Serum Thromboxane B₂ (TXB₂) Determination is Influenced by Sample Incubation Temperature in Healthy Beagle Dogs

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Abstract

The measurement of serum thromboxane B₂ (TXB₂) production by platelets is a specific test for assessment of platelet cyclooxygenase (COX-1) activity following administration of non-steroidal anti-inflammatory drugs (NSAIDs). The aim of this study was to investigate the influence of sample incubation at 37 °C for one hour on serum TXB₂ concentration in comparison with incubation at room temperature. A total of 54 blood samples for serum TXB₂ measurements were collected from six healthy beagle dogs into two separate serum tubes. While one group of tubes was incubated in a 37 °C water bath, the second group of tubes was left to coagulate at room temperature, both for one hour. Serum TXB₂ concentrations were measured by ELISA.

The mean concentration (± SD) of serum TXB₂ in the group of samples that were incubated at 37 °C was significantly (P < 0.0001) higher compared to the group of samples incubated at room temperature, 1098 ± 346 µg/l and 550 ± 257 µg/l, respectively.

The results of the study provide the information on serum TXB₂ concentration in healthy beagle dogs and demonstrate that validated methods for assessment of COX-1 activity by measurement of serum TXB₂ should be used in order to make results more reliable and comparable between different studies. The results of this study might be of great help in planning NSAID studies in dogs by providing the information that TXB₂ generation by platelets is influenced profoundly by incubation temperature.

NSAIDs, sample preparation, thromboxane B₂, dog

Non-steroidal anti-inflammatory drugs (NSAIDs) have been widely used to control acute and chronic pain and to manage oncologic diseases in both human and veterinary patients (Bergh and Budsberg 2005; Lascelles et al. 2005; Lees et al. 2004; Mathews 1996; Steinmeyer 2000). NSAIDs demonstrate both pain and inflammation inhibiting properties as well as several adverse effects such as inhibition of platelet function, gastrointestinal damage and renal impairment through inhibition of cyclooxygenase (COX), the rate limiting enzyme for synthesis of eicosanoids (prostaglandins, prostacyclins and thromboxanes) from arachidonic acid (Bergh and Budsberg 2005; Lascelles et al. 2005; Lees et al. 2004; Smecuol et al 2001; Steinmeyer 2000; Vane and Botting 1998; Vane 1971). COX exists in at least two isoforms, a constitutive cyclooxygenase-1 (COX-1) and inducible cyclooxygenase-2 (COX-2) (Fu et al. 1990; Masferrer et al. 1990; Vane et al. 1998). The therapeutic anti-inflammatory, analgesic, and antipyretic effects are mainly caused by the inhibition of COX-2 and the adverse effects are believed to result from inhibition of COX-1 (Bergh and Budsberg 2005; Brooks et al. 1999; Lees et al. 2004; Steinmeyer 2000; Vane and Botting 1998).

The inhibition of COX-1 in platelets by NSAIDs suppresses the formation of thromboxane A₂ (TXA₂), which is normally degraded to its stable and more easily measurable metabolite thromboxane B₂ (TXB₂) (Bergh and Budsberg 2005; Hamberg et al. 1975; Kamath et al. 2001; Steinmeyer 2000). Serum TXB₂ is predominantly derived from platelets, with leukocytes being another source (Higgs et al. 1983; Wallace and Ma 2001). In response to endogenous thrombin formation, i.e., clot formation, platelet COX-1 is...
maximally stimulated to produce TXA\(_2\), which is the major arachidonic acid metabolite produced by platelets, via COX-1, and one of the most potent platelet aggregating agent and vasoconstrictive substances known (Gilmer et al. 2003; Kamath et al. 2001; Smith 1989; Wallace and Ma 2001). Highly unstable TXA\(_2\) (\(t_{1/2}\) at 37 °C is 32 ± 2 seconds) is converted into a stable hydrolysis product TXB\(_2\) (Hamberg et al. 1975).

In contrast to extremely low TXB\(_2\) concentration in plasma (Jacoby et al. 2000; Knijff-Dutmer et al. 2002), an exceptionally high amount of TXB\(_2\) is produced ex vivo in blood after clotting under controlled conditions (Brideau et al. 1996; Patrignani et al. 1997). The measurement of serum TXB\(_2\) production by platelets following blood coagulation is a specific and most frequently used test for evaluation of platelet COX-1 activity in humans and other species (Brideau et al. 1996; Blain et al. 2002; Gilmer et al. 2003; Jones et al. 2002; Patrignani et al. 1997; Rinder et al. 2002; Sessions et al. 2005; Van Kralaij et al. 2002). Whole blood TXB\(_2\) production is measured from clotted blood samples obtained from the patient after administration of NSAID or after collection of blood into tubes preloaded with NSAID. Serum is then isolated by centrifugation and assayed for TXB\(_2\) by radioimmunoassay or ELISA. Validated methods that were developed (Brideau et al. 1996; Patrignani et al. 1997) for assessment of COX-1 and COX-2 activity (in vitro whole blood assays) recommended the incubation of samples during clotting at 37 °C for one hour, by which time TXB\(_2\) concentrations reach the plateau and thus enable the results to be more reliable and comparable between different studies.

Recently, we started a study to establish the safety of short-term use of meloxicam prior to general anaesthesia with isoflurane in healthy beagle dogs. While searching for the literature on normal values of TXB\(_2\) in canine serum and the measurement of serum TXB\(_2\) after NSAID administration, we found inconsistency in the published data, which might influence the results obtained. In some studies (Blain et al. 2002; Gilmer et al. 2002; Jones et al. 2002; McKellar et al. 1990; Rinder et al. 2002; Streppa et al. 2002) blood samples for serum TXB\(_2\) determination were incubated during blood clotting at 37 °C for one hour as recommended (Brideau et al. 1996; Patrignani et al. 1997), whereas other studies (Frelinger et al. 2006; Hashimoto et al. 2002; Morchon et al. 2006; Sunose et al. 2001a; Sunose et al. 2001b; Tanus-Santos et al. 2000) did not mention the incubation of samples. Instead of serum, plasma was used for TXB\(_2\) determination in a few studies (Jacoby et al. 2000; Knijff-Dutmer et al. 2002; Yamaoka et al. 1993).

Although validated methods for assessment of COX-1 activity by measurement of serum TXB\(_2\) have been established and recommended in order to make results more reliable and comparable, great inconsistency in the published data, especially in the veterinary medicine field regarding blood sample preparation for TXB\(_2\) determination still exists. Therefore, the aim of this preliminary study was to investigate the influence of sample incubation at 37 °C for one hour on serum TXB\(_2\) concentration in comparison with incubation at room temperature in healthy beagle dogs in order to avoid possible errors in the pre-analytical phase of testing and to provide information on serum TXB\(_2\) concentration in healthy beagle dogs.

**Material and Methods**

**Animals**

Six adult intact male beagle dogs, weighing between 16.5 and 22.6 kg, all free from clinical evidence of disease, belonging to a research colony at the Veterinary Faculty, University of Ljubljana, Slovenia, were used in this study. The dogs were considered in good health with normal findings on physical examinations and had laboratory results (data not shown) that were within the reference range for CBC and white cell differential count, serum biochemical analysis (urea, creatinine, total protein, albumin, alanine aminotransferase and alkaline phosphatase) and coagulation analysis (prothrombin time, activated partial thromboplastine time, concentration of D-Dimers). All laboratory analyses were performed prior to the study commencement. General procedure for animal care and housing were in accordance with the Animal Protection Act (Official Gazette, Republic of Slovenia, 43/2007). The dogs were housed by pairs in cages of appropriate size in a room with room temperature...
between 18 and 21 °C, fed a commercial dry and canned diet (Pedigree Pal, Mars Incorporated, USA) twice a day with unlimited access to water and walked in pairs at least 20 min three times per day. Social contacts between the caretakers and dogs were carried out during the day.

The study protocol was reviewed and approved by Ministry of Agriculture, Forestry and Food, Veterinary Administration of the Republic of Slovenia; license No 323-02-818/2005.

Blood sample collection, serum sample preparation and TXB₂ measurements

Blood samples for serum TXB₂ measurements were collected from the jugular vein from each of six dogs three times per day, at 7:00, 13:00 and 19:00 h, every time before meal, on three consecutive days, all together 54 samples. Samples were collected into two separate 4 ml serum tubes (Vacuette, Greiner bio-one, Kremsmuenster, Austria). One group of serum tubes was immediately placed in a 37 °C water bath and incubated for one hour to obtain maximal TXB₂ generation during coagulation. The second group of serum tubes was left to coagulate at room temperature for one hour. After one hour, the serum was separated by centrifugation at 1300 g for 10 min at 4 °C. Serum samples were stored at −70 °C until assayed. Serum TXB₂ concentrations were measured in two batches by use of an ELISA (Cayman Chemical Co, Mich., USA) according to the manufacturer’s instructions.

Statistical analysis

The results were evaluated by paired Student’s t-test (SPSS for Windows 8.0, SPSS 1998, Il, USA). The difference between the two groups was considered significant at P < 0.05. All values are reported as mean ± SD.

Results

The concentration of serum TXB₂ (mean ± SD) in the group of samples that were incubated at 37 °C for one hour was 1098 ± 346 μg/l. In the group of samples incubated at room temperature for one hour the concentration of serum TXB₂ was 550 ± 257 μg/l, the difference between the groups was significant (P < 0.0001). Serum TXB₂ concentrations in both groups of samples are presented in Fig. 1.

![Graph showing serum TXB₂ concentrations at 37 °C and room temperature](image)

Fig. 1. Serum thromboxane B₂, in two groups of samples; one was incubated at 37 °C for one hour, the second was incubated at room temperature for one hour

Serum TXB₂ concentrations were measured in two batches. Inter-assay variation (CV = 10%) was estimated by measurement of 20 serum samples in both batches. The difference between the batches was not significant (P = 0.88).

Discussion

The present study was conducted as a part of investigation on the safety of short-term use of meloxicam before general anaesthesia with isoflurane in healthy beagle dogs.

The post-clotting measurement of serum TXB₂ concentration is used as a specific test for assessment of platelet COX-1 activity following administration of NSAID. This method
is the most frequently used means of evaluating COX inhibitors mainly because this approach reduces variability due to sampling, which profoundly affects direct measurement of highly unstable TXA₂ (Blain et al. 2002; Brideau et al. 1996; Gilmer et al. 2003; Lees et al. 2004; Patrignani et al. 1997; Van Hecken et al. 2000). TXA₂ is normally converted to its stable product TXB₂ (Hamberg et al. 1975).

Although validated methods for in vitro whole blood assays for COX-1 and COX-2 have already been published (Brideau et al. 1996; Patrignani et al. 1997) and used by several research groups (Blain et al. 2002; Cryer and Feldman 1998; Gilmer et al. 2003; Panara et al. 1999; Sessions et al. 2005; Van Hecken et al. 2000), literature review on normal values of TXB₂ in canine serum and the measurement of serum TXB₂ revealed inconsistent data, especially on the preparation of serum samples and the methods of measurement used (RIA, ELISA). Therefore, we decided to investigate the influence of recommended sample incubation at 37 °C for one hour on serum TXB₂ concentration compared to incubation at room temperature in healthy beagle dogs. Our intention was to emphasize the importance of blood sample incubation at 37 °C, as it affects profoundly whole blood TXB₂ generation by platelets and thus serum TXB₂ concentration. As expected, the results of our study showed a significant difference in serum TXB₂ concentration between both groups of samples, 1098 ± 346 µg/l (incubation at 37 °C for one hour) versus 550 ± 257 µg/l (incubation at room temperature) for one hour. Serum TXB₂ concentrations in both groups of samples showed marked intra- and inter-individual variations, which is in accordance with previously reported results (Gilmer et al. 2003; Yamanaka et al. 1993).

In our study, mean serum TXB₂ concentration in samples incubated at 37 °C for one hour is in general agreement with studies that respect the same recommended conditions for incubation of canine blood samples (Gilmer et al. 2003; McKellar et al. 1990; Sessions et al. 2005). Dog serum TXB₂ levels are the highest among common domestic animals (887 ± 123 µg/l) as reported by McKellar et al. (1990). Gilmer et al. (2003) reported high intra- and inter-individual variations in serum TXB₂ concentration in canine serum. The mean value and standard deviation of TXB₂ determined in samples incubated at 37 °C in our study are very close to the mean basal value reported by this group, 1098 ± 346 µg/l and 1117± 383 µg/l, respectively. Unfortunately, we could not compare our results with other studies (Jones et al. 2002; Streppa et al. 2002) where TXB₂ was determined in canine serum samples at the same conditions as in our study, because serum TXB₂ was reported only as the percentage of change from the baseline.

While mean TXB₂ concentration in samples incubated at 37 °C for one hour was similar to the values of TXB₂ concentration in studies that incubated samples at the same conditions, mean serum TXB₂ concentration in samples incubated at room temperature (550 ± 257 µg/l) was not in accordance with the results of other research groups that used canine serum or plasma samples without incubation at 37 °C and reported TXB₂ concentrations of 1.5 µg/l or less (Hashimoto et al. 2002; Sunose et al. 2001a; Sunose et al. 2001b; Tanus-Santos et al. 2000; Yamanaka et al. 1993). In contrast to extremely low TXB₂ concentrations in plasma, an exceptionally high amount of TXB₂ is produced ex vivo in blood after clotting under controlled conditions that is incubation for one hour at 37 °C (Brideau et al. 1996; Patrignani et al. 1997). The great difference in TXB₂ concentrations obtained in our study after incubation of samples at room temperature and other studies that used plasma or serum samples without incubation at 37 °C might be ascribed not only to incubation of samples at possibly different room temperatures and time of incubation prior to centrifugation, but also to the type of sample for TXB₂ determination used.

The results of this preliminary study provide the information on serum TXB₂ concentration in healthy beagle dogs and will be used in our future study on the safety of short-term use of meloxicam prior to general anaesthesia with isoflurane in healthy beagle dogs. In conclusion, the results of the study demonstrate that validated methods for assessment of
COX-1 activity by measurement of serum TXB₂ should be used in order to make results more reliable and comparable between different studies. The results of our study might be of great help in planning NSAID studies in dogs by providing the information that TXB₂ generation by platelets is influenced profoundly by incubation temperature.

Vliv přípravy vzorků na koncentraci sérového thromboxanu B₂ (TXB₂) u zdravých psů plemene bígl

Měření produkce sérového thromboxanu B₂ (TXB₂) krevními destičkami je specifický test, kterým lze stanovit aktivitu COX-1 po podání nesteroidních protizánětlivých léků (NSAID). Cílem této studie bylo sledovat vliv inkubace vzorků při 37 °C po dobu 1 hodiny na koncentraci TXB₂ ve srovnání s inkubací při teplotě místnosti. Analýzováno bylo 54 vzorků krve odebrané řádně z drážděných psů plemene bígl vždy do dvou zkumavek. Zatímco jedna skupina vzorků byla inkubována ve vodní lázní při 37 °C byla 1098 ± 346 μg/l, zatímco ve vzorcích ponechaných při pokojové teplotě byla 550 ± 257 μg/l (p < 0,0001). Výsledky této studie poskytují data o koncentraci TXB₂ u zdravých psů plemene bígl a ukazují, že pro stanovení aktivity COX-1 měřením TXB₂ by se měly používat ověřené metody tak, aby bylo možné srovnávat výsledky různých studií. Naše údaje mohou být pomoci v plánování studií o NSAID; poukazují na skutečnost, že produkce TXB₂ trombocyty je pod vlivem inkubační teploty.

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References


