**Figure 46:** Cathepsin K inhibition alters different lineages and MHC molecules expression of CpG-stimulated BMNCs in horses. Mean±SEM % changes from control in mean fluorescence intensity (MFI) are shown for CD90, CD11b, CD2, CD4, CD5, MHC I, and MHC II. Statistical comparison results are denoted; (\*) when group is significant compared to the CatKI (0μM), P<0.05. (\*\*) when group is highly significant compared to the CatKI (0μM), P<0.01. (#) when group has a trend change compared to the CatKI (0μM), 0.10>P>0.05.

## **DISCUSSION:**

The results of this study indicated that CatK inhibition had a potent immunomodulatory effect on equine BMNCs *in vitro*. This effect was evident in non-stimulated cells exposed to the culture environmental conditions *in vitro*, and in the two inflammatory models; the LPS stimulation (TLR4 ligand) and the unmethylated CpG stimulation (TLR9 ligand).

In this study, CatK inhibition in equine BMNCs, via blockage of the active site of the enzyme CatK, markedly decreased pro-inflammatory cytokine secretion, expression of certain cell surface markers, and expression of MHC molecules in response to TLR4 and TLR9 agonists rendering the cells hypo-responsive to the inflammatory effect. However, both non-stimulated and stimulated BMNCs exhibited greater viability with lower apoptotic activity when CatK functions were impaired.

Both TLR4 and TLR9 signaling pathway share the myeloid differentiation factor (MyD88)-dependent pathway. Thus, a proteolytic function for CatK at one or more aspects of this pathway is plausible in horses as revealed by the CatK inhibition effect on BMNCs. Although TLR4 can additionally signal independently of MyD88 through TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF), which

results in 75% of the TLR4-induced inflammatory response in most animal species and human (15, 16), this pathway (the MyD88- independent) is of a negligible function in horses and does not contribute to their relatively higher sensitivity to LPS stimulation (13). Surprisingly, CatK inhibition modulated equine BMNCs inflammatory response to both TLR4 and TLR9, unlike mice dendritic cells, which found to be hypo-responsive to TLR9 but not TLR4 when a similar CatK functions were impaired (3).

The production of pro-inflammatory cytokines is an important consequence of TLRs-induced cell activation that mediates tolerance to microbial infection (17) and many of the pathophysiological effects including an increase in bone resorption activities (18). The major cytokines produced by the MyD88 pathway are IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-10. In this study, the availability of equine-specific cytokine analyses provided an accurate quantification for three of these major cytokines in a highly sensitive manner. We have detected a statistically significant decrease in the production of IL-1 $\beta$ , IL-6, and TNF $\alpha$  in the presence of a CatKI, which was CatKI concentration-dependent in both LPS and CpG inflammatory models. The decrease in cytokine protein secretion of IL-6, and TNF $\alpha$  was further confirmed by a similar significant decrease in mRNA levels of both genes in all experimental conditions. These results do confirm the proposed role of CatK in the MyD88 signaling pathway but do not exclude a possible role for CatK in the proteolytic activities required for extracellular secretion of these cytokines, including IL-1 $\beta$  by which secretion is an inflammasome-dependent. A similar inhibition in pro-inflammatory cytokines secretions (IL-12 and IL-23) was observed when rat dendritic cells



cultured in vitro with multiple concentration of VEL-0230. This inhibition was observed with CpG but not LPS stimulation (3). Interestingly, our result came in agreement with a recent study that reported a significant decrease in the pro-inflammatory mRNA (IL-23, IL-6, and TNF $\alpha$ ) and correspondent cytokines expression in response to both TLR4 and TLR9 stimulation in a mouse model of periapical disease using another CatKI, odanacatib (9).

The recognition of ligands by TLRs results in activation of monocyte-derived dendritic cells, and other antigen-presenting cells, to secrete pro-inflammatory cytokines that promote dendritic cell maturation, antigen uptake and presentation. Reducing the biological activities of pro-inflammatory cytokines can alleviate the brunt of attack of diseases and lesions mediated by pro-inflammatory cytokines. The biological activities of TNF $\alpha$  include the induction of other inflammatory factors, such as IL-1 $\beta$  and IL-6, activation of the inflammatory cells, increasing expression of the adhesion molecules, and increasing the production of nitric oxide and reactive oxygen species. Similar biological activities are also induced by IL-1 $\beta$ . Whereas IL-6 is mainly responsible for inducing B cell maturation, the synthesis of acute phase proteins, and fever (19). In this study, a significant reduction in the pro-inflammatory cytokines secretions as well as the expression of different equine BMNCs surface markers and MHC molecules were observed under the effect of one or two different CatKI concentrations.

The results of this study revealed a significant decrease in the % of cells positive for CD90, CD11b, CD2, CD4, CD5, MHCI, and MHCII molecules. All of which have

important functions in the innate and/or adaptive immune response. While the expression of CD90 had previously shown to be restricted to neuronal cells, a subset of CD34+ blood stem cells, fibroblasts, and activated endothelial cells in human (20, 21), it has been identified as an activation-associated cell adhesion molecule that can interact with a corresponding ligand on monocytes and polymorphonuclear cells leading to adhesion of these leukocytes to activated CD90-expressing endothelial cells (22). Meanwhile, the CD11b molecule is expressed on the surface of many lymphoid cells involved in the innate immune system, including monocytes, granulocytes, and macrophages. It mediates inflammation by regulating leukocyte adhesion and migration, and has been implicated in several immune processes such as phagocytosis, cell-mediated cytotoxicity, chemotaxis and cellular activation. Moreover, the CD11b molecule is also involved in the complement system (23). The CD2 is another adhesion molecule mainly found on the surface of T cells and natural killer (NK) cells, while CD4 is mainly found on the surface of T helper cells, monocytes, macrophages, and dendritic cells. Whereas CD5 is a molecule that primarily found on a subset of IgM-secreting B cells called B-1 cells, and also on T cells (24). Among different MHC classes identified, class II and I are of major importance. The MHCI molecule mediates cellular immunity by interacting with CD8 molecules on surfaces of cytotoxic T cells and destruction of infected and malignant host cells. Meanwhile, MHCII molecule mediates the establishment of the adaptive immune response by interacting with CD4 molecules on surfaces of helper T cells (25).

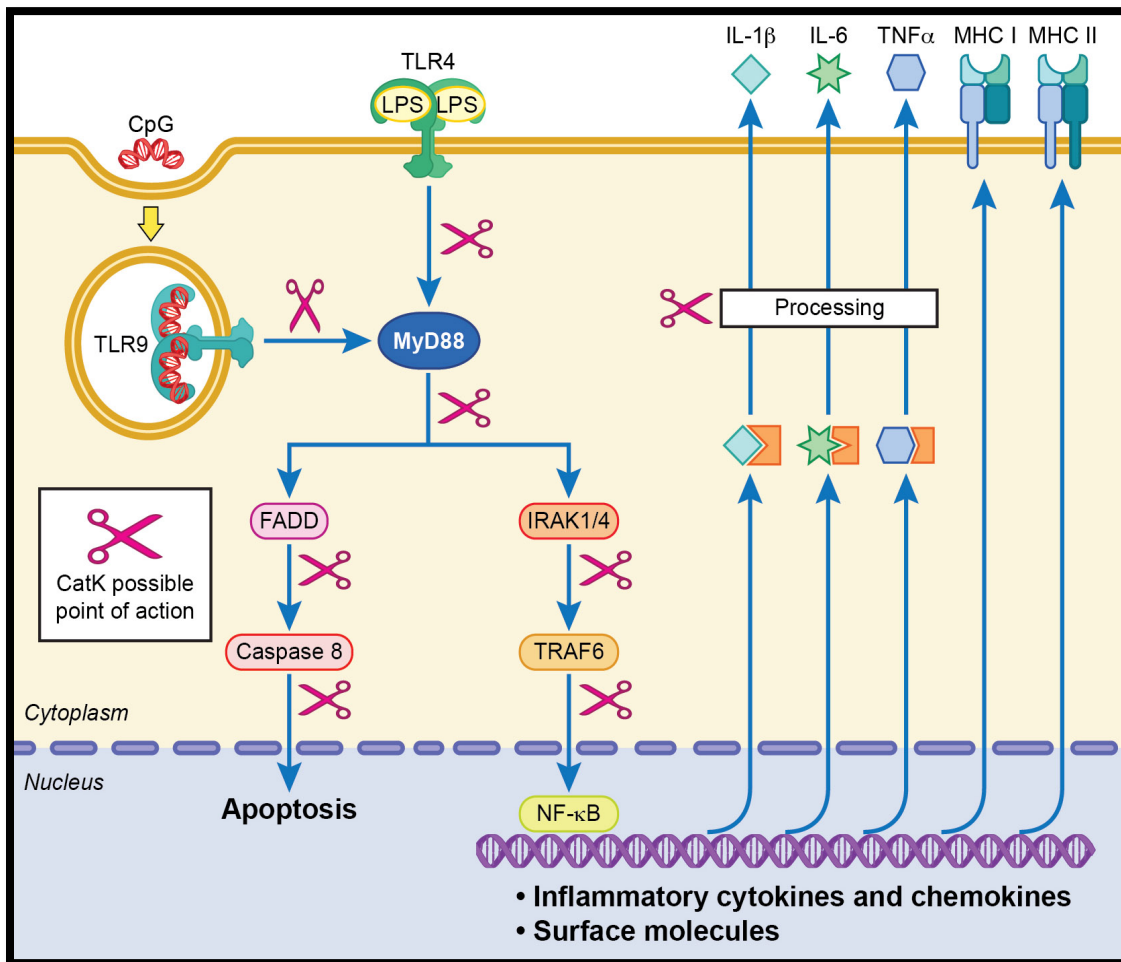
In a partial disagreement to Asagiri *et al.* (3), and in agreement with other previous

research (6, 26), our data provided evidence for an anti-inflammatory effect of CatK inhibition in equine BMNCs. While CatK depletion caused a dramatic reduction in the pro-inflammatory cytokines secretion of mice dendritic cells in response to TLR9 stimulation, it did not affect the formation, morphology and antigen presenting ability of these cells (3). Our data provided an evidence for decreased antigen presenting ability of equine BMNCs exhibited by the decrease in the percentage of MHCI and II positive cells under the effect of CatK inhibition. Similarly to our results, CatK inhibition revealed an anti-inflammatory role in mouse models for experimental autoimmune encephalomyelitis and psoriasis as CatK-knockout mice showed significant lower encephalomyelitis compared to wild type mice, and VEL-0230 topical treatment significantly ameliorated psoriatic skin lesions in a mouse model for Psoriasis (3, 6). Moreover, CatK, uniquely among other cysteine proteases, was found to have a kininase activity impairing symptoms of the bradykinin-dependant lung inflammatory diseases (26). Contrary to the anti-inflammatory role previously reported for CatK inhibition, CatK depletion significantly aggravated the chronic colitis in a mouse model suggesting an antimicrobial role for extracellular CatK within the gut mucosa (5). However, such antimicrobial activity for CatK was excluded within the immune response to influenza virus vaccine in vitro (27).

Cathepsin K inhibition represents a novel strategy for developing agents to treat osteoporosis, osteoarthritis, and other disorders characterized by increased bone resorption in humans. It has also been proposed for treatment of certain osteo-inflammatory conditions in horses (10). The anticipated outcome of a CatK

inhibitor would be to slow down the resorption of bone during certain biologic stress risers seen in inflammatory bone diseases, such as exercise-induced bone trauma. Such outcome is very likely to arise following the decrease in pro-inflammatory cytokines secretions by inflammatory cells which could possibly lead to attenuation in the induction of T helper 17 cells, which are an osteoclastogenic T cell subset known to play an important role in the inflammatory bone loss (4). Additionally, it is possible to utilize CatK inhibition to minimize the deleterious effects of TLR activation induced by microbial ligands in horses.

In conclusion, CatK inhibition in horses did affect BMNCs other than mature osteoclasts rendering them hypo-responsive to both TLR4- and TLR9-induced inflammation, and predicting a proteolytic activity for CatK within the MyD88 pathway and/or the following proteolytic events required for extracellular secretion of the produced cytokines (Fig. 47).



**Figure 47:** Possible points of action for Cathepsin K within TLR4- and TLR9-induced inflammatory response. Cathepsin K inhibition in horses did affect BMNCs other than mature osteoclasts rendering them hypo-responsive to both TLR4- and TLR9-induced inflammation, and predicting a proteolytic activity for CatK within the MyD88 pathway and/or the following proteolytic events required for extracellular secretion of the produced cytokines.

## **Chapter 5: Supplementary Data**

### **Altered Gene Expression By Cathepsin K Inhibition During Bone Marrow Chondrogenic, Osteogenic, And Osteoclastogenic Differentiation *In Vitro***

#### **INTRODUCTION:**

The crosstalk between the skeletal and immune systems is sophisticated and directly connected to the location and tight integration of bone marrow within bone. Bone marrow is the body's resource for progenitor cells of the lymphoid cell lines (immune system) and the osteoblastic and osteoclastic cell lines responsible for bone formation and turnover. Both systems share components and mechanisms in vertebrates, including ligands, receptors, signaling molecules and transcription factors. This dissertation investigated the effect of a potent, highly selective Cathepsin K (CatK) inhibitor on both bone and bone marrow cells to provide added insight into the mechanistic nexus by which CatK inhibition could provide both anti-inflammatory effects in immune mediated diseases and inhibit bone resorption. The signaling pathways by which CatK inhibition could have potential to treat

osseous inflammation and bone resorption associated with osseous inflammation have not been identified. We conducted a pilot *in vivo* experiment using fresh concentrated equine bone marrow cells and the CatK inhibitor (VEL-0230) in the same concentrations which previously had shown a significant inhibition of pro-inflammatory cytokine secretions and MHC molecules expression (Chapter 5). We hypothesized that one or more of the concentrations of VEL-0230 that suppressed expression of inflammatory markers would significantly alter gene expression of progenitor cells as they differentiate along chondrogenic, osteogenic, and osteoclastogenic pathways that would provide relevance into the effect of VEL0230 in bone.

#### **METHODS:**

Sternal bone marrow was isolated from 6 healthy horses, cultured in monolayer and adherent cells, bone marrow derived-mesenchymal stromal cells (BMD-MSCs), were driven down the chondrogenic and osteogenic pathways, while the non adherent fraction was driven down to the osteoclastogenic pathway as previously described (Chapter 4, material and methods section) under the effect of 0, 1, 10, and 100 uM of VEL-0230 in culture media. Analysis of selected specific gene expression relevant to the three pathways was subsequently performed for all experimental conditions.

***Gene expression analyses:***

Quantification of gene expression relevant to the chondrogenesis, osteogenesis, and osteoclastogenesis pathways by real-time reverse transcription polymerase chain reaction was performed for all experimental conditions. Total RNA was isolated using Guanidinium thiocyanate-phenol-chloroform extraction method (TRIzol<sup>®</sup> and Chlorophorm; Invitrogen). Complimentary (c) DNA was generated from 1 µg total RNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions using MJ Research PTC-150 Thermal Cycler (MJ Research, USA). Relative gene expression analysis was performed using equine specific custom primers (Primer3 software; Steve Rozen, Helen J. Skaletsky, 1998) for Collagen I, Aggrecan, Collagen II, Osteoclastin, Osteopontin, Alkaline phosphatase, TRAP, CatK and GAPDH. Real-time PCR was performed in a total volume of 50 µl using 96-well microwell plates, an ABI PRISM 7000 sequence detector and SYBR<sup>®</sup> Green PCR Master Mix (Applied BioSystems). Cycle threshold (Ct) values were obtained for all analyzed genes during log phase of the cycle and expression level was compared between different experimental conditions using  $2^{-\Delta\Delta Ct}$  calculated using the Ct values of cells cultured in regular DMEM media as control sample.

***Statistical analysis:***

Data for all outcomes were statistically analyzed using SPSS v.18.0 (IBM Corp., Armonk, NY, USA). The data was then graphed as mean with standard deviation



(SD). The normal distribution within the different variables was investigated using Shapiro-Wilk test. Comparison between different CatKI concentrations for each gene expression data was performed using the one-way analysis of variance with repeated measures for normally distributed data followed by Duncan's Multiple Range Test, and Friedman test for skewed data. Significance was determined when  $P < 0.05$ .

## **RESULTS:**

### ***Cathepsin K inhibition significantly up-regulated gene expression relevant to the chondrogenic, osteogenic, and osteoclastogenic pathways in vitro:***

Relative expression of genes related to the chondrogenesis pathway (Collagen I, Aggrecan, and Collagen II) normalized to GAPDH and reference samples are shown in Fig. 48. There were statically significant increases in the gene expression under all CatKI concentrations compared to the zero concentration. Relative expression of genes related to the osteogenesis pathway (Collagen I, Osteoclastin, Osteopontin, and Alkaline phosphatase) normalized to GAPDH and reference samples are shown in Fig. 49. There were statically significant increases in the gene expression under all CatKI concentrations compared to the zero concentration. Relative expression of genes related to the osteoclastogenesis pathway (TRAP and CatK) normalized to GAPDH and reference samples are shown in Fig. 50. There were statistically significant increases in the expression under all CatKI concentration compared to the zero concentration.

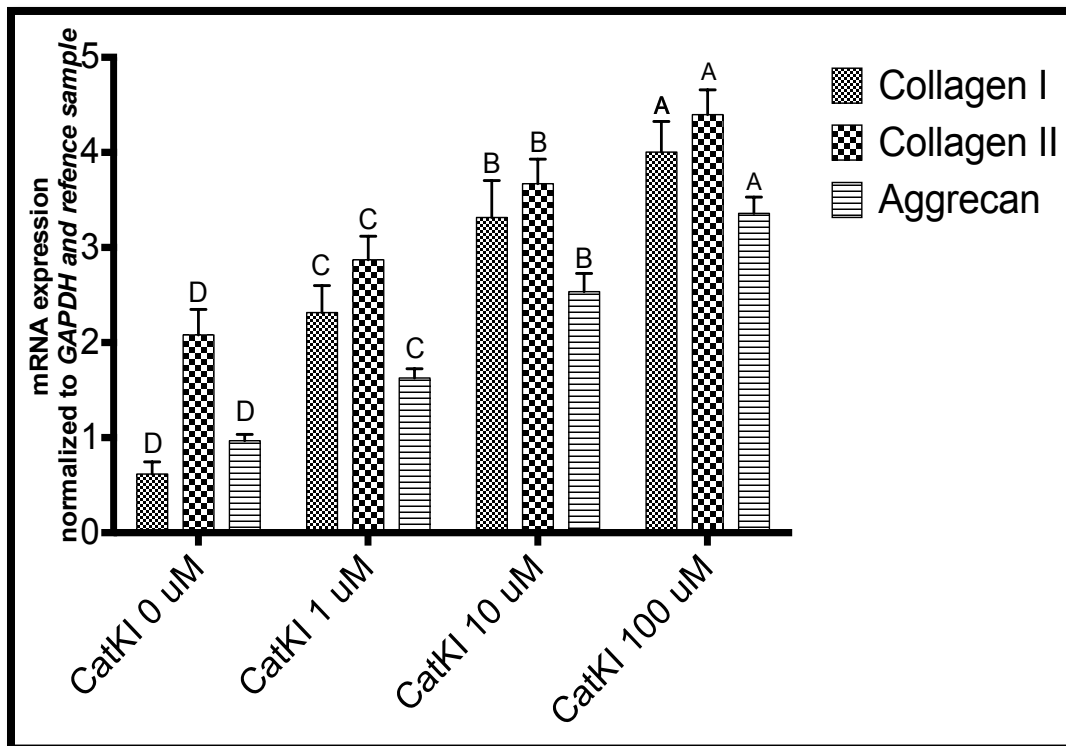
## **DISCUSSION:**

The increased gene expression of Collagen 1, Aggrecan, Collagen II, Alkaline phosphatase, Osteocalcin, Osteopontin, TRAP and CatK under the effect of VEL-0230 suggested an up-regulation of the differentiating cells ability to form cartilage (Collagen I, Collagen II and Aggrecan), bone (Collagen 1, Alkaline phosphatase, Osteocalcin and Osteopontin), and resorb bone (TRAP and CatK).

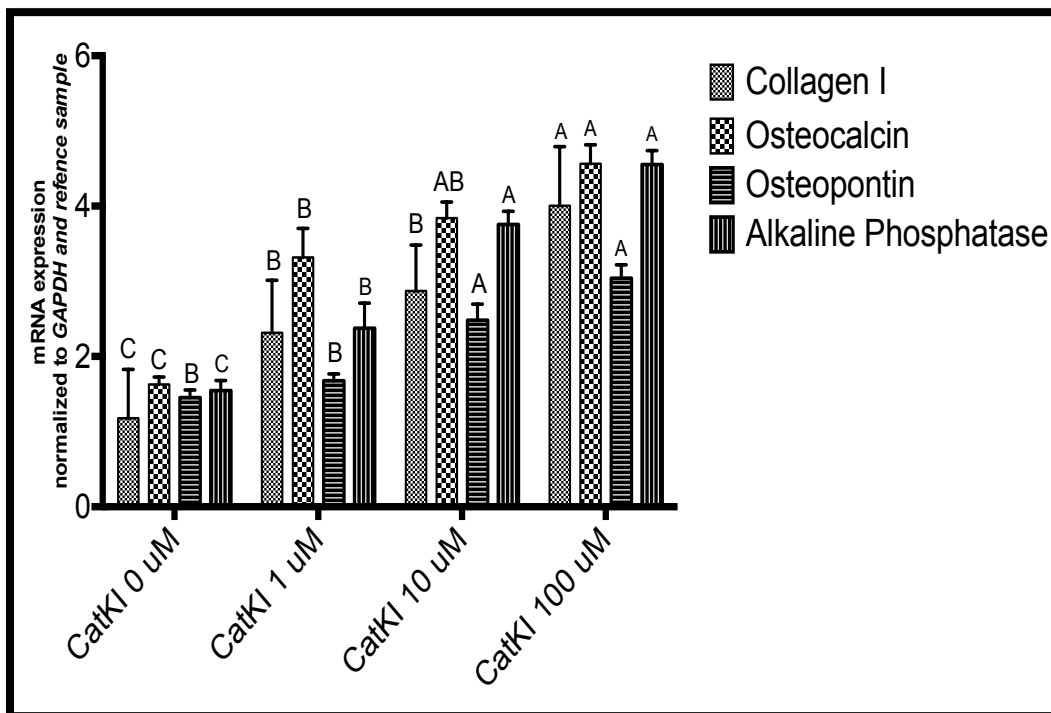
While type II collagen mRNA is primarily detected in cartilage and type I collagen is the primarily detected collagen in bone, levels of mRNAs for both types are detected in both bone and cartilage. Collagen is the main component of the intercellular matrix, in which numerous chondrocytes and osteocytes become embedded during chondrogenesis and osteogenesis. Aggrecan, known as cartilage-specific proteoglycan core protein or chondroitin sulfate proteoglycan 1, is another protein that is an integral part of the extracellular matrix in all cartilaginous tissues. Osteocalcin, a bone specific and vitamin K-dependent protein, and osteopontin, a phosphoprotein, have both been shown to be transcriptionally regulated during the phase in which the bone extracellular matrix begins to mineralize. Alkaline phosphatase is another molecule that is abundantly found on the surface of osteoblasts and has known to be primarily involved in multiple enzymatic activities, which promote osseous tissue mineralization. On the other hand, TRAP and CatK are two enzymes, which abundantly secreted by activated osteoclast to resorb bone

and are being used as marker for osteoclast differentiation *in vitro*.

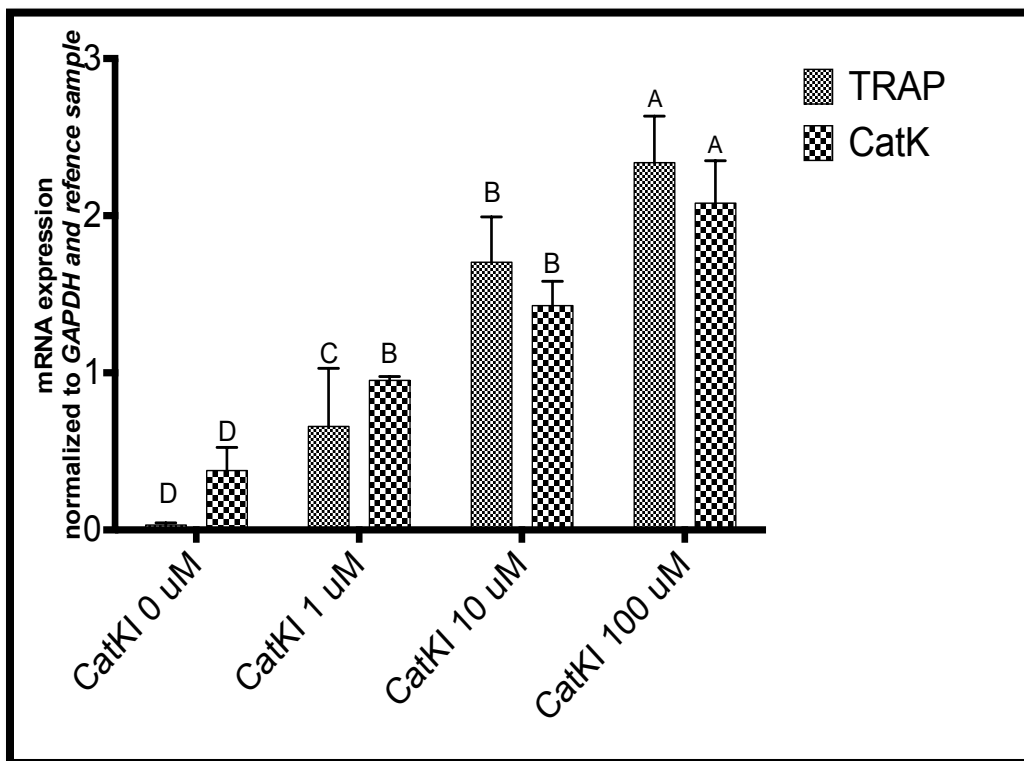
VEL0230 activated the pathways responsible for bone turnover in these bone marrow cells. Moreover, the magnitude of the changes in the gene expression was CatKI-concentration dependent, implying a causal relationship. These results may be explained as a feedback response to CatK depletion. CatK inhibition suppresses the cell's ability to resorb bone, which, in turn, could produce signals to up-regulate these same processes in these cells. Our data may suggest a potential for anabolic effects of CatK inhibition on bone and cartilage regeneration. Use of CatK inhibition over other current systemic, local, or regional treatments to moderate bone resorption and inflammation in equine osseous disorders may offer advantages that would require further study.



**Figure 48:** Analysis of gene relevant to the chondrogenic pathway (Collagen I, Collagen II, and Aggrecan) under different CatKI concentrations normalized to GAPDH and reference sample. Different letters refer to the significance between different CatKI concentrations within the same gene data.



**Figure 49:** Analysis of gene relevant to the osteogenic pathway (Collagen I, Osteocalcin, Osteopontin, and Alkaline phosphatase) under different CatKI concentrations normalized to GAPDH and reference sample. Different letters refer to the significance between different CatKI concentrations within the same gene data.



**Figure 50:** Analysis of gene relevant to the osteoclastogenic pathway (TRAP and CatK) under different CatKI concentrations normalized to GAPDH and reference sample. Different letters refer to the significance between different CatKI concentrations within the same gene data.

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**Chapter 2: Pharmacokinetics and bone resorption evaluation of a novel  
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**Chapter 3: Repeated Oral Administration Of A Cathepsin K Inhibitor  
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Young Exercising Horses With Evidence Of Maintained Bone Remodeling**

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#### **Chapter 4: Effect Of Repeated Oral Administration Of A Cathepsin K**

##### **Inhibitor On Equine Stem And Progenitor Cells Differentiation In Vitro.**

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## **Chapter 5: Cathepsin K inhibition renders equine bone marrow nucleated cells**

### **hypo-responsive to LPS and unmethylated CpG stimulation *in vitro***

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