# Effects of low and high dose intraarticular tiludronate on synovial fluid and clinical variables in healthy horses - a preliminary investigation

To determine effects of intraarticularly administered tiludronate on articular cartilage in vivo, eight healthy horses were injected once with tiludronate (low dose tiludronate [LDT] 0.017 mg, n=4; high dose tiludronate [HDT] 50 mg, n=4) into one middle carpal joint and with saline into the contralateral joint. Arthrocentesis of both middle carpal joints was performed pre-treatment, and 10 min, 24 h, 48 h, 7 and 14 days after treatment. Synovial nucleated cell counts and total solids, tiludronate, sulfated glycosaminoglycan (sGAG), chondroitin sulfate 846 epitope (CS-846, a measure of aggrecan synthesis), and collagen type II cleavage neoepitope (C2C) concentrations were determined. Histologic analysis of joint tissues and sGAG quantitation in cartilage was performed at 14 days in HDT horses. Data were analyzed by repeated measures non-parametric ANOVA and Wilcoxon signed-rank test. High dose tiludronate administration produced synovial fluid tiludronate concentrations of 2,677,500 ng/mL, exceeding concentrations that were safe for cartilage in vitro, and LDT administration produced synovial fluid concentrations of 1,353 ng/mL, remaining below concentrations considered potentially detrimental to cartilage. With HDT, synovial fluid total solids concentration was higher at 24 h and 7 days and sGAG concentration was higher at 48 h, compared to control joints. Synovial fluid CS-846 concentration was increased over pre-treatment values in HDT control but not in HDT treated joints at 24 and 48 h. All joints (HDT and LDT control and treated) showed a temporary decrease in synovial fluid C2C concentration, compared to pre-treatment values. Histologic features of articular cartilage and synovial membrane did not differ between HDT treated and control joints. High dose tiludronate treatment caused a transient increase in synovial total solids and temporarily

increased proteoglycan degradation in cartilage. Although clinical significance of these changes are questionable, as they did not result in articular cartilage damage, further investigation of the safety of intraarticular HDT in a larger number of horses is warranted.

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14    **Introduction**

15    Tiludronate, a non-nitrogen containing bisphosphonate, slows bone turnover by causing

16    apoptosis in osteoclasts (Rogers et al., 2011). This effect may be useful to treat conditions

17    associated with an increase in bone turnover, such as navicular disease or osteoarthritis. In fact,

18    some horses with navicular disease (Denoix, Thibaud & Riccio, 2003), osteoarthritis of the distal

19    tarsal joints (Gough, Thibaud & Smith, 2010) or the thoracolumbar facet joints (Coudry et al.,

20    2007) exhibited decreased signs of pain associated with their condition after systemic tiludronate

21    treatment. In humans, atypical subtrochanteric and femoral shaft fractures have been reported

22    after years of treatment (Nieves & Cosman, 2010). In horses, only anecdotal reports of side

23    effects are available to date, which have included colic, tachycardia, electrolyte imbalances, and

24    presumed association with renal failure. Concerns regarding side effects and the high cost of

25    systemic treatment constitute possible reasons why veterinarians have begun to administer

26    tiludronate locally via intraarticular injection or regional limb perfusion. However, no

27    information is available concerning safety, efficacy, or appropriate doses of these routes of

28    administration. Based on in-vitro results (Duesterdieck-Zellmer, Driscoll & Ott, 2012), the extra-

29    label use of tiludronate via intraarticular injection or regional limb perfusion, especially in high

30    doses, may result in deleterious effects for articular cartilage. While it is common belief among

31    veterinarians who have been using this drug in an extra-label fashion, that no untoward effects

32    exist, this view is scientifically unfounded. Further, lameness after treatment is likely being

33    attributed to the cause for the original lameness problem and not to negative effects of

34    tiludronate. Thus, it is imperative to investigate possible deleterious effects of extra-label routes

35    of administration for tiludronate in horses.

36    Little is known about tiludronate's effects on joint tissues after intraarticular

37    administration. Concentration-dependent effects of tiludronate on articular cartilage in-vitro

38   (Duesterdieck-Zellmer, Driscoll & Ott, 2012) raise concerns about the safety of higher doses

39   administered intraarticularly. More specifically, tiludronate concentrations of ≥19,000 ng/mL

40   increased chondrocyte apoptosis and release of glycosaminoglycans from equine articular

41   cartilage explants, suggesting that doses resulting in synovial fluid concentrations of ≥19,000

42   ng/mL may promote articular cartilage damage (Duesterdieck-Zellmer, Driscoll & Ott, 2012).

43   Anecdotally, the intraarticular dose of tiludronate is 50 mg per joint, which is likely to result in

44   synovial fluid concentrations above 19,000 ng/mL when injected into the middle carpal joint,

45   based on an estimated synovial fluid volume of 15 mL for this joint (Ekman et al., 1981). This

46   dose was chosen as the high dose in this study, whereas the low dose (0.017 mg of tiludronate per

47   joint) was chosen to result in synovial fluid concentrations of about 1,900 ng/mL. The objective

48   of this study was to determine effects of low dose tiludronate (LDT) and high dose tiludronate

49   (HDT) administered intraarticularly to healthy horses. We hypothesized that a single intraarticular

50   injection of HDT, but not of LDT would have a negative impact on clinical, clinicopathologic,

51   and biochemical indicators of joint health, compared to observations in contralateral control

52   joints injected with the same volume of saline. We further hypothesized that these negative

53   effects would result in histologically detectable articular cartilage damage in joints administered

54   HDT.

55   **Materials and methods**

56   Animals

57        After approval from the Institutional Animal Care and Use Committee, four horses (age

58   12-15 years, two Thoroughbreds, one Warmblood, one Quarter Horse) were enrolled to receive

59   LDT (ACUP 4034, approved 6/29/10) and a year later, four different horses (age 2-18 years,

60   Quarter Horses) were enrolled to receive HDT. All horses were without front limb lameness and

61    radiographic abnormalities of their carpal joints. They were housed in box stalls and offered free

62    choice grass hay and water during experiments.

63    Study Design

64       Baseline lameness examinations were conducted, followed by sedation with detomidine

65    (Dormosedan, Pfizer) and butorphanol (Torbugesic, Fort Dodge; both 0.01 mg/kg IV) for

66    arthrocentesis of both middle carpal joints (performed by KDZ and LM). After withdrawing up to

67    2 mL joint fluid (used to determine all synovial fluid variables as described below), 0.017 mg

68    tiludronate (Tildren, Ceva) in 1 mL saline was injected into one randomly assigned middle carpal

69    joint of four horses, and 1 mL saline was injected into the contralateral middle carpal joint (year

70    one, LDT). The other four horses (year two, HDT) were treated similarly, using an intraarticular

71    dose of tiludronate of 50 mg in 6 mL saline as treatment and 6 mL saline as control.

72    Randomization was performed via coin toss for each horse and investigators were blinded to

73    treatment allocation by an assistant removing the label from the syringes before injection. Both

74    joints were flexed and extended manually 15 times after injection. Aspiration of 0.5 mL joint

75    fluid (used purely for determination of tiludronate concentrations) was performed after 10 min on

76    both middle carpal joints. This resulted in removal of an estimated reduction of the effective

77    intraarticular dose of tiludronate by 3% for LDT and 2.5% for HDT based on a presumed

78    synovial fluid volume of 15 mL in the middle carpal joints (Ekman et al., 1981) and the

79    assumption that none of the saline used as diluent for tiludronate had been absorbed from the

80    joint space by 10 min post treatment. Repeat arthrocenteses of up to 3 mL joint fluid were

81    performed after 24 and 48 h, 7 and 14 days (used for determination of all synovial fluid variables

82    as described below). All horses treated with HDT were euthanatized on day 14 (Beuthanasia,

83    Merck; 1 mL/4.54 kg IV) and articular cartilage from the radial facet of both third carpal bones

84    and adjacent synovial membrane were collected. Investigators were blinded to treatment

85    allocations for all sample analyses.

86    Clinical variables

87         Horses underwent daily physical and lameness examinations (performed by KDZ and

88    LM), including palpation of both carpi for pain, effusion (none, mild, moderate, severe) and

89    development of edema, measurement of joint circumference of the middle carpal joints at the

90    level of a clipped mark on the lateral aspect of each middle carpal joint, and measurement of the

91    angle of maximum flexion of both carpi (Baseline Plastic Goniometer, Fabrication Enterprises).

92    For the latter, the angle present on the caudal/palmar aspect of the limb was measured. Thus, an

93    increase in angle of maximum flexion would indicate decreased range of motion of the carpal

94    joints. Horses were observed walking and trotting in a straight line, and trotting after flexion of

95    both carpi for 1 min. Lameness was categorized as sound, intermittently lame at the walk or trot,

96    consistently lame at the trot, or consistently lame at the walk. Responses to carpal flexion tests

97    were graded as positive when obvious lameness persisted for more than four strides (Ross, 2011).

98    Synovial fluid analyses

99         Synovial fluid samples were divided into 0.3 ml aliquots, of which one was placed in

100   EDTA containing tubes (Monoject, Covidien) for cytologic analysis. This aliquot was stored at 4

101   °C until cytologic analysis within 12 h of sample collection. All other aliquots were stored at -80

102   °C until sample analyses within 6 months of collection. All samples from year one (LDT) were

103   analyzed as one batch, and all samples from year two (HDT) were analyzed as one separate

104   batch.

105     Total solids concentration was measured via refractometry (E-line Veterinary,

106     Bellingham+Stanley). Total nucleated cell count was determined manually (Leuko-Tic,

107     Bioanalytic). Differential cell counts were obtained manually on Wright's-Giemsa stained

108     cytocentrifuge-prepared slides (CytoSpin* 4 Cytocentrifuge, Thermo Scientific).

109     Synovial fluid sulfated glycosaminoglycan (sGAG) concentration was determined

110     employing the 1,9-dimethylmethylene blue (DMMB) assay as described (Oke et al., 2003), with

111     some modifications. Briefly, samples were added to equal volumes of papain digestion buffer (2

112     mg/mL papain) and mixed at 65 °C for 3 h, followed by an additional 1:3 (v:v) dilution in papain

113     digestion buffer. Each sample was placed in triplicates into wells (5 μL/well) of a 96 well plate

114     (nunc Optical Bottom Plate non-treated, Thermo Scientific). Immediately before measurement of

115     absorbance at 540 nm, 245 μL of DMMB working solution (0.016 mg/mL DMMB) was added to

116     each appropriate well using a multichannel pipette. Sample concentrations were calculated from a

117     standard curve of chondroitin-6-sulfate (31.25-500 μg/mL; Chondroitin 6-sulfate from shark

118     cartilage, Sigma Aldrich).

119     Aggrecan synthesis in articular cartilage was estimated by quantitating chondroitin sulfate

120     846 epitope (CS-846) in synovial fluid by ELISA (IBEX Technologies), according to

121     manufacturer's instructions. Briefly, synovial fluid samples were centrifuged (16,000 x g for 2

122     min) and diluted 1:60 in Buffer III (provided by the manufacturer) and run in duplicates. A color

123     change was measured at 450nm (Multiskan Go, Thermo Scientific) and sample concentrations

124     were calculated from a standard curve of 20-1000 ng/mL CS-846.

125   Collagen type II degradation was estimated by quantitating collagen type II cleavage

126 neoepitope (C2C) in synovial fluid via ELISA (IBEX Technologies) according to manufacturer's

127 instructions. Briefly, synovial fluid samples were centrifuged (16,000 x g for 2 min) and diluted

128 1:2 in Buffer III (provided by the manufacturer) were run in duplicates. A color change was

129 measured at 450nm (Multiskan Go, Thermo Scientific). Sample concentrations were calculated

130 from a standard curve of 10-1000 ng/mL C2C.

131   Tiludronate concentration was determined by high performance liquid chromatography

132 (XBridge phenyl column, Waters) followed by mass spectrometry (Tarcomnicu et al., 2009; Zhu

133 et al., 2006). Tiludronate was methylated using 0.2 M trimethylsilyldiazomethane in acetone.

134 Sample concentrations were calculated from a standard curve of 0.5 – 64 ng/mL tiludronate.

135 Samples with tiludronate concentrations above the range of the standard curve were serial diluted

136 to fall within that range. Positive and negative control samples were run with each batch of

137 samples and deuterated tiludronate (Toronto Research Chemicals) was used as internal control in

138 each sample. The lower level of detection for this assay was 10 ng/mL.

139 Joint tissue analyses (n=4, HDT)

140   Cartilage samples from the radial facet of both 3rd carpal bones were digested with papain

141 (1 mL papain digestion buffer as described above per 10 mg cartilage wet weight) for ≥24 h until

142 no particulate matter was visible in the sample. Subsequently, sGAG content was determined via

143 DMMB assay as described above.

144   Histologic analysis on paraffin embedded sections of cartilage and synovial membrane

145 was performed by a board-certified veterinary pathologist (CVL) who was unaware of treatment

146 allocations. Hematoxylin and eosin stained cartilage sections were graded on a scale of 0-4 for

147  chondrocyte necrosis, chondrocyte clusters, cartilage fibrillation or fissures, and focal cell loss

148  (McIlwraith et al., 2010). Cartilage sections stained with toluidine blue (for proteoglycans) were

149  graded on a scale of 0-4 for loss of staining (McIlwraith et al., 2010). Hematoxylin and eosin

150  stained synovial membrane sections were graded on a scale of 0-4 for cellular infiltration with

151  lymphocytes and plasma cells, and intimal hyperplasia (McIlwraith et al., 2010). Scores were

152  recorded for three independent readings and the median score was determined.

153      Chondrocyte apoptosis was assessed on cartilage sections using the terminal

154  deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method (In Situ Cell Death

155  Detection Kit, AP, Roche) as described previously (Duesterdieck-Zellmer, Driscoll & Ott, 2012).

156  Data Analysis

157      Data were expressed as median and total range, other than tiludronate concentrations,

158  which were expressed as mean and standard error of the mean. To assess possible negative effects

159  of intraarticular administration of LDT or HDT on normal joints, differences between treated and

160  control joints within the same horse were determined using a two factor factorial model (dose of

161  tiludronate and treatment with saline or tiludronate) with repeated measures via non-parametric

162  ANOVA. To assess effects of LDT or HDT on normal joints over time, differences between

163  measurements prior to experimental treatment (baseline) and time points after experimental

164  treatment within the same joints (LDT control joints, LDT treated joints, HDT control joints, or

165  HDT treated joints) were determined. Comparisons between joints treated with HDT and joints

166  treated with LDT were not performed, as experiments were conducted in two different years and

167  samples were analyzed in different batches. Further, this comparison is of lower importance, as

168  the study did not aim at determining which dose was safer than the other. For variables in which

169  the main effect of time was not significant, median values for each treatment group (LDT control

170   joints, LDT treated joints, HDT control joints, HDT treated joints) were calculated by pooling all

171   time points. For those variables in which time significantly influenced the outcome, medians

172   were calculated for each time point separately. Data from post-mortem analyses in horses treated

173   with HDT was analyzed using the Wilcoxon signed-rank test. Statistical significance was set at

174   $P$=0.05.

175   A power analysis performed prior to the experiments estimated an 80% chance of detecting a

176   difference of 30% between treated and control joints within the same horse with a sample size of

177   n=4 and $P$<0.05.

178   **Results**

179       All physical examination variables remained within normal limits in all horses. No pain or

180   edema was evident upon palpation of any carpi, and no lameness or positive response to carpal

181   flexion was noted after tiludronate administration at any time. Mild to moderate effusion of the

182   middle carpal joints was common 1-3 days and 8 days after intraarticular injection of saline, LDT

183   and HDT (Table 1). Joints treated with HDT showed a greater degree of effusion than their saline

184   controls 1 day ($P$=0.0162), 3 days ($P$=0.0349), and 8 days ($P$=0.0349) after intraarticular

185   injection. In contrast, in the LDT group, joints treated with saline showed a greater degree of

186   effusion on day eight ($P$=0.0349) than joints treated with LDT. When comparing joint effusion

187   within experimental groups to baseline observations, HDT control joints showed increased

188   effusion 2 days after injection of saline ($P$=0.0008), whereas HDT treated joints showed

189   increased effusion 2 days ($P$=0.0264), 3 days ($P$=0.0264) and 8 days ($P$=0.0264) after treatment.

190   LDT control joints, showed increased joint effusion 1 day ($P$=0.0264), 2 days ($P$<0.0001) and 8

191   days ($P$=0.0008) after intraarticular injections, whereas LDT treated joints showed increased joint

192   effusion 1 day ($P$=0.0264) and 2 days ($P$<0.0001) after treatment.

193    Joint circumference did not differ over time ($P$=0.7326) nor between treated and control

194    joints ($P$=0.3735) in horses treated with HDT (28.3 [3.8] cm vs. 28.4 [2.7] cm – median and

195    range pooled over time) or LDT (29.0 [5.9] cm vs. 28.8 [6.5] cm – median and range pooled over

196    time).

197    Joint angle of maximum flexion was smaller in limbs treated with HDT than controls 10

198    ($P$=0.0135) and 13 days ($P$=0.0219) after treatment. There was no difference between treated and

199    control joints in horses treated with LDT (all $P$>0.3266) at any time. Joint angle of maximum

200    flexion was decreased at multiple time points compared to baseline measurements in HDT

201    treated, HDT control, LDT treated, and LDT control joints (Table 2). All differences were <10°,

202    and are unlikely to be clinically significant (Liljebrink & Bergh, 2010).

203    Synovial fluid concentrations of tiludronate are illustrated in Fig. 1. Tiludronate was

204    detectable in all joints treated with HDT throughout the study, whereas in joints treated with

205    LDT, tiludronate was not detectable after 24 h. Tiludronate was detectable in two HDT control

206    joints 24 h after treatment, albeit below the lower margin of quantitation for the assay.

207    Tiludronate was not detectable in any other samples from control joints or in any baseline

208    samples.

209    Synovial total nucleated cell count did not differ between treated and control joints in

210    horses treated with HDT or LDT ($P$=0.1165). Total nucleated cell counts exceeded the reference

211    interval of 0-500 cells/μL (Mahaffey, 2002) in three HDT treated joints, including one heavily

212    blood contaminated sample, and in one HDT control joint 7 days after treatment. All other values

213    remained within that reference range. When compared to total nucleated cell counts prior to

214    intraarticular injection, cell counts were increased 24 h, 7 days and 14 days (all $P$<0.0001) after

215    treatment in HDT treated joints and 24 h, 48 h,7 days and 14 days (all $P<0.0042$) after treatment

216    in HDT control joints. Total nucleated cell counts in samples from joints treated with LDT

217    decreased 7 days after treatment compared to baseline values ($P=0.0124$), and no change over

218    time was found for LDT control joints (all $P>0.7$).

219    The percentage of neutrophils among nucleated cells in synovial fluid samples remained

220    within the reference interval of $\leq10\%$ (Mahaffey, 2002) in the majority of samples (79%). The

221    remaining samples had 12-68% neutrophils, which was considered to be within normal limits due

222    to the very low total nucleated cell counts in these samples (Mahaffey, 2002). The percentage of

223    neutrophils among nucleated cells in synovial fluid samples did not differ over time ($P=0.0871$)

224    nor between treated and control joints ($P=0.3386$) in horses treated with HDT (4.5 [68] % vs. 5.5

225    [55] % – median and range pooled over time) or LDT (2 [20] % vs. 5 [25] % – median and range

226    pooled over time).

227    Total solids concentration in synovial fluid (Fig. 2) was higher in HDT treated joints than

228    HDT control joints but there was no difference between LDT treated and LDT control joints.

229    Compared to baseline values, total solids increased in HDT treated and HDT control joints for the

230    first 2 days. In LDT treated joints, total solids concentration was decreased at the end of the

231    study, compared to baseline values and there was no difference compared to baseline in LDT

232    control joints.

233    Synovial fluid sGAG concentration (Fig. 3) was greater in HDT treated than HDT control

234    joints on day 2 of the study, but there was no difference between LDT treated and LDT control

235    joints. Compared to baseline values, sGAG concentration tended to increase ($P=0.0513$) and then

236    decreased over time in HDT treated joints, whereas no increase compared to baseline was seen in

237    the HDT control joints, and instead, values decreased compared to baseline. In LDT treated or

238    control joints, sGAG concentration did not change compared to baseline.

239        Synovial fluid CS-846 concentration (Fig. 4) was lower in HDT treated joints compared

240    to HDT control joints, whereas there were no differences between LDT treated and LDT control

241    joints. In HDT control joints, synovial fluid CS-846 concentration increased and then returned to

242    not different from baseline levels again and this was not seen in HDT treated joints, as there was

243    no change compared to baseline. In LDT treated and LDT control joints, no change in synovial

244    fluid CS-846 concentration compared to baseline was found.

245        Synovial fluid C2C concentration (Fig. 5) was lower in HDT treated than control joints,

246    as well as in LDT treated compared to LDT control joints. All joints, HDT treated and HDT

247    control joints as well as LDT treated and LDT control joints showed a temporary decrease in C2C

248    concentration, compared to baseline values.

249        No significant difference was found between joints treated with HDT and control joints

250    for sGAG content of articular cartilage (308.0 [15.9] µg/mg vs. 308.6 [46.8] µg/mg respectively;

251    $P$=1.0) or % of apoptotic chondrocytes (2.2 [1.2] % vs. 1.7 [0.6] %; $P$=0.5637). Histologic scores

252    for joint tissues were not different between joints treated with HDT and control joints (Table 3).

253    **Discussion**

254        The target tissue for tiludronate has traditionally been bone (EMEA, 2001), but

255    tiludronate also exerts direct effects on cartilage and chondrocytes (Duesterdieck-Zellmer,

256    Driscoll & Ott, 2012; Emonds-Alt, Breliere & Roncucci, 1985), especially after extra-label local

257    administration via regional limb perfusion or intraarticular injection, since these routes of

258     administration potentially result in high synovial fluid concentrations. In-vitro data suggests that

259     synovial fluid tiludronate concentrations of 19,000 ng/mL and above promote chondrocyte

260     apoptosis and release of sGAGs from articular cartilage matrix, whereas concentrations of 1,900

261     ng/mL and below may ameliorate sGAG release and chondrocyte apoptosis in equine joints

262     (Duesterdieck-Zellmer, Driscoll & Ott, 2012). Subsequently, the dose for the LDT group was

263     chosen to produce synovial fluid tiludronate concentrations approximating 1,900 ng/mL. The

264     dose for HDT was selected based on anecdotal reports of clinical use and was suspected to

265     generate synovial fluid tiludronate concentrations exceeding those considered safe for articular

266     cartilage. As suspected, HDT resulted in synovial fluid concentrations much higher than 1,900

267     ng/mL for at least 48 h after intraarticular injection. This was accompanied by increased synovial

268     fluid sGAG concentration, resulting either from increased degradation and/or increased turnover

269     of sGAGs in articular cartilage (de Grauw, van de Lest & van Weeren, 2009), which would be

270     associated with an increased sGAG synthesis. To estimate synthesis of sGAGs, synovial CS-846

271     concentrations were determined, as this epitope has been used as a biomarker for aggrecan

272     synthesis (McIlwraith, 2005; Poole et al., 1994; Rizkalla et al., 1992). Aggrecan synthesis

273     appeared to remain unchanged in joints treated with HDT compared to measurements prior to

274     treatment. Thus, increased synovial sGAG concentrations in joints treated with HDT in the

275     present study were most likely associated with cartilage matrix degradation and not with an

276     increased turnover of sGAGs. Increased concentration of sGAG in synovial fluid has also been

277     reported due to repeated arthrocentesis in horses (van den Boom et al., 2005). However, it is

278     unlikely that the increase in sGAG concentration 48 h after intraarticular injection of HDT was

279     caused by repeated arthrocenteses, as sGAG concentration in contralateral control joints that had

280     undergone the same repeat arthrocentesis protocol as the treated joints were significantly lower

281     than in tiludronate treated joints. Further, repeat arthrocentesis caused a more modest increase in

282     synovial fluid sGAG concentration of about 20 µg/mL (van den Boom et al., 2005) compared to

283     what was found after HDT in this study (increase in synovial fluid concentratin of about 200

284     μg/mL). Nevertheless, sGAG content in articular cartilage was not found to be different between

285     joints treated with HDT and control joints 14 days after treatment, though this may reflect the

286     small number of horses investigated. A power analysis performed prior to the experiments

287     estimated an 80% chance of detecting a difference of 30% between treated and control joints

288     within the same horse with a sample size of n=4 and P<0.05. Another explanation for the lack of

289     difference in sGAG content between HDT treated and control joints may be that tiludronate

290     concentration in HDT treated joints remained within a range that has been shown to decrease

291     sGAG release from articular cartilage matrix in vitro (Duesterdieck-Zellmer, Driscoll & Ott,

292     2012) for at least the last seven days of the study. This may have allowed for increased retention

293     of sGAGs, thus ameliorating earlier sGAG losses.

294     Similar to what was observed in HDT control joints in this study, aggrecan synthesis,

295     measured as CS-846 concentration in synovial fluid, has been reported to increase with repeated

296     arthrocentesis after 24 and 48 h in horses (Lamprecht & Williams, 2012). This increase was

297     ameliorated in joints injected with HDT in our study, suggesting that high synovial fluid

298     concentrations of tiludronate may prevent chondrocytes anabolic response to mild insults such as

299     repeated arthrocenteses. Exercise has also been shown to increase aggrecan synthesis by

300     chondrocytes (Lamprecht & Williams, 2012), but it is impossible to state if intraarticular injection

301     of HDT would ameliorate this response in a similar fashion.

302     Another major protein of articular cartilage matrix is collagen type II (Vachon et al.,

303     1990), and it is accepted that collagen type II degradation in cartilage is irreversible (Catterall et

304     al., 2010; Jubb & Fell, 1980). The impact of repeat arthrocenteses on synovial fluid C2C

305     concentration appears to be variable over time in young horses (Lucia et al., 2013). After multiple

arthrocenteses over a time period of 12 h, synovial fluid C2C concentration was increased at 24

h, decreased at 7 days and unchanged at 14 days compared to baseline values. In the present

study, no increase in C2C was observed in LDT or HDT treated or control joints. Similarly to

what was reported in young horses (Lucia et al., 2013), a decrease in C2C was seen in all

experimental groups between 24 h and 14 days after treatment. Thus, changes in type II collagen

cleavage are likely attributable to the effect of repeat arthrocenteses, as opposed to tiludronate

treatment, although these results should be viewed in light of the small sample number used in

this study.

Synovial fluid total solids concentration increased at 24 and 48 h in both treated and

control joints of HDT horses. While this was likely due to effects from repeated arthrocenteses

(Francoz, Desrochers & Latouche, 2007; Jacobsen, Thomsen & Nanni, 2006) and saline

injections (Wagner, McIlwraith & Martin, 1982) for the control joints, total solids concentration

increased to a much greater extent in HDT treated joints than in control joints. Thus, intraarticular

administration of HDT was associated with a significant increase in synovial fluid total solids

concentration, most likely due to increased synovial total protein concentration. Increased

synovial protein concentration in HDT treated joints was attributed to be most likely caused by

synovial inflammation, as these joints were also palpable effused 24 and 48 h after treatment, and

in clinical cases, this observation is usually associated with a synovial inflammatory response

(Mahaffey, 2002). However, as synovial fluid prostaglandin $E_2$ concentration was not determined

in this study, we are unable to prove this assumption. It is unlikely that the high tiludronate

concentration in samples from HDT treated joints increased the total solids readings on the

refractometer, as spiking of blank synovial fluid samples with similar concentrations of

tiludronate did not change total solids readings (data not shown). Finally, while an increase in

sGAG concentration can affect total solids readings on the refractometer (data not shown), the

330   increased total solids concentration 24 h after HDT treatment cannot be explained with this

331   phenomenon, as sGAG concentratin was not elevated at that time point.

332         An unexpected finding was a significant elevation of the total nucleated cell count in both,

333   HDT treated and HDT control joints 7 days after treatment. This is unlikely to be due to repeat

334   arthrocenteses, as no arthrocenteses had been performed for the previous 5 days and elevation of

335   nucleated cell counts in synovial fluid due to repeat arthrocenteses have been reported to occur

336   within 24 – 48 h after initial arthrocentesis in horses (White et al., 1989) and calves (Francoz,

337   Desrochers & Latouche, 2007) and it has been suggested that joints can adapt to repeated

338   arthrocenteses over time, resulting in fewer alterations of total protein concentration and

339   nucleated cell counts after 24 h (Francoz, Desrochers & Latouche, 2007; White et al., 1989).

340   Further, there was no increase in total nucleated cell count 7 days after treatment in horses treated

341   with LDT. Thus, the cause for this increase in total nucleated cell count is unclear.

342         Although LDT resulted in synovial tiludronate concentrations that remained within a

343   range shown to be safe and beneficial for articular cartilage in vitro, these concentrations were

344   maintained only for 24 to 48 h, which may not be sustained enough to provide lasting beneficial

345   effects. However, it is possible that this dose is sufficient to affect subchondral bone remodeling

346   in treated joints if tiludronate diffuses through hyaline and calcified cartilage into adjacent

347   subchondral bone, although it is unknown whether or not this occurs. Investigations to determine

348   tiludronate content in subchondral bone after intraarticular administration are necessary to answer

349   this question.

350         Interestingly, tiludronate was detectable in two control joints of horses treated with HDT,

351   although concentrations were below the linear part of the standard curve for the tiludronate assay.

352 This finding is most likely explained by absorption of tiludronate from synovial fluid by synovial

353 capillaries, followed by redistribution into peripheral tissues, including distant joints. In support

354 of this, radioactivity from technetium 99 medronate, a radioactively labeled bisphosphonate, was

355 detectable in peripheral plasma within 5 min after intraarticular injection, peaked at about 45 min,

356 and was still detectable 24 h post intraarticular injection (Dulin et al., 2012). Alternatively,

357 contamination of the two samples from HDT control joints may have occurred during sample

358 processing.

359        The first hypothesis, that a single intraarticular injection of HDT, but not of LDT would

360 have a negative impact on variables of joint health, compared to contralateral control joints was

361 statistically confirmed in this preliminary investigation. However, the observed changes are of

362 questionable clinical significance and further investigation with a greater number of horses is

363 warranted. The second hypothesis that intraarticular injection of HDT would result in

364 histologically detectable articular cartilage damage was rejected, but the low power of this

365 preliminary study has to be taken into consideration when interpreting this result.

366 **Conclusions**

367        This study suggests that intraarticular administration of a high dose of tiludronate (50 mg)

368 may result in temporary elevation of synovial fluid total solids and sGAG concentration. Further,

369 this dose may prevent chondrocytes' short-term anabolic response to mild insults such as repeated

370 arthrocenteses. However, a high dose of tiludronate did not appear to negatively affect cartilage

371 or synovial membrane as assessed via histologic analysis 2 weeks after treatment. Thus, the

372 clinical significance of these findings remains questionable and further study of possible negative

373 effects of a high dose of tiludronate administered intraarticularly in a larger number of horses is

374 warranted.

375   Intraarticular administration of a low dose of tiludronate (0.017 mg) did not appear to

376 impact assessed variables of joint health, although this finding has to be interpreted with caution,

377 due to the small number of horses investigated.

378 **Acknowledgements**

379   The authors thank Drs. Stacy Semevolos and Erica McKenzie for their critical review of

380 this manuscript.

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Number of horses with middle carpal joint effusion before and after intraarticular administration of saline, LDT or HDT

Table 1: Number of horses with middle carpal joint effusion before and after intraarticular administration of saline, LDT or HDT.

1Table 1:  Number of horses with middle carpal joint effusion before and after intraarticular

2administration of saline, LDT or HDT.

| day of study | LDT control joints n=4 degree of joint effusion | | LDT treated joints n=4 degree of joint effusion | | HDT control joints n=4 degree of joint effusion | | HDT treated joints n=4 degree of joint effusion | |
|---|---|---|---|---|---|---|---|---|
| | mild | moderate | mild | moderate | mild | moderate | mild | moderate |
| 0[a] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1[b] | 3 | 0 | 3 | 0 | 0 | 0 | 1 | 1 |
| 2[b] | 2 | 2 | 2 | 2 | 4 | 0 | 4 | 0 |
| 3 | 1 | 0 | 2 | 0 | 2 | 0 | 4 | 0 |
| 4 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 |
| 5 | 0 | 0 | 0 | 0 | 2 | 0 | 2 | 0 |
| 6 | 1 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 7[b] | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 4 | 0 | 2 | 0 | 2 | 0 | 4 | 0 |
| 9 | 2 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 11 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| 13 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14[b] | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

4Notes.

5[a]Observations were made prior to any intraarticular injections or arthrocenteses.

6[b]Days with repeat arthrocenteses. Observations were made prior to that day's arthrocenteses.

Median joint angle of maximum flexion and total range in horses treated with either LDT or HDT in one middle carpal joint and with saline in the contralateral joint.

1Table 2:  Median joint angle of maximum flexion and total range in horses treated with either

2LDT or HDT in one middle carpal joint and with saline in the contralateral joint.

| day of study | LDT control joints | | LDT treated joints | | HDT control joints | | HDT treated joints | |
|---|---|---|---|---|---|---|---|---|
| | median | total range | median | total range | median | total range | median | total range |
| 0[a] | 11 | 6 | 13 | 8 | 4 | 6 | 3.5 | 3 |
| 1[b] | 8.5* | 3 | 9* | 4 | 3 | 2 | 4.5 | 6 |
| 2[b] | 10.5 | 6 | 8* | 4 | 2.5 | 3 | 2 | 1 |
| 3 | 6.5* | 2 | 7* | 6 | 2* | 1 | 2 | 2 |
| 4 | 9* | 4 | 7.5* | 3 | 2* | 2 | 2* | 2 |
| 5 | 8* | 1 | 8* | 3 | 2* | 1 | 2 | 1 |
| 6 | 8* | 7 | 8* | 5 | 1* | 2 | 1* | 3 |
| 7[b] | 11 | 6 | 11 | 3 | 2* | 4 | 2.5 | 6 |
| 8 | 14 | 7 | 12.5 | 3 | 2* | 1 | 2.5 | 3 |
| 9 | 11.5 | 3 | 10 | 4 | 0* | 2 | 2 | 3 |
| 10 | 11.5 | 3 | 12 | 6 | 0.5*‡ | 2 | 3‡ | 3 |
| 11 | 11.5 | 4 | 11.5 | 2 | 2* | 3 | 2 | 0 |
| 12 | 9 | 6 | 9* | 4 | 1* | 2 | 1.5 | 3 |
| 13 | 7* | 3 | 7* | 2 | 2*‡ | 2 | 3.5‡ | 3 |
| 14[b] | 8* | 4 | 9* | 2 | 0.5* | 2 | 1* | 2 |

4Notes.

5[a]Measurements were taken prior to any intraarticular injections or arthrocenteses.

6[b]Days with repeat arthrocenteses. Measurements were taken prior to that day's arthrocenteses.

7*Significantly different from baseline measurement on day 0 (*$P<0.05$). However,

8differences in angulation of <10° are unlikely to be clinically significant (Liljebrink & Bergh,

92010).

10‡Significant difference between HDT treated and control joints (‡$P<0.022$). However,

11differences in angulation of <10° are unlikely to be clinically significant (Liljebrink & Bergh,

122010).

Median histologic scores of articular cartilage and synovial membrane 14 days after intraarticular injection of 50 mg tiludronate or saline

Table 3: Median histologic scores of articular cartilage and synovial membrane 14 days after intraarticular injection of 50 mg tiludronate or saline.

1    Table 3:  Median histologic scores of articular cartilage and synovial membrane 14

2days after intraarticular injection of 50 mg tiludronate or saline.

| Histologic variable | Treatment | Median score[a] | Range | *P*-value |
|---|---|---|---|---|
| Chondrocyte necrosis | tiludronate | 0 | 0 | N/A |
|  | saline | 0 | 0 |  |
| Cluster formation | tiludronate | 1 | 1 | >0.999 |
|  | saline | 1 | 1 |  |
| Fibrillation/ fissuring | tiludronate | 0.5 | 1 | >0.999 |
|  | saline | 1 | 1 |  |
| Focal chondrocyte loss | tiludronate | 0 | 0 | N/A |
|  | saline | 0 | 0 |  |
| Cartilage toluidine blue stain uptake | tiludronate | 1.5 | 1 | >0.999 |
|  | saline | 1 | 2 |  |
| Synovial cellular infiltration (lymphocytes and plasma cells) | tiludronate | 1 | 2 | 0.25 |
|  | saline | 0 | 1 |  |
| Synovial intimal hyperplasia | tiludronate | 0.5 | 1 | >0.999 |
|  | saline | 0.5 | 1 |  |

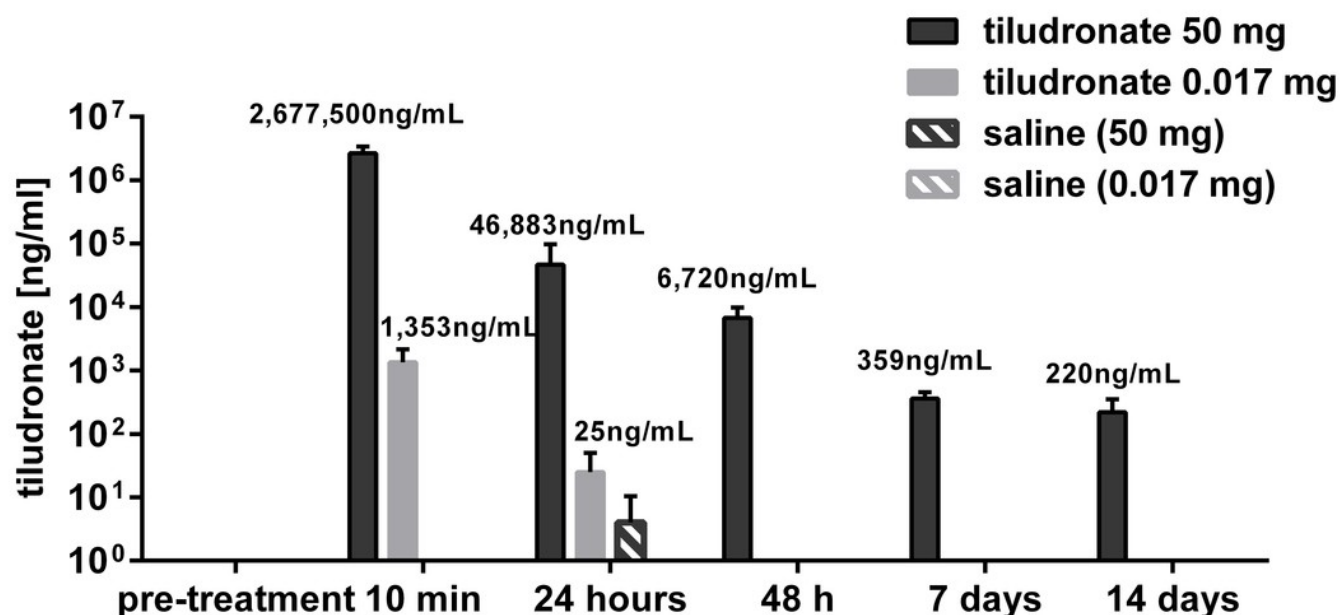
3Notes.

4[a]Histology was scored on a scale of 0-4, with 0 representing normal histology (McIlwraith et

5al., 2010).

# Figure 1

Graph of synovial fluid tiludronate concentration over time.

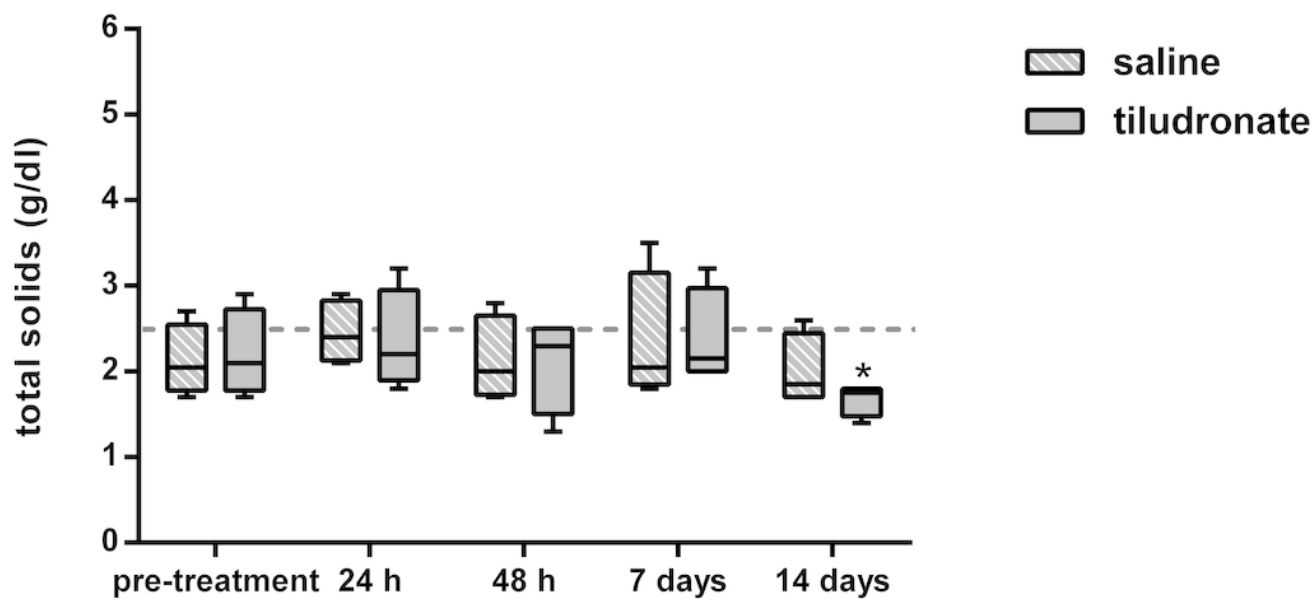
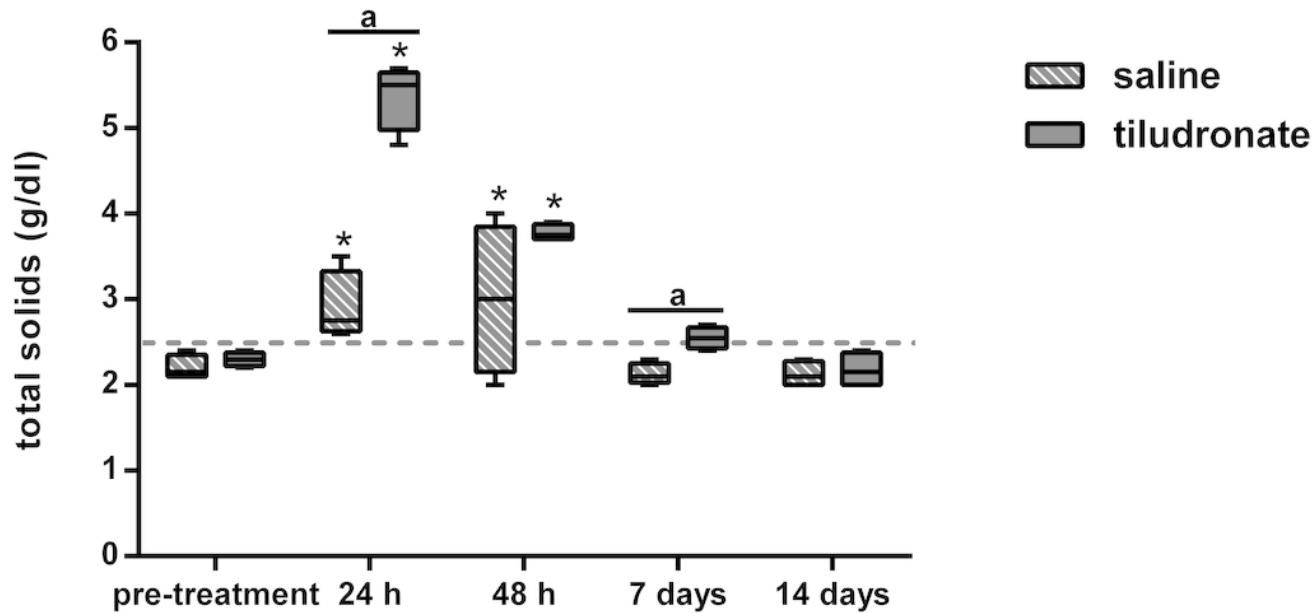
Fig. 1: Semi-log bar graph of mean synovial fluid tiludronate concentration over time. Tiludronate was injected at a dose of 0.017 mg in 1 ml saline (A) or at a dose of 50 mg in 6 ml saline (B) into normal middle carpal joints of 4 horses, respectively. The contralateral joints were injected with an equal volume of saline to serve as control joints. Tiludronate concentratin was determined using HPLC followed by mass spectrometry. Error bars show +95% confidence interval of measurements from 4 horses.

# Figure 2

Box-and-whisker plot of synovial fluid total solids concentration over time.

Fig. 2: Box-and-whisker plot of synovial fluid total solids concentration over time. Tiludronate was injected at a dose of 0.017 mg in 1 ml saline (A) or at a dose of 50 mg in 6 ml saline (B) into normal middle carpal joints of 4 horses, respectively. The contralateral joints were injected with an equal volume of saline to serve as control joints. Total solids concentration was determined via refractometry. The dotted horizontal line represents the upper limit of the normal reference interval (Davidson and Orsini, 2007).Whiskers represent the minimum and maximum values. Asterisks indicate significant difference compared to pre-treatment measurements in the same joints (*P<0.05). The superscript "a" indicates significant difference between treated and control joints (P<0.05).

**A**

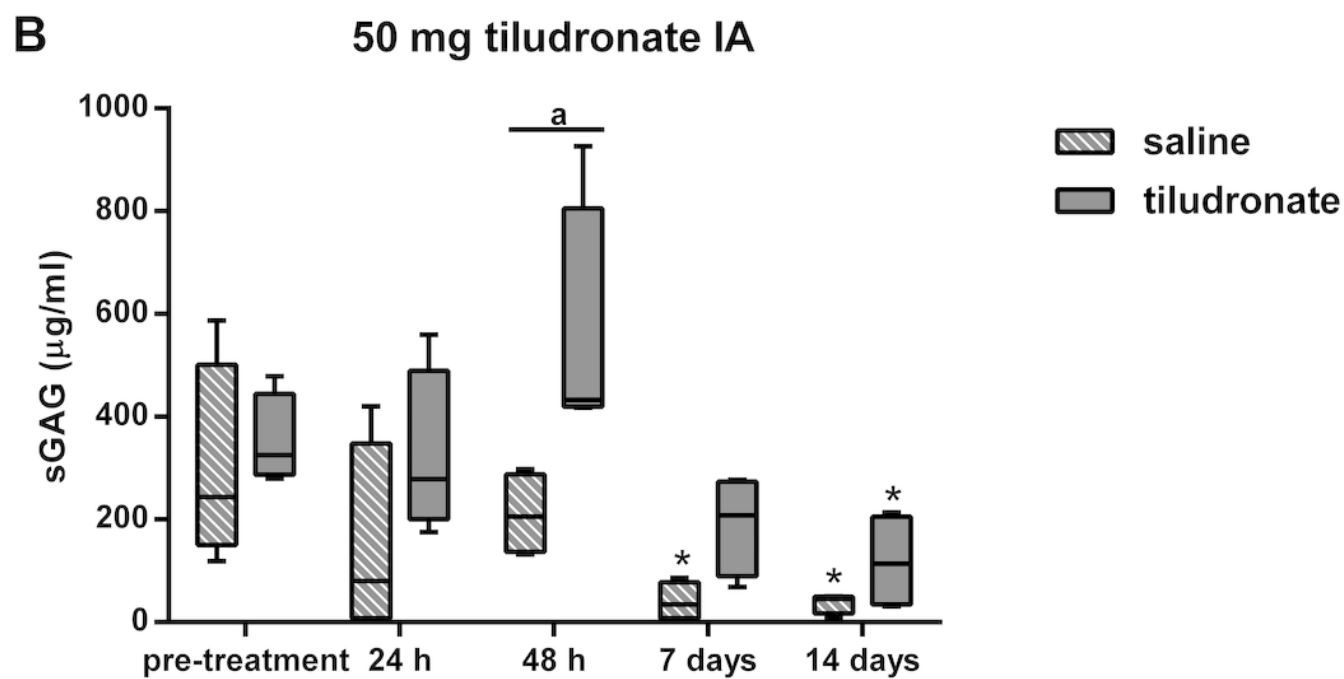
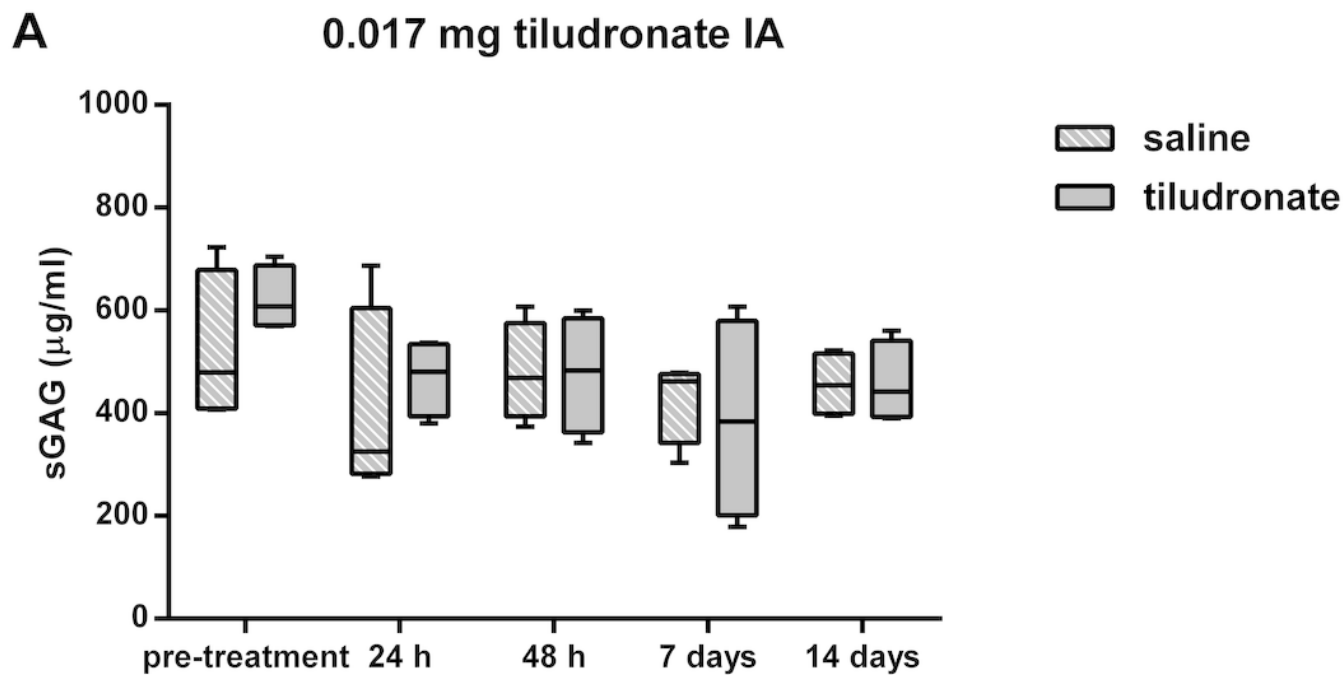
**0.017 mg tiludronate IA**

**B**

**50 mg tiludronate IA**

# Figure 3

Box-and-whisker plot of synovial fluid sulfated glycosaminoglycan (sGAG) concentration over time

Fig. 3: Box-and-whisker plot of synovial fluid sulfated glycosaminoglycan (sGAG) concentration over time. Tiludronate was injected at a dose of 0.017 mg in 1 ml saline (A) or at a dose of 50 mg in 6 ml saline (B) into normal middle carpal joints of 4 horses, respectively. The contralateral joints were injected with an equal volume of saline to serve as control joints. Sulfated glycosaminoglycans were quantitated using the 1,9-dimethylmethylene blue (DMMB) assay. Whiskers represent the minimum and maximum values. Asterisks indicate significant difference compared to pre-treatment measurements in the same joints (*$P < 0.05$). The superscript "a" indicates significant difference between treated and control joints ($P < 0.05$).

**A**  **0.017 mg tiludronate IA**

**B**  **50 mg tiludronate IA**

# Figure 4

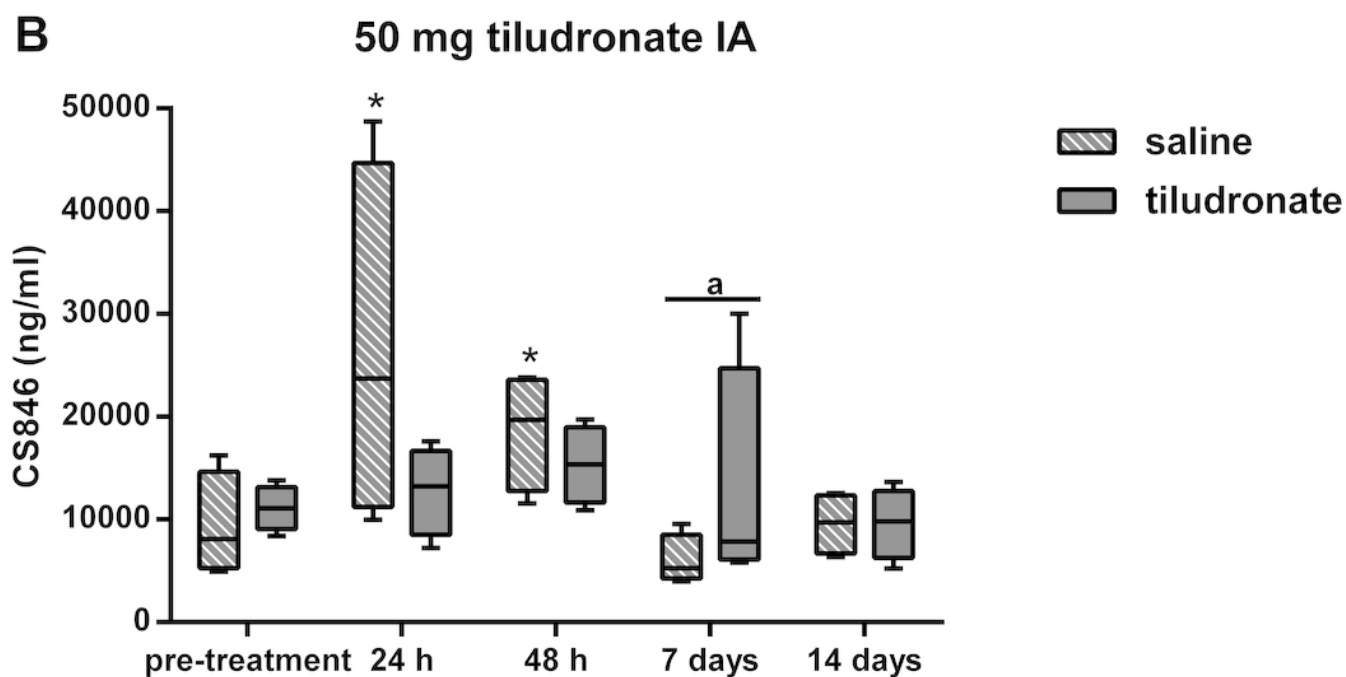
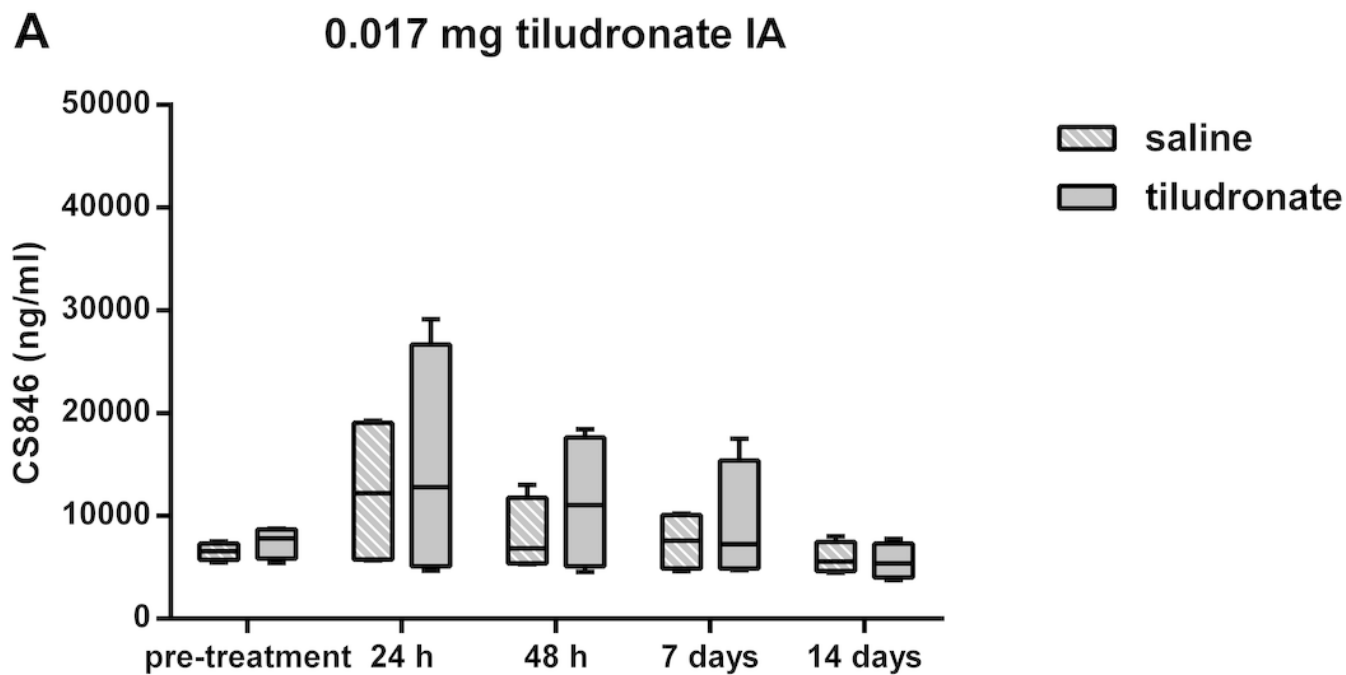
Box-and-whisker plot of synovial fluid concentration of chondroitin sulfate 846 epitope (CS-846) over time

Fig. 4: Box-and-whisker plot of synovial fluid concentration of chondroitin sulfate 846 epitope (CS-846) over time. Tiludronate was injected at a dose of 0.017 mg in 1 ml saline (A) or at a dose of 50 mg in 6 ml saline (B) into normal middle carpal joints of 4 horses, respectively. The contralateral joints were injected with an equal volume of saline to serve as control joints. Chondroitin sulfate 846 epitope was quantitated using ELISA to estimate aggrecan synthesis. Whiskers represent the minimum and maximum values. Asterisks indicate significant difference compared to pre-treatment measurements in the same joints ($*P<0.05$). The superscript "a" indicates significant difference between treated and control joints ($P<0.05$).

**A**

### 0.017 mg tiludronate IA

**B**

### 50 mg tiludronate IA

# Figure 5

Box-and-whisker plot of synovial fluid concentration of collagen type II cleavage neoepitope (C2C) over time

**A**

## 0.017 mg tiludronate IA

**B**

## 50 mg tiludronate IA