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Short-Term Increase of Serum Troponin I and Serum Heart-Type Fatty Acid-Binding Protein (H-FABP) in Dogs Following Administration of Formoterol

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Abstract

In this paper, changes in serum levels of the cardiac biomarkers troponin I and the heart-type fatty acid-binding protein (H-FABP) following administration of a long-acting β_2-sympathicomimeticum (LABA = long-acting beta-agonist) to dogs were measured.

We measured troponin I in dogs in a four week repeated-dose study with inhalative administration of formoterol (13 µg/kg/d) and a glucocorticoid/formoterol combination (143/16 µg/kg/d). The medians of troponin I increased within three days in both groups, far beyond the cut-off level (0.1 µg/L), but returned to baseline levels on study day 9. The increase was more pronounced in the formoterol-only group (3.29 µg/L) compared to the glucocorticoid/formoterol combination group (1.32 µg/L).

In a second study, we measured serum troponin I as well as serum H-FABP levels in several samples over seven days in dogs, receiving a single inhalative dose of a glucocorticoid/formoterol combination (120/12 µg/kg/d). The median of the troponin I concentration increased above the cut-off level within two hours, that of H-FABP within four hours. The medians of both parameters were temporarily above the cut-off levels even on study day 7.

Both studies were conducted according to national animal welfare guidelines.

To our knowledge, this is the first report which shows a corresponding increase of troponin I and H-FABP in dogs treated with formoterol. Both parameters are more sensitive to detect a drug-induced cardiac injury compared to total LDH, total CK as well as CK MB activity. However, it is recommended to take at least three blood samples per day to assess a temporary increase of troponin I.

Key Words

Formoterol, glucocorticoid, troponin I, heart-type fatty acid-binding protein, CK isoenzymes, dog

Introduction

The Expert Working Group on Drug-Induced Cardiac Toxicity of the Nonclinical Studies Subcommittee, reporting to the Advisory Committee for Pharmacological Sciences of the Center for Drug Evaluation and Research of the FDA, mentioned the serum troponins as specific, sensitive and robust biomarkers of myocardial damage which can be used for human as well as common laboratory animal samples (Wallace et al., 2004):

The troponin complex is bound to tropomyosin, which is located on the contractile actin filaments in the striated muscle. Troponin consists of three subunits: the calcium binding troponin C (TnC), troponin T (TnT) as bridging protein towards tropomyosin, and troponin I (TnI) which controls the binding of myosin to actin filaments.

The structure of TnT and TnI - but not that of TnC - in the cardiac muscle cells is different compared to that of skeletal muscles so that several immunoassays have been established to detect specifically the cardiac forms of the troponins in serum (Panteghini et al., 2004; Uettwiller-Geiger et al., 2002; Collinson et al., 2001; Datta et al., 1999).

A small amount of TnT and TnI (< 10 %) is not fixed to the actin filaments but is floating freely in the cytosol of muscle cells (Mair, 1997, Remppis et al., 1995, Voss et al., 1995).

Drug-induced cardiac histopathological findings have been correlated with serum cardiac troponin measurements in a number of species including rat, rabbit, mouse and dog (Pinelli et al., 2002; Machackova et al., 2001; Bertinchant et al., 2000; Adamcova et al., 1999; Herman et al., 1999; Bertsch et al., 1997; O'Brien et al., 1997a, 1997b, 1998; Bleuel et al., 1995).

In rats, the course of the serum troponin levels was assessed after administration of beta-adrenergic agonists (isoproterenol, orciprenaline; Bertinchant et al., 2000; Bertsch et al., 1997).

Fatty acid–binding proteins (FABPs) constitute a family of cytoplasmatic, non-enzymatic proteins with a size of about 15 kD, which are responsible for the intracellular buffering and transport of long-chained fatty acids (Glatz et al. 2002). The heart-type fatty acid-binding proteins (H-FABPs) are abundant in the heart muscle. Its concentration in the skeletal muscle is ten-fold lower than in the heart muscle. Even in the kidney, liver, small intestine and brain (Yoshimoto et al. 1995, Pelsers et al. 2004) low levels of H-FABP exist. After myocardial damage in humans, the H-FABPs are quickly released from the muscle cells. The increasing blood levels correlate well with the size of the myocardial infarction (Kleine et al, 1992; Glatz et al., 1994). The H-FABPs are cleared from the circulation by the kidneys with a plasma half-life in humans of 20 minutes (Glatz et al. 2002).

The serum levels of H-FABP after experimentally induced myocardial infarction were determined in the blood of mice (Aartsen et al, 2000) as well as the urine of rats (Volders et al, 1993), but to our knowledge not, as yet, in the dog.

In the following studies, the clinical and the histopathological findings are correlated with the serum troponin I levels during repeated administration of a substance combination containing the beta-adrenergic agonist formoterol to Beagle dogs.

Additionally, we describe the circadian course of serum troponin I and H-FABP levels in Beagle dogs after single administration of the above mentioned substance combination.

Materials and Methods

Laboratory Assays

Troponin I:

We applied a direct chemiluminescence troponin I immunoassay, using the ADVIA Centaur instrument, Bayer Diagnostics, Dublin, Ireland. The assay is established for the use of human serum samples. In order to test if the troponin I of canine serum samples can be measured reliably, we performed a short validation procedure:

Intra-assay precision: The troponin I levels (range 0.48 – 13.64 µg/L) in four canine serum samples were assessed in triplicate. The coefficients of variation were calculated to be < 3.1 %.

Inter-assay precision: The troponin I levels (range 0.46 – 13.37 µg/L) in four canine sera were measured in triplicate in 3 different test runs. The coefficients of variation were calculated to be < 10.0 %.

Linearity: Two canine serum samples (troponin I levels 2.67 and 32.54 µg/L) were diluted 1 : 2 and 1 : 4 with the troponin I diluent which was provided by the producer of the assay kit, and the troponin I levels were assessed in the serum samples as well as in the dilutions. The deviations of the measured troponin I levels compared to the calculated ones were < 16.8 %.

Recovery: Three canine serum samples (troponin I levels 0.01 - 1.81 µg/L) were spiked 1 + 1 and 2 + 1 with 2 other samples and the troponin I levels of the neat serum samples as well as the mixed samples were measured. The deviation of the calculated troponin I levels compared to the measured ones were < 14.8 %.

Reference range: The troponin I reference range was estimated from 158 serum samples of Beagle dogs (strain: Hsd Cpb: DOBE), Harlan Winkelmann, Germany, aged 9 – 12 months. The median of the troponin I values was calculated as 0.01 µg/L, the 97.5 percentile as 0.10 µg/L.

Creatin kinase (CK) and lactate dehydrogenase (LDH) activity:

The CK- as well as the LDH- activity was measured according to the standard methods of the IFCC. The reference ranges of the serum samples of Beagle dogs were estimated for the CK as < 190 U/l and for LDH as < 260 U/l (both measured at 37 °C with the Cobas Integra 400 instrument, Roche, Germany).

CK isoenzymes:

The CK isoenzymes were separated by agarose gel electrophoresis followed by an enzymatic formazane staining method. This procedure was performed semi-automatically with a Hydrasys instrument, Sebia, France. Three CK isoenzyme bands, the CK BB, CK MM_1 and the CK MM_2 isoenzyme, can be detected in serum samples of healthy dogs. The intensity of the isoenzyme bands was quantified by densitometry using the Hyrys 2 instrument, Sebia, France.

This method was only validated for human serum samples, and we therefore performed a short validation with canine serum samples:

Intra-assay precision: The CK isoenzymes of three canine serum samples were measured in triplicates. The coefficients of variation of each isoenzyme (CK activities 17 – 197 U/l) were calculated to be < 8.5 %.

Detection limit: The following criteria for the detection limit of a CK isoenzyme were set: The band on the agarose gel can be detected visually, and can be measured by

the densitometer with a coefficient of variation for intra-assay precision of < 10 %. Under these conditions, the detection limit was estimated as 7 U/l.

Inter-assay precision: The CK isoenzymes of two canine serum samples were assessed in duplicates on three different agarose gels. The coefficient of variation for each isoenzyme (CK activities 17 – 117 U/l) was < 12.2 %.

Linearity: Two canine serum samples were diluted 1 : 2 with 0.9 % NaCl solution. The CK isoenzymes in the undiluted samples as well as in the dilutions were measured. The deviation of the calculated activity of each isoenzyme compared to the assessed activity was < 15.2 % (CK activity of the isoenzymes 11 – 66 U/l).

Recovery: Three canine serum samples were spiked 1 + 1 with a different sample and the CK isoenzymes were assessed in the neat as well as the mixed samples. The deviations of the measured compared to the calculated isoenzyme activities were < 10.8 % (CK activity of the isoenzymes: 72 – 150 U/l).

Reference range: The CK isoenzymes of 60 serum samples of healthy Beagle dogs, Harlan Winkelmann, Germany, aged 12 to 24 months were measured. The following reference ranges were established (median, 2.5 and 97.5 percentiles): CK BB: 56 – 138 U/l; CK MB: < 7 U/l; CK MM_2: < 78 U/l; CK MM_1: 13 – 43 U/l.

H-FABP:

The H-FABP levels in canine serum samples were assessed using a sandwich ELISA designed for the measurement of H-FABP in rodents (HyCult, Biotechnology, Utrecht, The Netherlands). According to the supplier, a sufficient cross-reactivity to H-FABP exists between rats, mice and dogs. When using canine samples the assay was performed with the following modifications:

For the calculation of the H-FABP concentrations, the mouse standards were used. The canine serum samples were diluted 1 : 10 with the sample diluent provided with the kit. The incubation time of the samples in the precoated wells was prolonged to 18 – 20 hours at 2 – 8 °C.

Intra-assay precision: The H-FABP levels (range: 7.1 – 10.6 µg/L) in three canine serum samples were measured in quadruplicate. The coefficients of variation were < 9.3%.

Inter-assay precision: The H-FABP levels (range: 7.5 – 10.3 µg/L) in three canine serum samples were assessed in three different test runs in triplicates. The coefficients of variation were < 12.6%.

Linearity: Three 1 : 10 prediluted canine serum samples were further diluted with sample diluent 1 : 2 and 1 : 4. The H-FABP levels were measured in the prediluted samples (range: 20.5 – 29.1 µg/L) as well as in the further dilutions. The deviations of the measured H-FABP concentrations in the dilutions compared to the calculated values were < 16.0 %.

Recovery: Three canine serum samples (range: 5.7 – 17.9 µg/L) were spiked with two different canine serum samples (7.5/9.6 µg/L). The deviations of the assessed H-FABP levels in the mixed samples compared to the calculated ones from the H-FABP concentrations in the neat samples were < 16.0%.

Preliminary reference range: The diurnal course of the H-FABP levels in the serum samples of four healthy Beagle dogs, Harlan Winkelmann, Germany, aged about 24 months, was assessed. The following preliminary reference ranges were established (< 97.5 percentiles): morning peak (10 am): < 11.7 µg/L; range in the late afternoon (6 pm) < 8.2 µg/L (see Figure 3).

Study design

All studies were conducted as regulatory toxicology studies according to the ICH guidelines and in compliance with national animal welfare guidelines.

The objective of the two studies was the demonstration of the safe administration of the selected pharmaceutical compounds for humans. There is a sufficient safety margin between the applied doses to the dogs in the two studies and the intended pharmaceutical doses in humans. The selected compounds can safely be administered to humans under indicated conditions.

1st study (four week repeated dose):

40 Beagle dogs (20 females and 20 males; aged 9 to 12 months; Harlan Winkelmann, Germany) inhaled different pharmaceutical substances for 30 minutes once daily for 4 weeks. Animals were assigned to the following 4 groups:

Table 1

Clinical examinations: The behaviour of all dogs was observed daily. Clinical examinations including control of the pulse rate, body temperature, the muscle tone, sensitivity, reflexes, vision and hearing, as well as percussion and auscultation were performed prior to study initiation as well as about 1.5 and 24 hours after the start of the administration of the test substances in week one and four, respectively. At the same time points, electrocardiograms (ECG) were recorded. Additional ECGs were registered in individual dogs at further time points depending on clinical findings.

Blood samples were taken from all animals 14 and 7 days prior to the first substance administration as well as on Days 3, 9, 15 and 29 of the study. The venipuncture was performed on the V. cephalica antebrachii in the morning (prior to the substance administration and feeding of the dogs). After clotting, the blood samples (ca. 1ml)

were centrifuged (2000 x g; 10 minutes), and the separated serum was used the same day to assess troponin I, CK and LDH activity as well as the CK isoenzymes, as described above.

Histopathological evaluation: All dogs were sacrificed and exsanguinated on study day 29, with the exception of 4 animals each from the groups 1 and 4, which were necropsied after 4 further weeks without administration of the substance combination (recovery period). A complete post-mortem examination was performed and organs and tissues were sampled and fixed in 8% buffered formalin for at least 24 hours. Seven areas from the heart (right and left atrium, right and left auricle, right and left ventricle, septum) were trimmed, embedded in paraffin (Paraplast^{TM}), cut at a thickness of approximately 2 - 4 µm and stained with hematoxylin and eosin (H&E) using a standard procedure.

Statistics: In general, it is assumed that the troponin I values in a reference population don't have a standard Gaussian distribution. The Kolmogorov-Smirnov test applied to the troponin I values of the samples prior to the substance administration in this study confirmed this assumption. Therefore, we performed the non-parametric Mann-Whitney U test in order to compare the troponin I values in the various animal groups as well as in the treated dogs with and without ventricular extrasystoles, AV blocks and tachycardia. In addition, we used the Mann-Whitney U test to compare troponin I, as well as total CK and total LDH activity values of the male and female dogs in the various groups at each sampling date.

2nd study (single dose):

In this study, we administered a single glucocorticoid/formoterol fumarate dihydrate combination dose (intended dose: 120/12 µg/kg) via inhalation for 30 minutes to 7

Beagle dogs (3 females and 4 males; aged 28 to 60 months; Harlan Winkelmann, Germany). Blood samples were taken just before substance administration and 0.5, 1, 1.5, 2, 4, 6, 8 and 24 hours after the start of the inhalation. At the corresponding time points we also drew blood samples on study days 4 and 7. The venipuncture (ca. 0.5 ml blood) and the serum separation were performed as described for the 1st study, but the serum was frozen in aliquots immediately at -80°C, and thawed once for the measurement of troponin I and H-FABP as described above.

Reference ranges of healthy dogs:

To examine if a diurnal course of the troponin I and H-FABP levels exists in serum of healthy individuals, we determined both analytes in seven blood samples taken at the same time points as described in the 2nd study, using 4 Beagle dogs without clinical cardiac symptoms.

Results

1st study (four week repeated dose):

Clinical pathology: Comparing troponin I as well as total CK and total LDH activity values between male and female dogs of the various groups at each sampling date, there was found no gender difference (Mann-Whitney U test: $p > 5$ %). Therefore the values of both sexes were put together.

After three days, the medians of the troponin I concentrations in the serum samples of the dogs treated with 13 µg/kg/d formoterol (median 3.29 µg/L) as well as the dogs treated with the glucocorticoid/formoterol combination (143/16 µg/kg/d; median 1.32 µg/L) were significantly elevated compared to the troponin I medians of the vehicle control group (median 0.03 µg/L) as well as the group of dogs which were treated with the glucocorticoid only (118 µg/kg/d; median: 0.03 µg/L). The maximum troponin value on study day 3 in the formoterol group was 12.38 µg/L, and in the glucocorticoid/formoterol combination group 34.93 µg/L (Figure 1).

The troponin I median of the formoterol-only group was significantly higher compared to that of the glucocorticoid/formoterol combination group (Mann-Whitney U test: $p < 0.1$ %).

The medians of the enzyme activity of CK and LDH were not elevated above the reference values (CK: 190 U/L; LDH 260 U/L) in either the serum samples of the formoterol group or in those of the glucocorticoid/formoterol group. Nevertheless, single values were outside the reference range, but even in these samples we found no heart specific CK MB isoenzyme in the agarose gel electrophoresis (Table 2).

Although the daily administration was continued, the serum troponin I values returned to below the cut-off value (0.1 µg/L) on study day 9. Nevertheless, troponin levels were still above the cut-off level in single dogs (maximum troponin I value in the

formoterol group: 0.09 µg/L; in the glucocorticoid/formoterol combination group: 0.93 µg/L). On study day 29, the troponin I values of all dogs were below the cut-off value.

Clinical examinations: A red discolouration of mucosal tissue, scrotum and the skin of the abdominal region was noted in dogs of the formoterol-only group and the combination group.

Reduced activity was observed in several animals of the formoterol-only group and the combination group. On the first day of administration, a lateral recumbency lasting between 2.5 and 4 hours was seen in dogs of both groups.

Analysis of the ECG revealed a sinus tachycardia (values greater than 160 beats per minute) 1.5 hours after the start of the administration on Day 4 and 22 in the formoterol-only group and the combination group. In both groups, visual assessment of the ECG showed premature ventricular depolarization and escape depolarization, as well as AV blocks.

The dogs with ventricular extrasystoles during the first study week in the formoterol-only group and the glucocorticoid/formoterol combination group showed a significantly higher troponin I median (12.38 µg/L; N = 3) compared to the troponin I median of the individuals without this ECG finding (1.36 µg/L; N = 17; Mann-Whitney U test: $P < 1$ %). When comparing the troponin I medians of the dogs with and without tachycardia as well as with and without AV blocks, such significant difference could not be observed.

Nevertheless, the female dog of the combination group with the highest troponin I level (34.93 µg/L) showed a lateral recumbency lasting for up to 5 hours after administration on Days 1 to 3. It was accompanied by forced respiration on Day 2. In the ECG, ventricular extrasystoles were observed from Day 4 onwards.

Histopathological evaluation of the heart: Mild or moderate focal necrosis of the myocardium, predominantly in the papillary muscle of the left ventricle, associated with fibrosis and sometimes with mononuclear cell infiltration, was observed in 5 of 8 dogs (with a higher incidence in females) of the formoterol-only group. After the four week recovery period, mild or moderate focal fibrosis (without necrosis) was observed in the myocardium of 3 of 4 dogs in the substance combination group (Figure 2).

Table 2

Figure 1

Figure 2 a - b

2nd study (single dose):

In this study, the course of the troponin I values after an intended single dose of glucocorticoid/formoterol fumarate dihydrate (120/12 µg/kg/d) was investigated. We took a set of blood samples at different time points during the day of drug administration to 7 dogs as well as during the study days 4 and 7. Additionally, H-FABP as a second sensitive cardiac marker was measured in the serum samples.

The troponin I concentration increased in all samples two hours after the administration of the glucocorticoid/formoterol substance. The peak of the troponin I median was found eight hours after dosing. Thereafter, the median decreased until the next morning (24 hpa), but was still above the cut-off value. On study day 4, the troponin I median was still slightly elevated and even on Day 7, a slight increase in the medians at certain time points during the day was observed, with a peak in the afternoon.

Following glucocorticoid/formoterol administration there was no increase in H-FABP prior to 4 hours. On Day 4 and Day 7, there was still an increase in the median of the H-FABP serum levels at certain time points.

We measured the troponin I and the H-FABP concentrations in serum samples of four dogs without clinical cardiac symptoms taken at different time points during the course of one day. The troponin I values were below the cut-off value (0.1 µg/L) without any circadian cycle. The serum H-FABP concentrations were higher in the morning (peak at 10 am: 97.5 percentile: < 11.7 µg/L) than in the afternoon (6 pm: 97.5 percentile: < 8.2 µg/L; see Figure 3).

Figures 3 a - c

Discussion

Formoterol is a long-acting β_2-sympathicomimeticum. When administered in high doses, it causes tachycardia by stimulating the β_1-adrenergic receptors in the heart. Additionally, formoterol maintains the tachycardia by decreasing the blood pressure in the peripheral blood vessels. As a consequence of the increased heart rate, the time interval of the diastolic phase, which is necessary for the blood supply of the heart muscle, is shortened and causes an ischemic situation, which affects the muscle cells. The leakage of the cell membrane and release of intracellular molecules is a first sign of this cell damage.

The cytoplasmatic fraction of the small cardiac troponin I molecule (22.5 kD) passes through the gaps of the cell membrane and can therefore be found in the circulation. The troponin molecules are eliminated from the blood by the kidneys (Collinson, 2001). The estimated half-life of troponin T in the circulation is 120 minutes, while that of troponin I is yet unknown (Katus, 1991; Wallace et al., 2004).

After an acute myocardial infarction, the troponin fraction bound to the actin filaments is also released into the circulation because of a degradation of the muscle fibres per se. In this case, increased troponin levels can be found in the plasma for up to 21 days (Collinson, 2001).

In the four week repeated dose study, there was a large increase in the median of the plasma troponin I levels during the first three days of formoterol administration, which returned to the cut-off level at the latest on study day 9. This may indicate, that in this study only the cell membranes of the heart muscle cells were affected, not the muscle fibres themselves.

Nevertheless, significant clinical symptoms as well as effects in the ECG could be observed in dogs treated either with formoterol only or with the

glucocorticoid/formoterol combination. The dogs showing ventricular extrasystoles seemed to have higher troponin I levels compared to those without this ECG finding.

In addition, the histopathological evaluation showed lesions in the myocardium of the Beagle dogs treated with formoterol only as well as in those which received the substance combination for four weeks. However, these focal lesions, which are typical of β-agonists (Hoffmann, 2001), were mild to moderate and did not occur in all dogs. Nevertheless, these morphological findings in the heart could be correlated to the observed clinical effects.

Neither the CK nor the LDH activity in the serum was elevated in the dogs with increased troponin I serum levels. This supports the hypothesis of small gaps in the muscle cell membranes, because both enzyme molecules are considerably larger (CK 86 kD; LDH 135 kD) compared to the troponin I molecule. This observation is confirmed by Bertinchant (2000), showing similar results in isoproterenol-induced cardiac injury in rats.

The median of the troponin I serum concentrations in the dogs which were treated for three days with a combination of a glucocorticoid and formoterol was not as high as that in the individuals treated with formoterol only. This might be due to the fact that the corticoid substance has a stabilizing effect on the cell membranes.

To support this hypothesis, we measured the serum troponin I levels in dogs after single administration of a glucocorticoid/formoterol combination in the same dose-range as used in the repeated dose study. The medians of the troponin I concentrations increased above the cut-off level 2 hours after substance administration. This time interval was also observed in isoproterenol-induced cardiac injury in rats (Bertinchant, 2000). This was a considerably earlier increase as described for acute myocardial infarction in humans (about 6 hours, Wu, 2000;

Bodor, 1994). Although the medians of the troponin I concentrations decreased up to study day 4, they were still above the cut-off level. On Day 7, the serum troponin I medians were below the cut-off level in the morning, but increased above this value in the afternoon. This finding is in contrast to that of orciprenaline-induced tachycardia in rats (Bertsch, 1997). In this study, the serum troponin I and T concentrations in the rats returned to baseline values after 96 hours, however the authors analyzed only one blood sample on Day 4.

The theory of a physiological circadian variation in troponin I levels in dogs could be excluded by the measurement of troponin I in a set of blood samples of four dogs without clinical cardiac symptoms taken at the same time points during the day as the samples in the mentioned time-course study. All troponin I values of the healthy individuals were below the cut-off value (0.1 µg/L).

The temporary increase of the troponin I levels in the dogs 6 days after administration of a single dose of the glucocorticoid/formoterol combination may be explained as follows: The plasma half-life of formoterol fumarate in dogs after inhalation is about 6 to 7 hours (Sasaki et al., 1982; own unpublished data). Therefore, any pharmacological level of the substance one week after dosing can be excluded. However, it is possible, that the heart muscle cell membranes are still affected after this time interval. If there is an increase in endogenous catecholamines in these individuals during the day, this may lead to repeated leakage of the cell membranes with subsequent release of troponin I. Of course, this assumption has to be confirmed in further studies. Nevertheless, the canine hearts seem to adapt to a repeated dosage of formoterol, because in the repeated dose study the medians of the serum troponin I values decreased to below the cut-off level up to Day 9 of the study.

Additional to the assessment of the troponin I levels, we measured the H-FABP levels in the blood samples of the single dose study. Although the small H-FABP protein (15 kD) is not as specific for cardiac injury as the troponins, it is thought to be more sensitive. The increase in H-FABP in the serum can be detected 1 to 3 hours after an acute myocardial infarction in humans. The level should return to the reference value within 24 to 36 hours (Glatz, 2002). However, some publications challenge the sensitivity of H-FABP in humans with acute myocardial infarction (Wu, 2000).

In the present time-course study in dogs after the glucocorticoid/formoterol administration, the medians of the serum H-FABP levels did not increase before 4 hours after dosing. Therefore, in this study the serum level of this biomarker reached the cut-off level later compared to troponin I. Moreover, the increase in the H-FABP serum level in relation to the baseline concentration was very slight compared to that of troponin I. In view of the results of this study, H-FABP is not superior to troponin I with regard to the sensitivity of diagnosing a cardiac injury in the dog.

Similar to the course of troponin I serum levels 6 days after dosing of formoterol, H-FABP levels were at least temporarily increased above the cut off level. This supports the hypothesis of a leakage of the heart muscle cell membranes.

Nevertheless, it may be possible, that the H-FABP serum levels are influenced by the activity of the skeletal muscles, because the H-FABP concentration of these is one tenth compared to that of the heart muscle (Glatz, 2002). Indeed, the H-FABP values in the serum samples of the four healthy dogs were higher in the morning than in the afternoon. This may correlate with the physical activity of the dogs, but more H-FABP measurements in diurnal sets of samples of healthy dogs are necessary to confirm these reference ranges.

Based on the results of this study, it is recommended to take at least three blood samples during the day (e.g. 2, 4 and 8 hpa) in order to assess a temporary increase in troponin I. In addition, our results provide some evidence that H-FABP can be used as a biomarker in dogs for assessing drug-induced cardiac injury. In contrast to the serum levels in humans, the H-FABP serum levels in dogs do not seem to increase prior to the troponin levels after myocardial damage.

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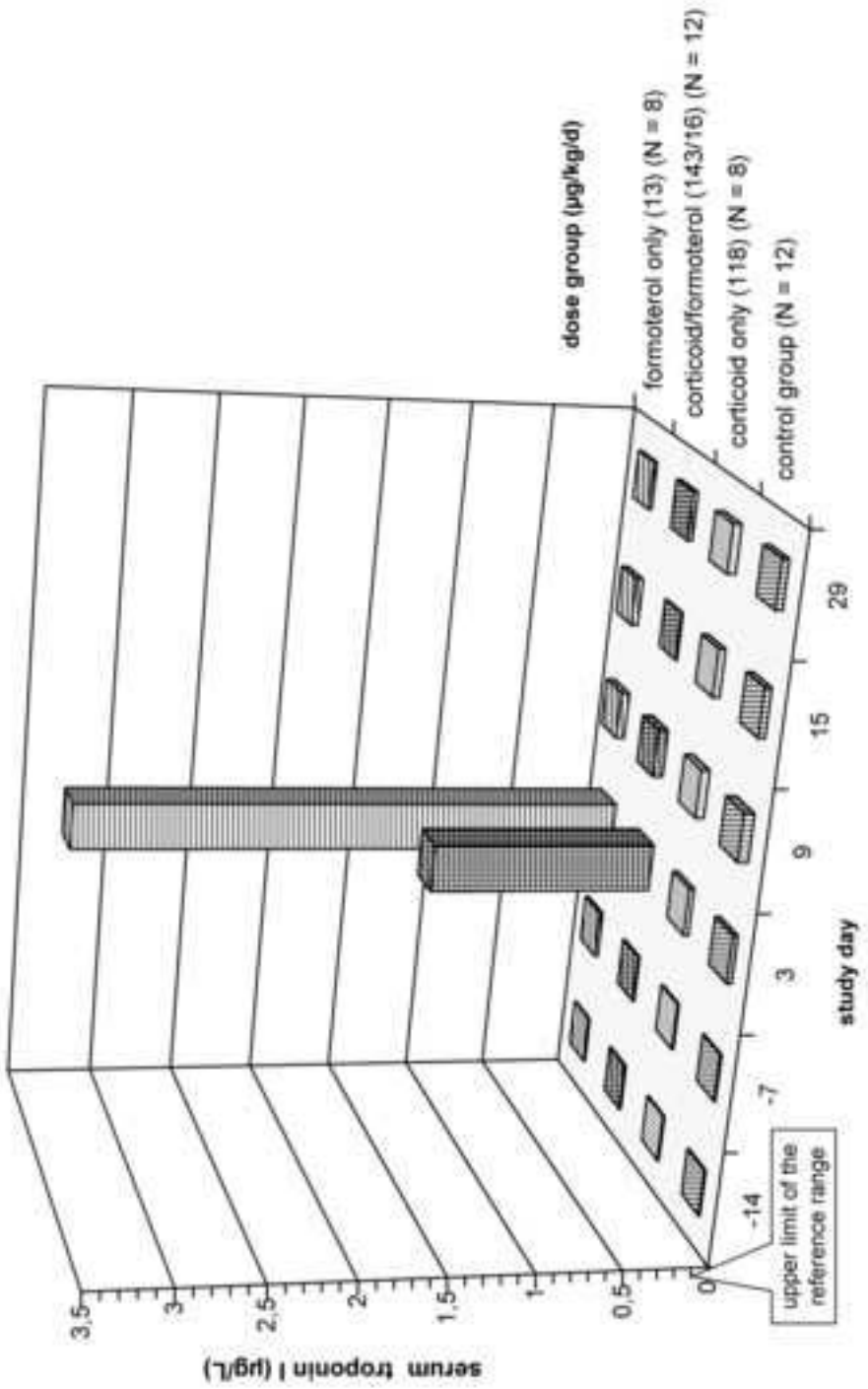
serum troponin I (µg/L)
3,5
3
2,5
2
1,5
1
0,5
0
upper limit of the reference range
-14
-7
3
9
15
29
study day
dose group (µg/kg/d)
formoterol only (13) (N = 8)
corticoid/formoterol (143/16) (N = 12)
corticoid only (118) (N = 8)
control group (N = 12)

Figure 1

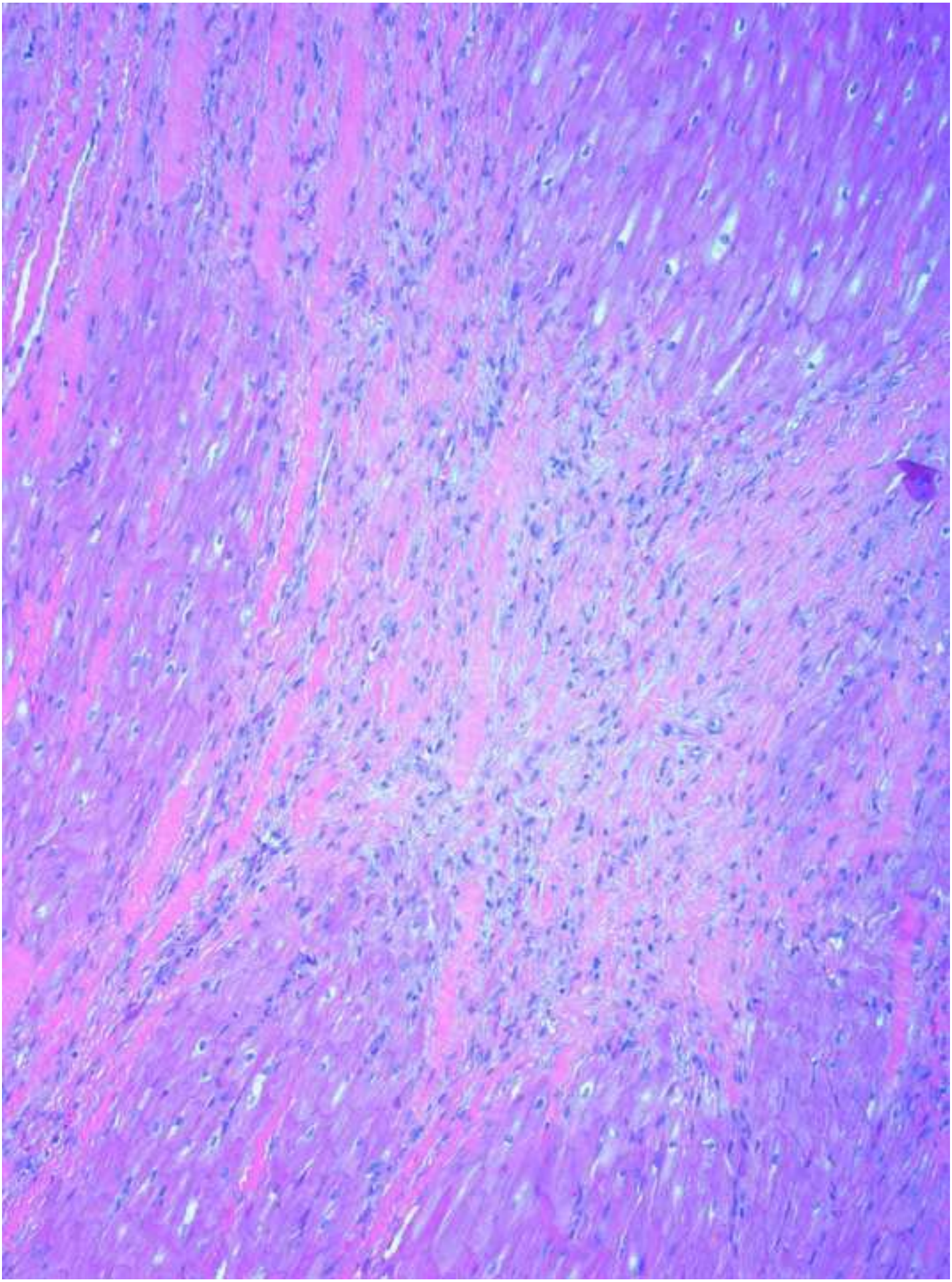

Figure 2a

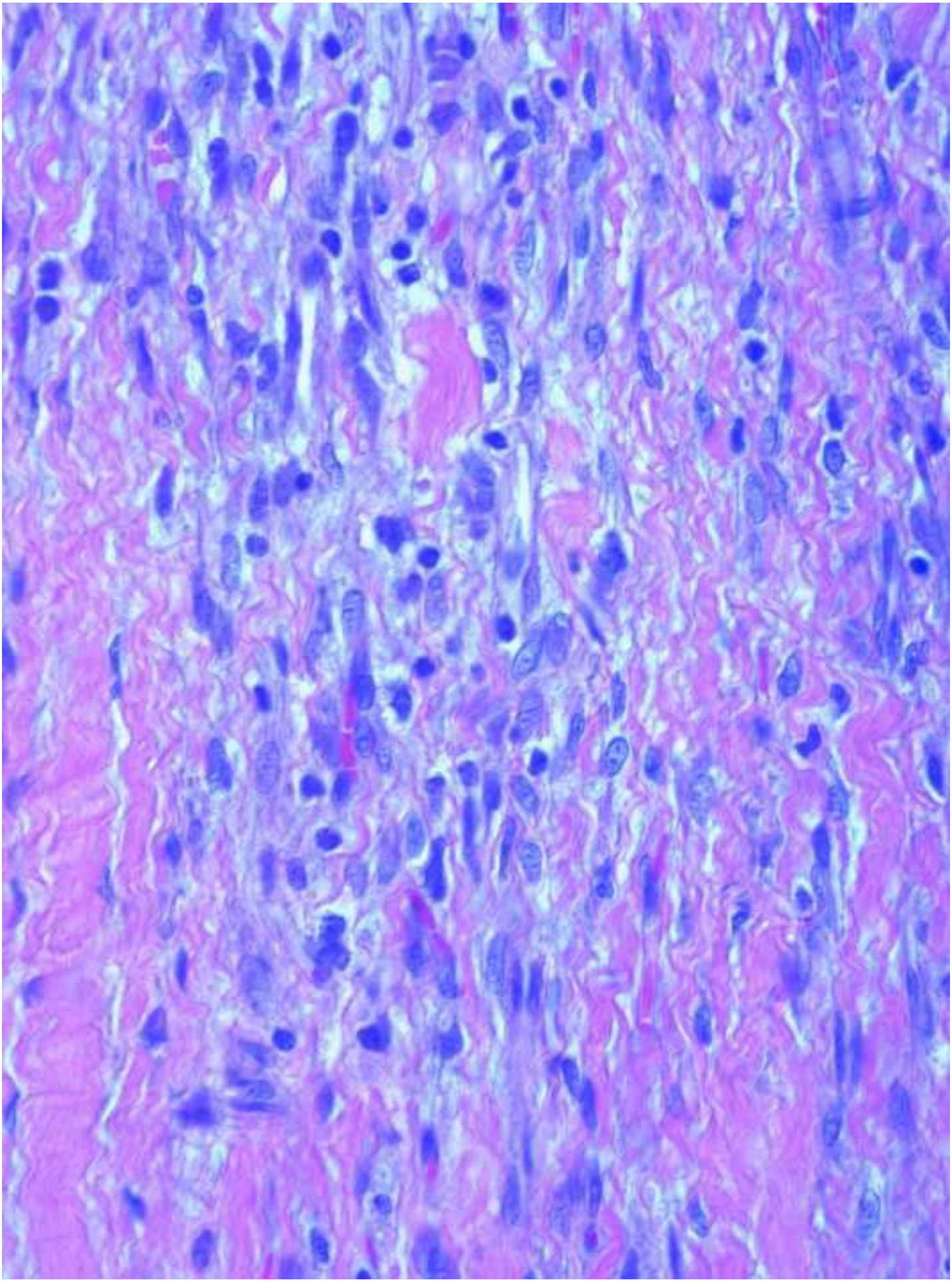

Figure 2b

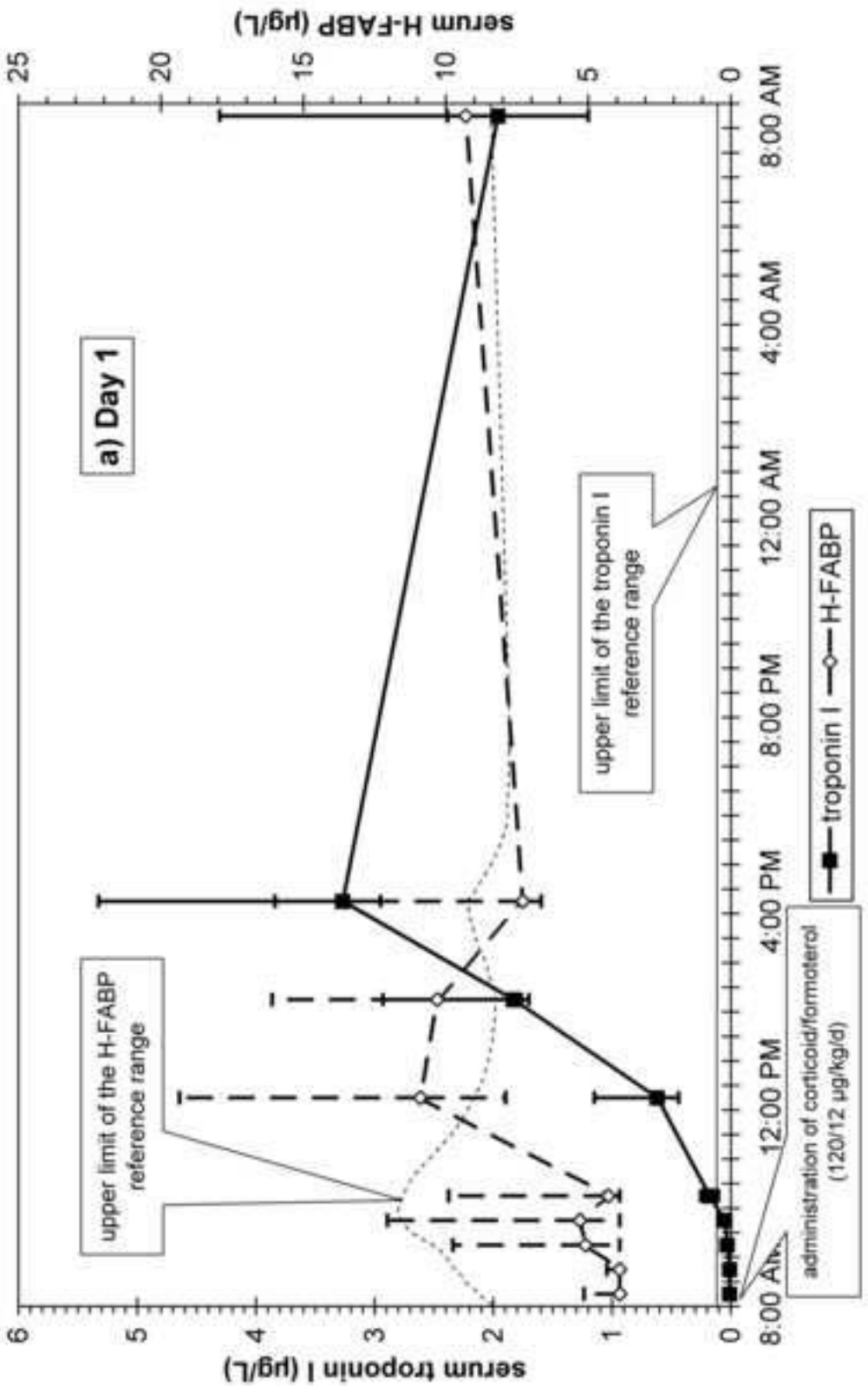
a) Day 1
serum H-FABP (µg/L)
serum troponin I (µg/L)
upper limit of the H-FABP reference range
upper limit of the troponin I reference range
administration of corticoid/formoterol (120/12 µg/kg/d)
troponin I
H-FABP
8:00 AM
12:00 PM
4:00 PM
8:00 PM
12:00 AM
4:00 AM
8:00 AM

Figure 3a

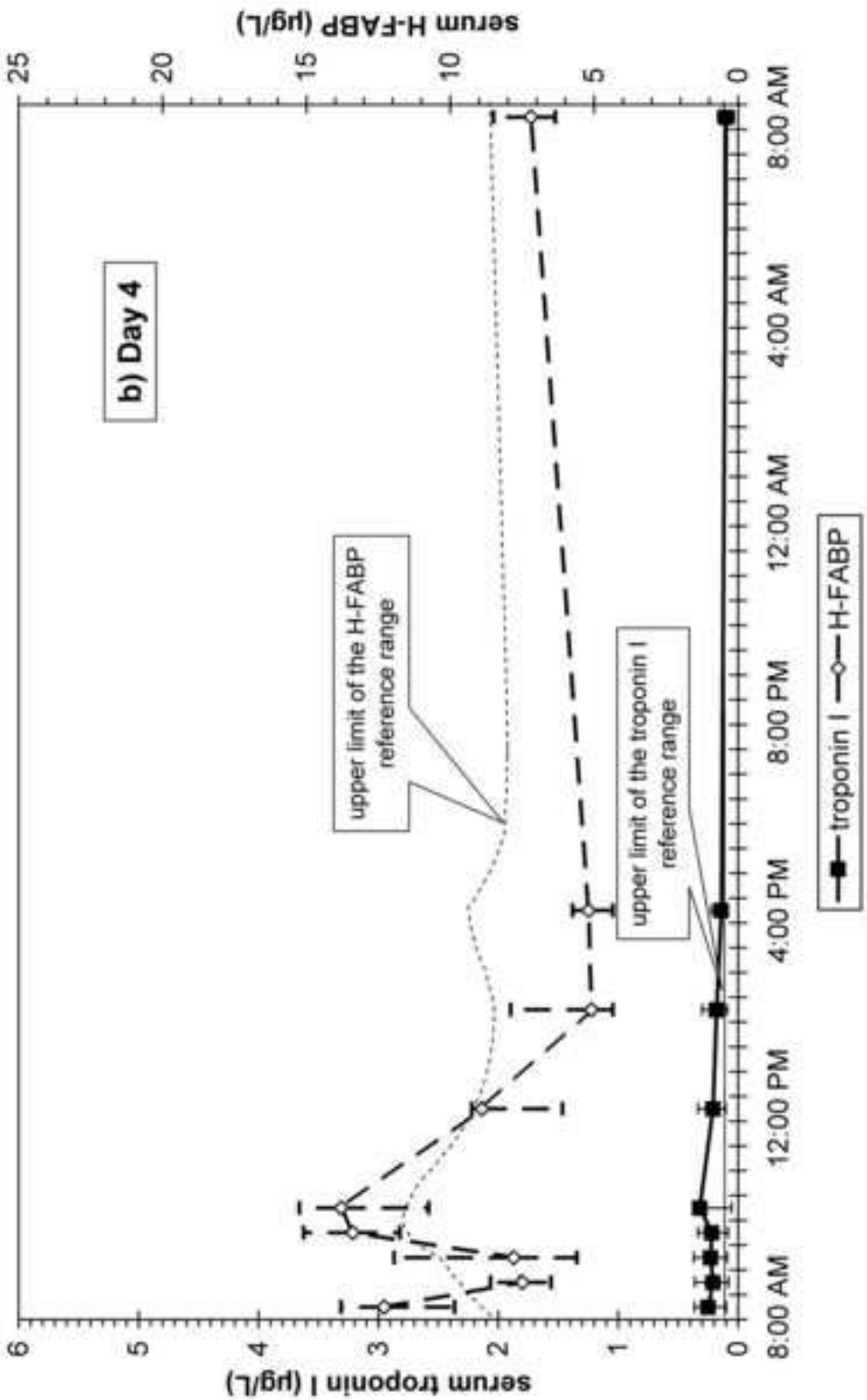

Figure 3b

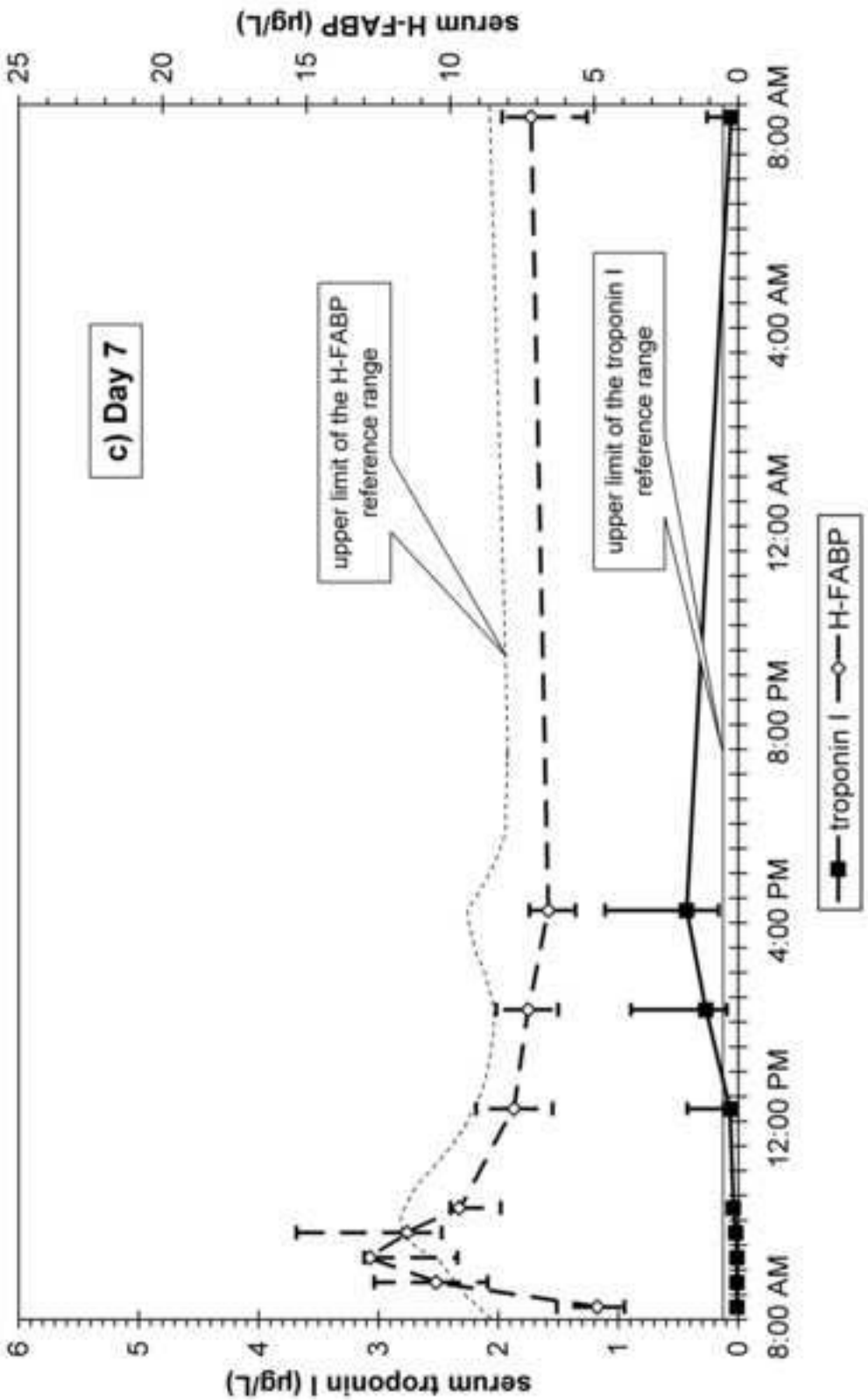

Figure 3c

Figure legends

Figure 1: 1st study (four week repeated dose); medians of the serum troponin I levels of Beagle dogs treated with formoterol or a glucocorticoid only, a combination of both substances or a vehicle substance, once daily for 29 days. Blood was taken from all dogs on Days 14 and 7 before starting the substance administration, as well as on Days 9, 15 and 29 of the study.

Figures 2 a – b: Heart of a dog showing focal fibrosis in the myocardium after administration of formoterol (13 µg/kg/d) for 29 days (H&E; Fig. 2a x100, Fig. 2b x400)

Figures 3 a – c.: 2nd study (single dose); serum troponin I and serum H-FABP concentrations (medians, 2.5 and 97.5 percentiles) in 7 Beagles after a single dose of a glucocorticoid/formoterol (120/12 µg/kg/d) substance at 8.00 AM: a) the day of substance administration, b) Day 4 and c) Day 7

Table 1: Assigned dog groups in the 4 week repeated dose study.

Group	Glucocorticoid; achieved dose [µg/kg/d]	Formoterol fumarate dihydrate; achieved dose [µg/kg/d]	Number of dogs males/females
1	-- (vehicle control)	-- (vehicle control)	6/6*
2	118	--	4/4
3	--	13	4/4
4	143	16	6/6*

* 2/2 dogs of each group in a 4 week recovery period

Table 2: 1st study (four week repeated dose); medians and maximum values of total CK and total LDH activities (in U/l, 37 °C) of Beagle dogs treated with formoterol or a glucocorticoid only, a combination of both substances or a vehicle substance (controls), once daily for 29 days. Blood was taken from all dogs on Days 14 and 7 before starting the substance administration, as well as on Days 9, 15 and 29 of the study.

group	dose [mg/kg/d]	N	study day	-14	-7	3	9	15	29
CK, total [U/l, 37 °C] (reference range < 190 U/l)									
vehicle controls	--	12	median	169	170	154	161	161	150
			maximum	248	258	249	257	239	310
corticoid only	118	8	median	172	144	108	133	145	137
			maximum	190	229	140	190	243	161
corticoid/formoterol	143/16	12	median	153	164	188	139	112	109
			maximum	189	211	*1223	337	241	150
formoterol only	13	8	median	135	152	116	104	110	177
			maximum	220	223	156	135	168	341
LDH, total [U/l, 37 °C] (reference range < 260 U/l)									
vehicle controls	--	12	median	99	116	72	88	85	76
			maximum	179	309	168	252	137	266
corticoid only	118	8	median	86	76	34	70	194	96
			maximum	140	191	89	309	110	255
corticoid/formoterol	143/16	12	median	83	96	83	99	64	34
			maximum	113	200	245	349	235	83
formoterol only	13	8	median	79	109	40	61	54	54
			maximum	204	169	55	81	64	107

*The sample with this high LDH activity did neither have an increased total CK activity nor showed any CK MB band in the isoenzyme electrophoresis, but the troponin I value was increased (0.97 µg/l).