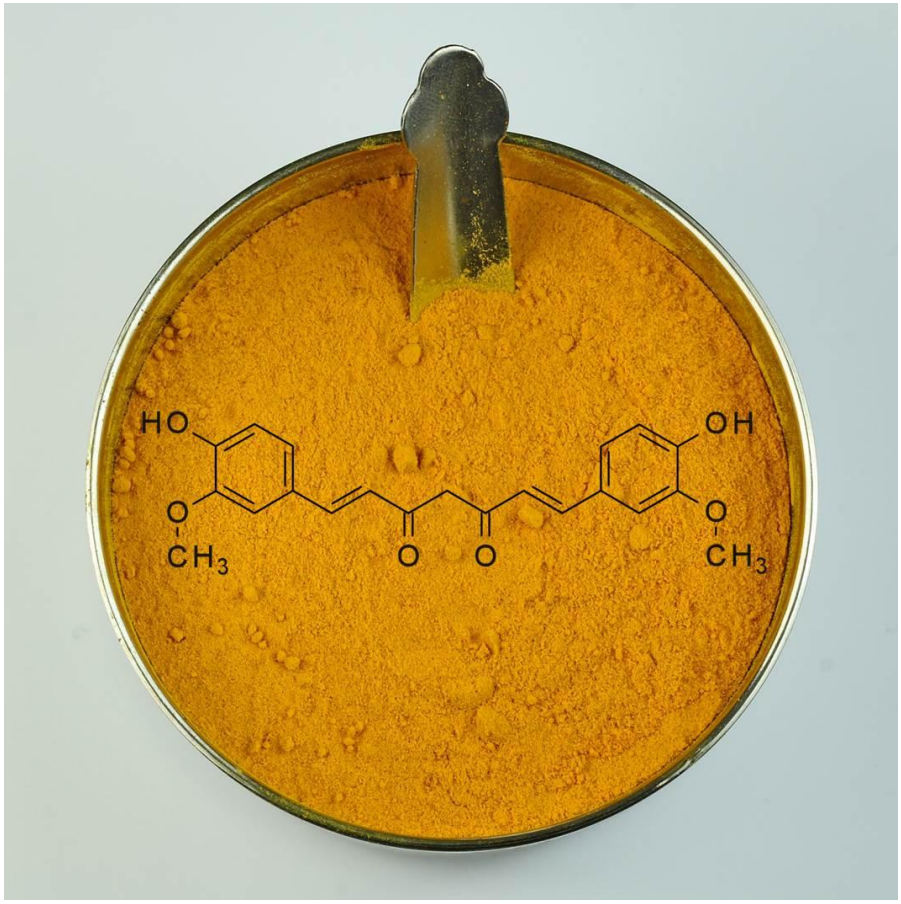
Curcumin as an anti-inflammatory medicine: effects on PGE2 and IL-6 in LPS stimulated canine whole blood



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Abstract

Treatment of inflammatory bowel disease (IBD) in dogs commonly consists of modification of the diet and immunomodulation by means of prednisolone or metronidazole. Unfortunately, these therapeutics have their drawbacks and negative side-effects and their effect has not always been proven. A more natural and safe alternative might be curcumin, a major constituent of curry powder, prepared from the roots of *Curcuma longa*. Various preclinical and clinical studies show that curcumin might help in all sorts of chronic diseases, including neoplastic, neurological, cardiovascular, pulmonary, metabolic and psychological diseases. An experiment was set up to test the possible attenuating effects of curcumin in dogs with IBD. Canine whole blood was triggered to inflammation by lipopolysaccharide in the presence of various concentrations of curcumin (1nM, 10 nM, 100 nM, 1 µM, 10 µM). Besides standard curcumin, Meriva® was tested, a curcumin-phosphatidylcholine complex that has been shown to be absorbed better. After 24 hours of incubation with LPS and curcumin or Meriva®, the pro-inflammatory mediators prostaglandin E2 (PGE2) and interleukine 6 (IL-6) were measured. The results showed that curcumin and Meriva® in a 10 µM concentration can attenuate inflammation by decreasing PGE2 production, as demonstrated in a model of whole canine blood stimulated with LPS. IL-6 levels seemed not to be influenced by curcumin or Meriva®. Considering the small sample size of our study and the intrinsic biological variation in our model, it should however be noted that no firm conclusions can be drawn from our results. Former research is ambiguous about the effect of curcumin on both PGE2 and IL-6, but curcumin has shown to be a promising cure for several diseases and ailments. There is strong suggestion that human IBD is one of the diseases that might be alleviated by curcumin, and hopefully this goes for canine IBD as well. Our experiment was set up as a small step towards a possible clinical trial testing the effects of curcumin on canine IBD. Future research will hopefully facilitate the use of this age-old medicine in the veterinary clinic.

Introduction

Inflammatory bowel disease (IBD) is not only relatively common in humans, manifesting as Crohns' disease or ulcerative colitis, but can also occur in dogs. It manifests as a chronic enteropathy, defined as so when it lasts more than three weeks and when mucosal inflammation is histologically demonstrated and other possible causes of enteritis have been excluded (Cerquetella *et al.*, 2010). Several types of IBD are seen in dogs; they are classified with regard to the specific location and the predominant cells infiltrating the intestinal wall. Types occurring in the small intestine are for instance lymphocytic-plasmacytic enteritis, eosinophilic enteritis, protein-loosing enteropathy, granulomatous enteritis. The large intestine can be affected by lymphocytic-plasmacytic colitis, eosinophilic enterocolitis, histiocytic colitis and granulomatous colitis. IBD can also occur in both large and small intestine, and even in the stomach. IBD seems to affect mostly middle-aged dogs, without sex predisposition. There are breed predispositions however, with most often very specific IBD forms occurring in specific breeds: examples are histiocytic ulcerative colitis in Boxers, protein-loosing enteropathy in Soft-Coated Wheaten Terriers, immunoproliferative enteritis in Basenjis and so-called Lundehund diarrhoea. A recent study identified Weimaraner, German shepherd dog, Border Collie, and Boxer as the most susceptible canine breeds in the south-eastern UK for developing IBD (Kathrini *et al.*, 2011).

Since the intestinal mucosa is in contact with the resident microbial flora, food and passing microbes, local immunity has a very important barrier function. IBD is thought to develop as a result of a deregulation of mucosal immunity, perhaps due to a loss of tolerance to antigens like food components or bacteria (Allenspach *et al.*, 2011). The specificity of the immune system is partly based on pattern recognition receptors, that are able to recognize pathogens by their molecular pattern. Toll-like receptors (TLRs) make up an important part of these pattern recognition receptors. When they are stimulated TLRs start their pro-inflammatory action by starting a cascade of cytokines. Three specific TLRs (2,4 and 9) were recently found to be up-regulated in dogs with IBD. In humans, the same was demonstrated for TLR4. Some polymorphisms in Toll-like receptors have been shown to be associated with IBD in dogs. They were located in the TLR4 and TLR5 genes (Kathrani *et al.*, 2011).

A Toll-like receptor like TLR4 can induce nuclear factor κB (NF-κB), the central transcription factor of the immune system, that in turn can stimulate pro-inflammatory cytokines and other mediators of inflammation. In biopsy samples from dogs with IBD, a higher NF-κB activation was found in lamina propria macrophages as compared to the controls (Luckschander *et al.*, 2010). Although cytokines seem to be involved in the immunoreactions leading to IBD, studies disagree on whether specific cytokine expression is increased in dogs with IBD. In a study on German shepherd dogs with IBD, mRNA expression of cytokines like IL-2, IL-5, TNF-α, and TGF-ß were shown to be higher than in controls (Jergens *et al.*, 2009; Schmitz *et al.*, 2012). Other mediators are likely involved in IBD as well.

Prostaglandin E2 (PGE2) is a lipid mediator involved in inflammation (Koeberle *et al.*, 2009). Its biosynthesis starts with arachidonic acid being converted to PGH2 by cyclooxygenases, COX-1 and COX-2. PGH2 is subsequently transformed to PGE2 by PGE2 synthases. The cytosolic PGE2 synthase is the more constitutive form and preferentially couples to COX-1, whereas the microsomal PGE2 synthase is linked to COX-2. The latter two enzymes can be induced by several pro-inflammatory stimuli. PGE2 has been found to be produced in excess in IBD, and enhances the development and function of IL-17 producing CD4 T helper cells (Barrie *et al.*, 2011). The resulting Th17-mediated chronic inflammation has recently received attention as an important mechanism in human IBD.

A very important mediator in the Th17 reaction is interleukin 6 (IL-6), a cytokine that regulates the balance between Th17 cells and Treg cells (Kimura *et al.*, 2010). The latter cells are the regulatory T cells that have an immunosuppressive function. One of its most important anti-inflammatory cytokines is IL-10. Blocking this Treg cytokine led to the development of spontaneous inflammation of the gastrointestinal tract in mice. As a regulator of the Treg/Th17 balance IL-6 shifts the immune system towards a more pro-inflammatory state by inducing development of Th17 cells and inhibiting the production of Treg cells (Mudter *et al.*, 2007). In various animal models of IBD a neutralizing antibody against the IL-6 receptor could suppress colitis (Atreya *et al.*, 2000; Yamamoto *et al.*, 2000). Recently however, Schmitz *et al.* (2012) could not find evidence of the contribution of Treg and Th17 to canine IBD, as no involvement of their signature cytokines (like IL-10 and IL-17 respectively) was seen in IBD affected dogs.

Treatment of IBD in dogs involves modification of the diet, and possibly immunomodulation by means of prednisolone or metronidazole. Metronidazole also has an antimicrobial action that might contribute to its positive effect. Other possible immunomodulating or anti-inflammatory drugs that can be used are azathioprine, cyclosporine, chlorambucil, cyclophosphamide and 5-aminosalicylates. Unfortunately, all those therapeutics have their drawbacks and negative side-effects. Besides, the effect on IBD has not always been proven.

A more natural and safe alternative might be curcumin. Curcumin is a major constituent of curry powder, prepared from the roots of *Curcuma longa* (Aggarwal, 2009a). In traditional Indian medicine (Ayurveda) it has been used for at least 4000 years for several ailments; traditional Chinese and Arabian medicine mention the use of turmeric as well. Curcumin's modern use is as spice and colorant (E100). Its active ingredient is diferuloylmethane, a compound in which two oxy-substituted aryl units are connected by a seven carbon chain (Stankovic, 2004). Besides curcumin (making up 77% of the curcuminoids in turmeric), the analogues demethoxycurcumin (17%) and bis-demethoxycurcumin (3%) are found naturally. In these molecules one or two methoxy groups are absent, respectively (shown in *figure 1*).

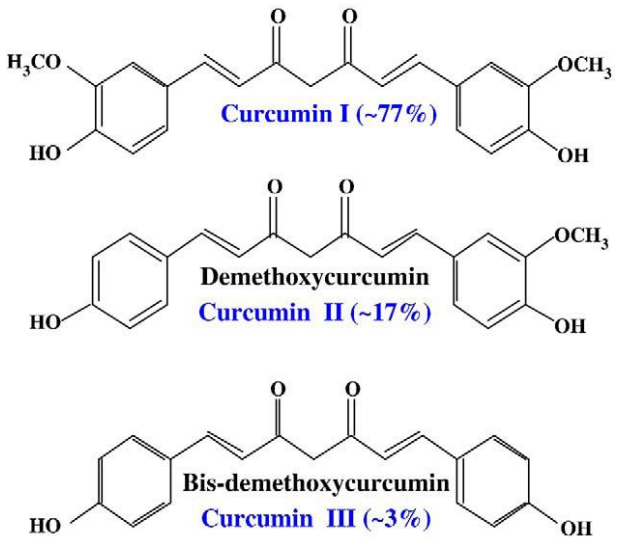

Figure 1: Curcumin and the two analogues that are most common in turmeric (Srivastava et al., 2011).

Vogel and Pelletier were the first to isolate curcumin in 1815 and its chemical structure was determined in 1910 by Milobedzka and Lampe (Wahlang *et al.*, 2010). Its use in medicine has long been described only sporadically, with publications in 1937 (use in biliary disease), 1949 (its antibacterial action), and 1972 (as an antidiabetic), but since 1990 publications on curcumin have increased exponentially from about 10 per year to almost 700 in 2011. Various preclinical and clinical studies show that curcumin might help in all sorts of chronic diseases, including neoplastic, neurological, cardiovascular, pulmonary, metabolic and psychological diseases (Aggarwal *et al.*, 2009a; Epstein *et al.*, 2010). Curcumin is considered to be very safe in human trials: daily doses up to 12 g are well tolerated, with only some diarrhea as a minor side effect (Jurenka *et al.*, 2009). Drawback of curcumin however is its low bioavailability, due to poor absorption in the guts, a large first pass effect, and a rapid elimination (Wang *et al.*, 1997). Elimination occurs mainly through glucuronidation and sulphation in the liver, and partly by reduction to tetrahydrocurcumin. In a phase II trial testing curcumin for pancreatic cancer, plasma levels were found to be only 22-41 ng/mL, even at high oral doses (Shen *et al.,* 2012). Several strategies have been invented to improve curcumin's bioavailability, for instance using nanoparticles (Sasaki *et al.*, 2011), heat treatment (Kurien *et al.*, 2009), cyclodextrin (Ali *et al.*, 2012), micelles (Setthacheewakul *et al.*, 2010; Zhang *et al.*, 2012) or liposomes (Cuomo *et al.*, 2011). An example of the latter is Meriva® (Belcaro *et al.*, 2010): a curcumin-phosphatidylcholine complex that has been shown to be absorbed better. Peak plasma levels and area under the plasma concentration time curve values for curcumin after administration of Meriva® were fivefold higher than the equivalent values seen after unformulated curcumin (Marczylo *et al.*, 2007). Besides a better absorption and bioavailability, pharmacokinetics were better as well using Meriva ®: the half-life in plasma was almost doubled compared to normal curcumin (Gupta *et al.*, 2011).

Curcumin has shown its benefits in the several animal models for IBD that have been developed: MDR gene-deficient mice, induction with trinitrobenzene sulphonic acid, 2,4-dinitrochlorobenzene or dextran sodium sulphate, or IL-10 knock out mice (Larmonier *et al.*, 2008). In all of these models curcumin has shown a protective effect against gut inflammation. Curcumin seems to have numerous pharmacological targets, but its anti-inflammatory action is mainly attributed to its interference with the arachadonic acid cascade and blockade of NF-κB (Aggarwal *et al.*, 2009b).

Although several publications have shown the antioxidant and anti-inflammatory effects of curcumin in vitro, so far only two clinical trials have been performed regarding IBD (Ali *et al.*, 2012). In the first, by Holt *et al.* in 2005, 5 UC and 5 CD patients received 550 mg curcumin TID for 3 months. It lead to clinical improvement in 9 of them. They also needed less medication when they took curcumin . The other study, by Hanai *et al.* in 2006, had 89 participants with quiescent UC. They received 1g of oral curcumin BID for 6 months, leading to clinical improvement. At the end of the study 2% of the patients taking oral curcumin had relapsed, compared to 21% in the placebo group. With such results, one would expect that many more trials are on the way, but there are only a few. One is a phase I clinical trial (completed; results are pending, www.clinicaltrials.gov) in which the tolerability of curcumin in pediatric patients with IBD was studied. Another trial studies the efficacy of Coltect, a supplement for UC patients, containing curcumin, green tea and selenium.

The translation from the many positive effects of curcumin *in vitro* to using it as a possible cure in the clinics seems to be hard and time-consuming. In the present study an attempt was made to proof that curcumin is a promising candidate for use in dogs with IBD. As a first step towards this goal, the anti-inflammatory effects of curcumin were to be demonstrated in a whole blood assay using canine blood triggered to inflammation by bacterial lipopolysaccharide (LPS). To show the potency of curcumin in attenuating inflammation, the inflammatory mediators PGE2 and IL-6, that are also involved in IBD, were measured.

Materials & Methods

- Chemicals

Curcumine (92% CURCUMIN, 99% TOTAL CURCUMINOIDS) was obtained from Sigma-Aldrich (prod.nr. C7727). Meriva®/Curcuvet® (20.1% of total curcuminoids) was kindly provided by Indena. LPS from E. coli 0111:B4 was obtained from Sigma-Aldrich: cell culture tested, purified by gel-filtration chromatography, γ-irradiated (prod. Nr. L4391). RPMI, HBBS, and DMSO were kindly provided by Jeanette Wolfswinkel of the Genetics lab.

- Blood samples

Blood was collected from 6 healthy test dogs of the Faculty of Veterinary Medicine. 15 mL of blood was drawn from each dog; 14 mL was collected in a 10 mL and 4 mL Li-heparine blood tube, and 1 mL was put in a EDTA-tube for microscopic examination. Blood collection was performed by veterinary interns, as approved by the Dier-Ethische Commissie.

- Incubation assay

Fresh blood was collected from a dog into warmed sterile vacuum tubes containing Li-heparin. 14 mL of heparinized whole blood was directly added to 28 mL of warm (37°C) RPMI medium. 2.8 mL of this suspension was brought into 14 polypropylene 5-mL tubes (or 12-well plates, in one case). 100 µL of test solution was then added: either a Meriva solution, a curcumin solution or plain HBBS. Meriva® and Curcumin solutions were prepared from a solution of 55 mg of Meriva® and 11 mg curcumin respectively in 10 mL of DMSO. This stock solution was diluted 10 times with HBBS to obtain the X solution, for the tubes with highest curcumin-concentration. The X solution was diluted 10 times to obtain the I solution; the 100, 10 and 1 solutions were produced in a similar way by diluting the preceding solution. The mixtures of blood:RPMI:test compound were incubated for 30 minutes at 37°C. 100 µL of lipopolysaccharide (LPS) solution or HBBS was then added to the tubes: the LPS solution was prepared by dissolving 1 mg of LPS in 333 µL in HBBS, and transferring 20 µL of this solution to 2 mL of HBBS. The assay tubes were then incubated in a 5% CO_2-incubator at 37°C for 24 hours. After incubation the sunken blood cells were resuspended and a 20 µL was taken for microscopic examination on leukocyte viability. For that purpose 20 µL of 1% Trypan Blue solution was added. After 3-5 minutes a drop of this mixture was microscopically examined in a haemocytometer to differentiate between dead (blue) and live (unstained) leukocytes. The tubes containing the blood:RPMI suspensions were centrifuged for 5 minutes at 4000 × *g*. The supernatant of each tube was then divided to 5 Eppendorf tubes, about 400 µL each. The Eppendorf tubes were labeled accordingly, put in a box, and stored at -80°C.

- Measurement of PGE_2

The samples were thawed for analysis. A PGE_2 high sensitivity EIA kit (Enzo® Life Sciences) was used, and the analysis steps were performed according to the protocol. For the first (pilot) assay serial dilutions of 1, 10, 100, 1000 and 10 000 times were made by adding 20 µL of the foregoing dilution to 180 µL of assay buffer (belonging to the kit). For the second assay only 1000 × solutions (for Meriva® and Curcumine preparations and the LPS blank) or 100× solutions (for the other 3 blanks) were used. For the former 10 µL of plasma was added to 990 µL of HBBS; for the latter 20 µL of plasma was added to 180 µL of HBBS. For the final dilution 30 µL of these solutions were added to 270 µL of assay buffer. The plates were incubated for 20-22 hours with antibody and conjugate, then washed, after which substrate was added. A 1-hour incubation followed, then stop solution was added and the plate measured using a

BioRad iMark™ Microplate reader. The computer used Microplate Manager 6.1 to interpret and structure the data.

- Measurement of IL-6

The samples were thawed for analysis. A Quantikine® Canine IL-6 immunoassay was used, and the analysis steps were performed according to the protocol. Dilution of the samples was not necessary and incubation time was a total 4½ hours. A BioRad iMark™ Microplate reader and Microplate Manager 6.1 were used.

- Statistics

Microsoft Excel 2007 was used for calculations and preparing the tables.

Results

- General/cell viability

Assesing the cell viability was done by staining the blood cells with Trypan Blue. Unfortunately, white blood cells were very hard to find in the samples that had been incubated for 24 hours. Only a few white blood cells were seen, stained as well as unstained, roughly in the same amount. Using an Advia 120 hematology analyzer it could be shown that the incubated blood samples did actually contain lymphocytes still, albeit in somewhat smaller numbers than references for canine blood prescribe (corrected for the dilution with RPMI). With such low numbers of visible cells, no conclusions could be made regarding viability.

- PGE2

PGE2 was measured in the thawn serum samples. Fourteen distinct samples were present (*see table 1*).

Table 1: The fourteen different samples used in the whole blood assay.

Name of the sample	Meriva	Curcumin	LPS
M1	1 nM	-	1 µg/mL
M10	10 nM	-	1 µg/mL
M100	100 nM	-	1 µg/mL
MI	1 µM	-	1 µg/mL
MX	10 µM	-	1 µg/mL
C1	-	1 nM	1 µg/mL
C10	-	10 nM	1 µg/mL
C100	-	100 nM	1 µg/mL
CI	-	1 µM	1 µg/mL
CX	-	10 µM	1 µg/mL

L	-	-	1 µg/mL
M	10 µM	-	-
C	-	10 µM	-
B	-	-	-

In a pilot experiment the necessary dilutions were explored. The samples containing no LPS seemed to be best measured at a 1000x dilution; for all the other samples that was found to be a 10 000x dilution. The samples of dog 4 were used for this experiment; the results are shown for the dilutions that worked (*see figure 2*).

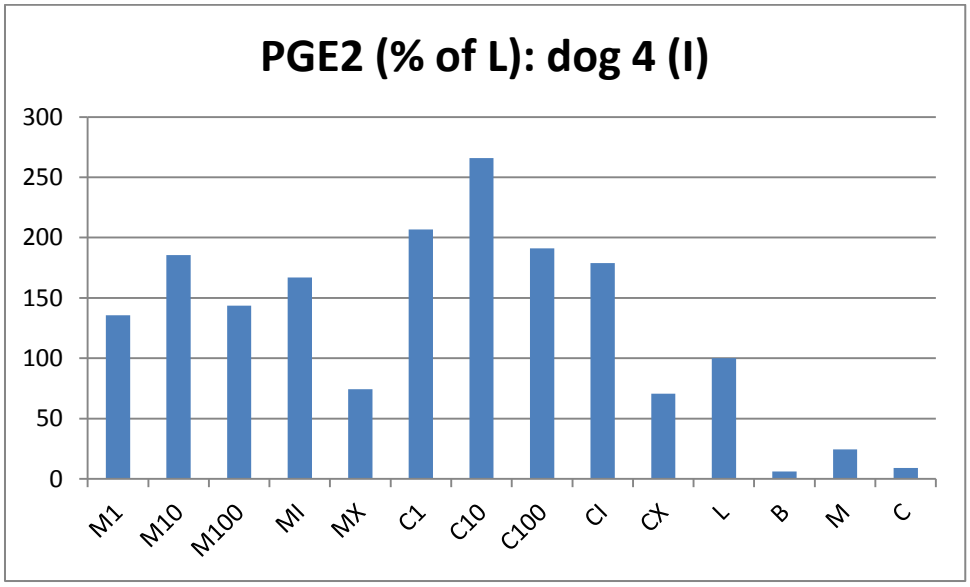

Figure 2: PGE2 levels as measured in the samples of dog 4, expressed as a percentage relative to LPS control (L).The blank and other (negative) controls (B, M and C) show only a relatively small amount of PGE2, whereas both the highest concentration of Meriva®, and of curcumin seem to decrease the production of PGE2 in the canine blood after LPS stimulation. In the other concentrations of curcumin or Meriva® however the PGE2 production was higher than in the LPS control. The measured PGE2 concentration in this L sample was 118 000 pg/mL. In this pilot analysis measurements were performed only once in the sample dilutions used (1000x for B, M and C; 10 000x for the LPS challenged samples).

A new series of measurements was done on the samples of dog 2 and 3, and dog 4 again. Samples of dog 2 and 3 were measured twice; the samples of dog 4 once. The results are shown in the *figures 3, 4* and *5*.

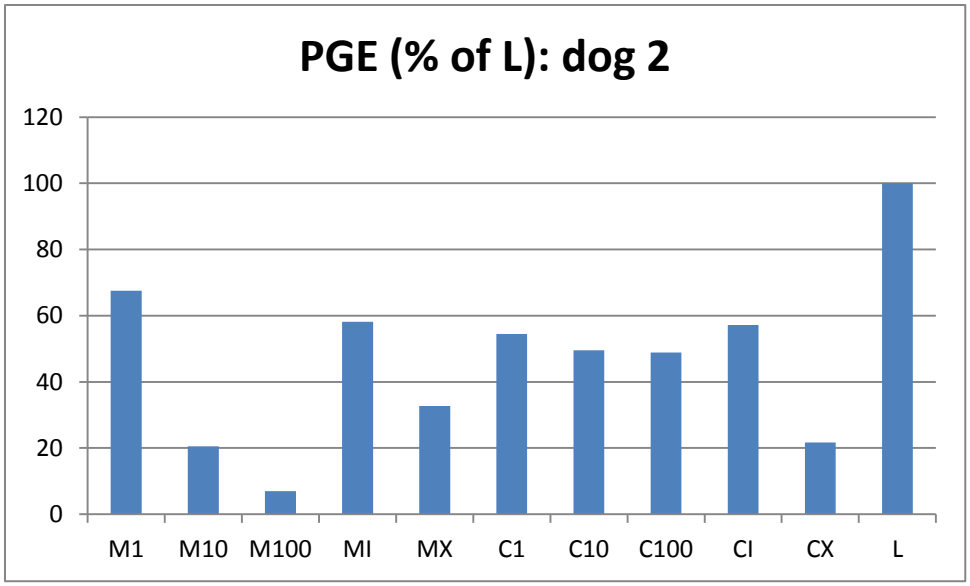

Figure 3: In dog 2 the PGE2 production in the LPS control was 115 000 pg/mL. In the rest of the samples the amount of PGE2 was lower, especially in the highest concentration of Meriva and curcumin, but typically even more so in the Meriva samples containing 10 and 100 nM. The latter value was below the calibration range of the PGE2 test. PGE2 levels in the blanks (B, M and C) were above the calibration range.

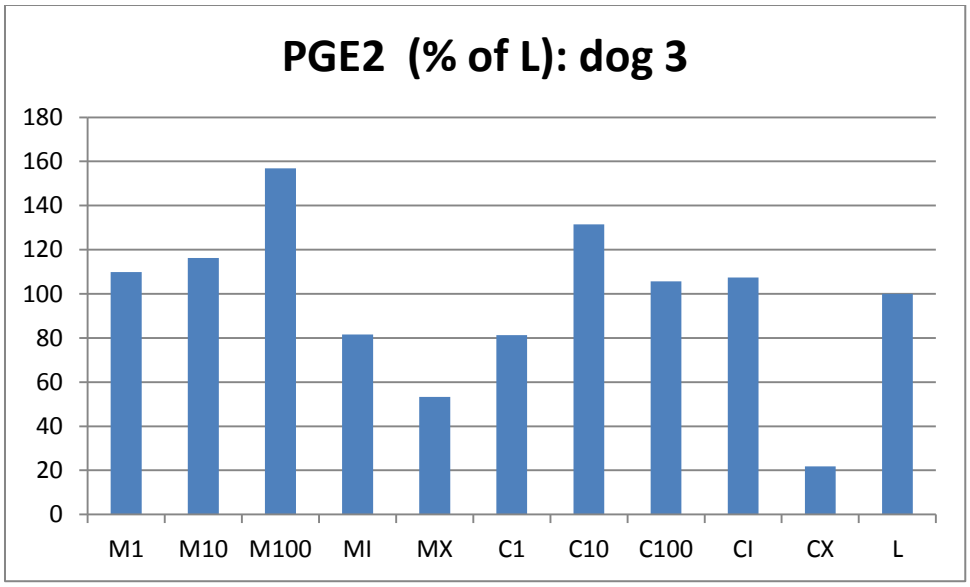

Figure 4: In dog 3 the PGE2 level in L was 37 000 pg/mL, much lower than measured in the other dogs before. The pattern reminds of the first test of the dog 4 samples (shown before), with lower levels of PGE2 for the highest concentration of Meriva® or curcumin, and the other samples comparable to the LPS control. The quantity in CX was below calibration range; B, M and C had levels above calibration range.

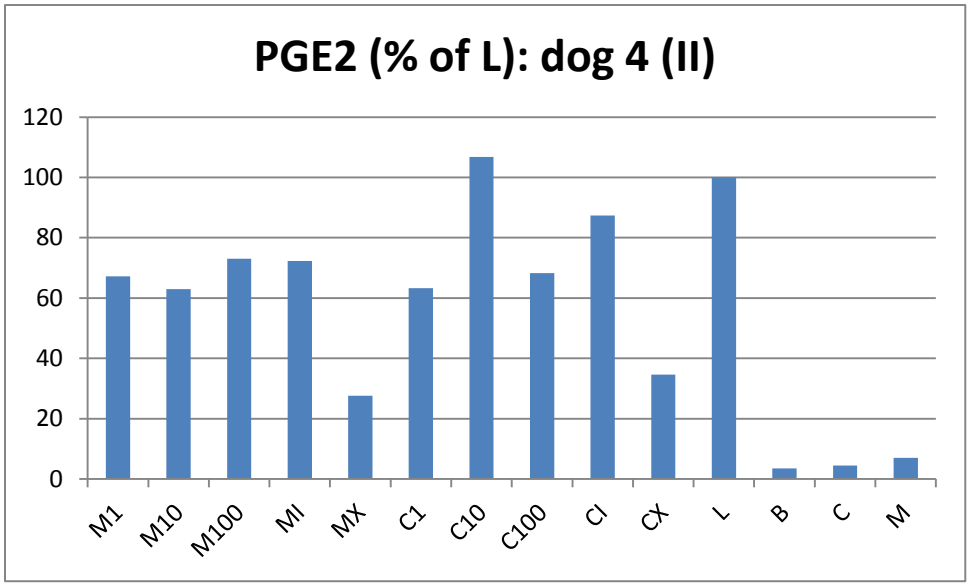

Figure 5: In the second measurement of the dog 4 samples the PGE2 level in L was 175 000 pg/mL, which is somewhat higher than measured in the first analysis. Again, the PGE2 levels are lowest in the MX and CX sample. Typically however, in this trial only the C10 is higher than the LPS control, whereas in the first measurement of dog 4 almost all samples with curcumin/Meriva ® showed higher PGE2 amounts than L.

- IL-6

IL-6 was measured using a Quantikine® Canine IL-6 Immunoassay. The thawed serum samples did not need to be diluted. Values for dog 4 and 5 (*figure 7 and 8*) are based on double measurements; triple measurements were done on the samples of dog 3 and 6 (*figure 6 and 9*).

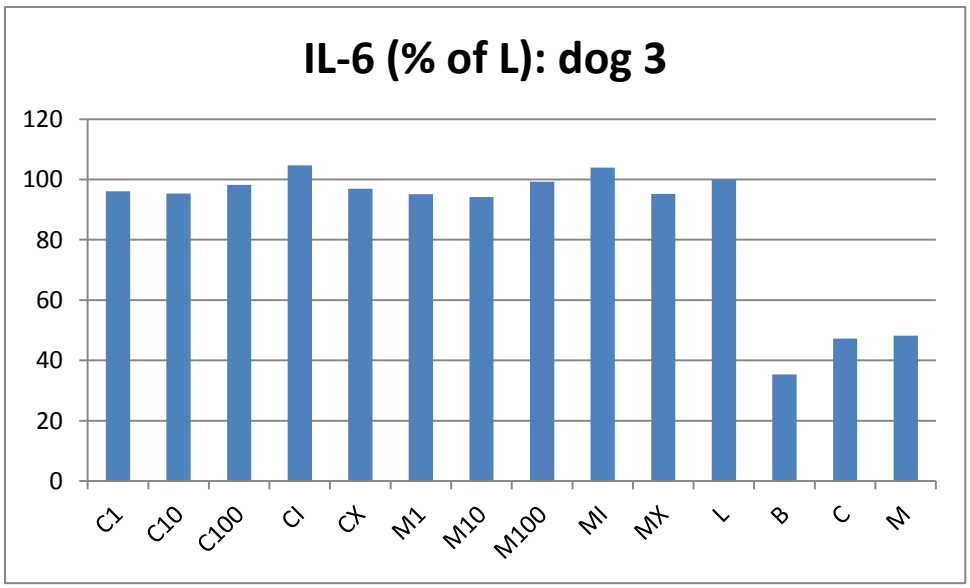

Figure 6: Dog 3 shows IL-6 levels with only little variation in the samples that are stimulated with LPS. IL-6 in the LPS challenged sample L was 1200 pg/mL.

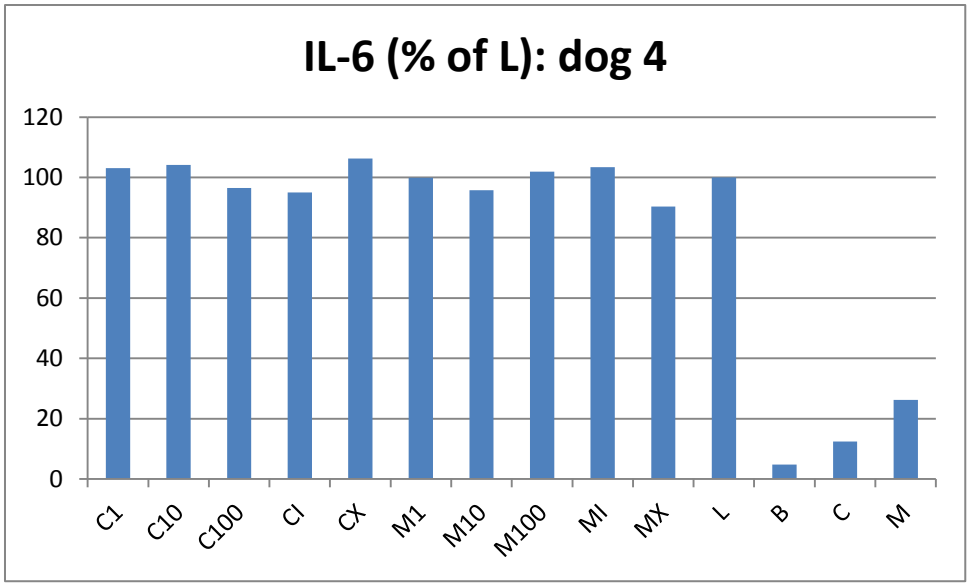

Figure 7: The IL-6 amounts in dog 4 are roughly two times lower than in dog 3 (LPS sample: 600 pg/mL), but the pattern is the same: high levels in the LPS stimulated samples, regardless of the presence of curcumin of Meriva®, and some IL-6 production in B, C and M.

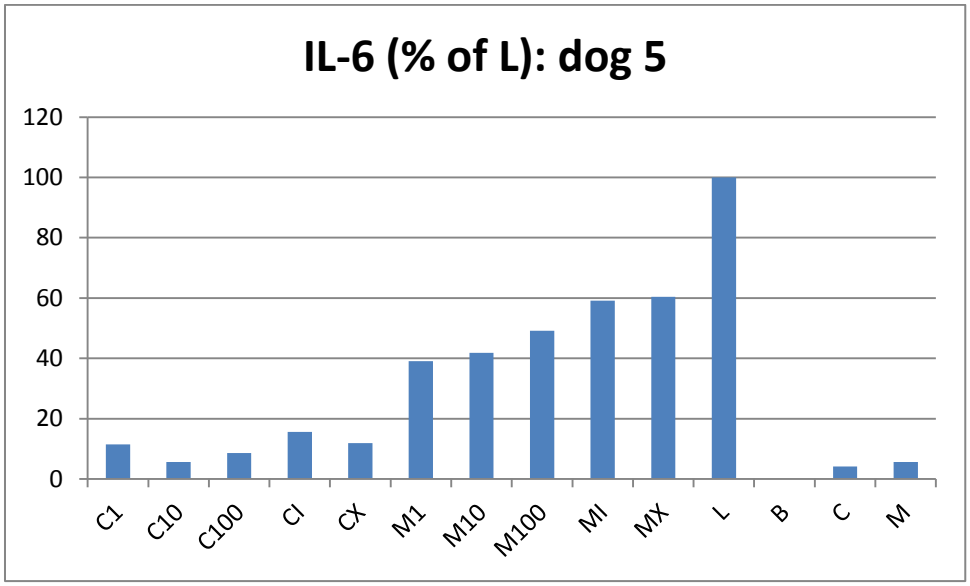

Figure 8: Dog 5 shows a completely different pattern of IL-6 production: quite high in the LPS control, low in the curcumin samples, and about half the LPS level in Meriva samples. The IL-6 amount in the L sample was 1000 pg/mL.

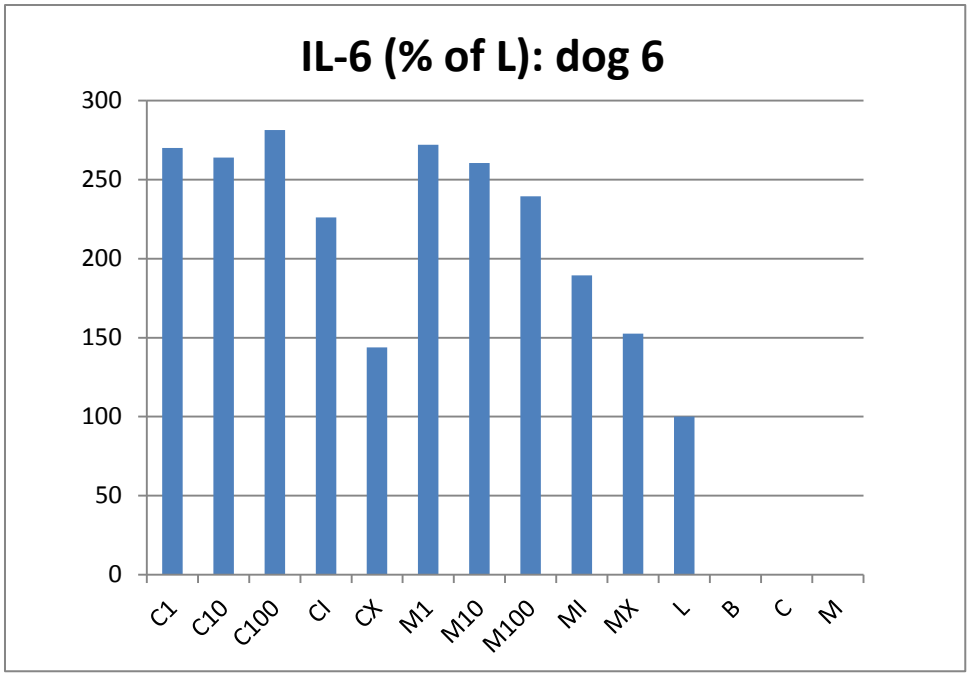

Figure 9: Dog 6 even had another pattern: low in the LPS control (relative as well as absolute: 200 pg/mL), slightly higher at the highest concentrations of Meriva® and curcumin, with increasing IL-6 levels towards the lower curcumin and Meriva® concentrations. IL-6 in B, C and M was below measuring threshold.

Discussion

In our assay, the Trypan Blue exclusion test was used to determine the number of viable cells present in the blood sample after the 24-hour incubation. Live cells possess intact cell membranes that exclude trypan blue, whereas dead cells do not. The test can be disturbed by protein-rich media, which gives a background stain, and by letting the cells incubate for longer than 5 min with the Trypan Blue solution, as cells might die because of that (Strober *et al.*, 2001).

But even with those notices the Trypan Blue Exclusion test did not work out on our blood samples. Starting with the basic protocol, adaptations were made in incubation time, dye/sample ratio, sample preparation, but still only low numbers of leukocytes could be found, stained or unstained. Even skilled laboratory workers could not find them, although the hematology analyzer of the UVDL lab revealed that the canine blood contained an amount of leukocytes that fell only slightly below reference values. Probably the leukocytes adhered to the sides of the polypropylene containers, but no evidence was found for that hypothesis.

With this unfunctional Trypan Blue Exclusion we could not test the viability before and after the incubation with curcumin and LPS. Thus, we could not see whether LPS, curcumin or some other compound caused cell death during the incubation. Based on former research we can however conclude that curcumin is well tolerated by cells. Larmonier *et al.* (2011) used the Trypan Blue exclusion test to assess the cell viability of

neutrophils with different concentrations of curcumin. At 10, 25 and 50 μM more than 95% of the cells remained viable after 2 hours incubation; at 100 μM curcumin it decreased to 70%. Lou *et al.* (2010) studied the viability of A2780 cells (ovarian cancer) to curcumin by using a MTS assay. Cells were incubated with curcumin for 72 hours. 1 μM of curcumin led to 90% viable cells; 5 μM gave 80% viability; 10 μM 50%, and at 30 μM only 15% of the cells survived. Maier *et al.* (2010) also used the Trypan Blue method to count viable cells in human blood. Curcumin was used in concentrations of 0,01; 0,1; 1;10 and 50 μM; those concentrations were reported not to affect cell viability in 48 hours.

To test the curcumin and Meriva we used a whole blood assay. This is considered to be a very relevant pharmacologic test system: compounds that would fail in vivo because of unfavorable intracellular availability, degradation or high plasma protein binding indeed show low activity in this assay (Laufer *et al.*, 2008). There is however the variability of different donors with regard to metabolism and different enzyme expression. A whole blood assay can also be more costly than a simple enzyme-based assay.

As a solvent for the curcumin DMSO was used, in a final concentration of 0,33% in the blood samples. Brideau *et al.* (2001) states that even up to 5% DMSO does not affect COX-2. Laufer *et al.* (2008) affirms the same for aspirin: in their study COX-2 mediated PGE2 synthesis was left almost unaffected by the addition of aspirin. We added aspirin to the blood mixture to select for curcumin's effect on COX-2 and PGHS-2. PGHS-1, which is present in the platelets, is irreversibly acetylated by aspirin, so its contribution to COX products is aborted (Wilson *et al.*, 2004). Acetylsalicylic acid is quickly hydrolyzed by esterases in the blood, so it will not affect PGHS-2 from the monocytes, which is induced later. Several researchers have however found that aspirin addition to a whole blood assay did not significantly change PGE2 (Young *et al.*, 1996). Patrignani *et al.* (1994) reports a PGE2 contribution by the platelets of 1 to 2%.

In our assay levels of PGE2 were measured to see if curcumin is able to reduce the production of this inflammation mediator in LPS stimulated canine whole blood. A PGE2 enzyme immunoassay was used to measure the samples of 3 dogs. Different reaction patterns were seen in every dog, and even big differences in two independent measurements of one dog's samples. It is hard to explain the way PGE2 levels are influenced by curcumin in every single dog, so as an illustration of the general pattern all measurements were put together to produce the diagram below (*figure 10*).

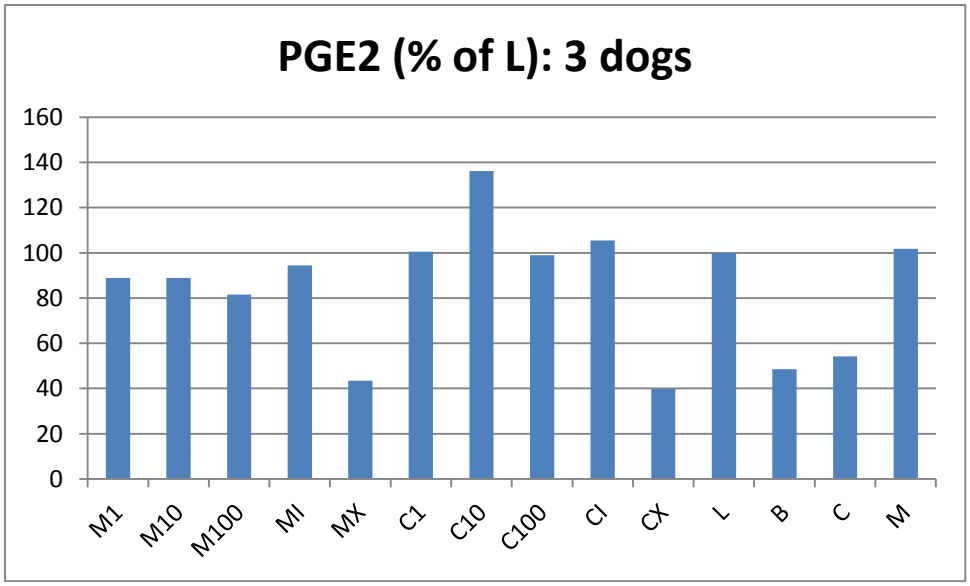

Figure 10: Summative diagram summarizing the effect of curcumin on PGE2 production, as found in this experiment.

The PGE2 level in the LPS sample is used as reference: in the three dogs the measured amount varied from 37 000 pg/mL to 175 000 pg/mL. Generally, only the MX and CX sample, containing 10 µM of Meriva® or curcumin respectively, had significantly lower PGE2 levels. The curcumin thus seems to have an attenuating effect on the LPS triggered PGE2 production. The other curcumin and Meriva® samples contained roughly the same amount of PGE2 as the LPS control, although C10 is typically high. No explanation was found for this. Another curiosity in this pattern is the high PGE2 production in the blood sample only containing Meriva®. Perhaps the liposome preparation of curcumin can stimulate PGE2 production.

Unfortunately, our results are no firm foundation for the statement that curcumin can influence inflammation, or more specifically: PGE2 production in LPS stimulated blood cells. We are however not the first to test the effect of curcumin on PGE2 formation in whole blood. Koeberle *et al.* (2009) preincubated human whole blood with curcumin for 5 min, after which LPS was added. PGE2 was then measured after 5 hours of incubation. In this first assay 0.03 µM of curcumin decreased the PGE2 formation to about 85%, 0.3 µM to 70%, 3 µM to 60%, and 30 µM to 45%. In the second assay of Koeberle *et al.*, human blood was first stimulated for 16 hours with LPS. Then it was incubated for 10 min with curcumin, after which arachadonic acid was added as substrate for COX-2. Only at the higher curcumin concentrations of 3 and 30 µM, PGE2 was found to be decreased significantly (20 and 15% respectively). This was done to demonstrate that the reduction of PGE2 formation in the first assay was mainly due to reduced expression of microsomal PGE2 synthase. Once the PGE2 synthase has been induced curcumin only inhibits PGE2 production by direct inhibition of COX-2, at higher concentrations.

Shah *et al.* (2010) performed a similar whole blood assay and found an effect of curcumin on PGE2 levels as well. In the presence of 20 µM of curcumin, PGE2 production was reduced to half of the amount found in the LPS control (900 pg/mL).

Typically, an earlier study by Plummer *et al.* (2001) showed that curcumin decreased PGE2 induction in a human whole blood assay at a 1 μM concentration, but not at 5, 10 or 20 μM concentration. The researchers concluded that curcumin may have a narrow therapeutic window, but this is not confirmed by other studies.

In a 2007 article for example Jin *et al.* state they found a dose-dependent inhibition of PGE2 production using curcumin concentrations of 5, 10 and 20 μM. It should be mentioned however that Jin *et al*. performed their research in microglia instead of human whole blood; the effect of curcumin might differ between cell types.

In adipocytes Gonzales *et al*. (2008) has shown that curcumin decreases the PGE2 secretion to about 50% at a concentration of 20 μM. This is in accordance with the results of Shah *et al*. (2010), as mentioned before.

In our assay curcumin concentrations of 1 nM, 10 nM, 100 nM, 1 μM and 10 μM were used. The data suggest that curcumin attenuates PGE2 production at the 10 μM concentration, but not at the lower concentrations. A more significant effect might be seen at a concentration of 20 μM or higher, but whether these are realistic plasma concentrations for curcumin is questionable. In a phase II trial of curcumin given at a daily dose of 8 g, plasma peak levels (Cmax) were about 0,3 μM (Dhillon *et al*., 2008).

Some data even suggest that high doses of curcumin can stimulate PGE2 production. Guimaraes *et al.* (2012) assessed whether curcumin could help in periodontal disease. Curcumin was administered to rats by oral gavage (30 and 100 mg/kg) and the gingival tissue was challenged by LPS injection. Curcumin completely blocked PGE2-s mRNA expression, and no PGE2 was detected. Interestingly, the higher dose of curcumin induced PGE2-s mRNA expression in healthy gingival tissue.

Some other researchers have found curcumin could induce PGE2 production as well, rather than decreasing it. In a study by Jiang *et al.* (2006) colitis was induced in rats with TNBS acid. Curcumin was supplemented in a dose of either 30 or 60 mg/kg, and was shown to decrease the level of COX-2. PGE2 however was increased in the rats that were fed curcumin. They had less severe colitis and a better survival rate. Although PGE2 is an inflammatory mediator, it also stimulates repair of epithelium (Lejeune *et al.*, 2010) and suppresses neutrophil function and mast cell degranulation (Ungaro *et al.*, 2009).

Jung *et al.* (2010) used murine bone marrow-derived dendritic cells in his study, and found that COX-2 expression and PGE2 production were increased when those cells were treated with curcumin. BMDCs were pretreated with curcumin (0, 1, 5, 10 and 25 μM) for 2 h and then stimulated with 200 ng/ml LPS for 6 h and 12 h to detect COX-2 expression and to detect PGE2 production, respectively. Both showed a concentration-dependent increase, with 10 μM curcumin dose leading to a double COX-2 and PGE2 expression. In LPS-activated macrophages PGE2 can down-regulate production of the pro-inflammatory cytokines IL-12 and TNF-α, thus act as an immunosuppressant.

All those results of former research show that it is still unclear in what way curcumin influences PGE2 production. In our experiment no evident action was found either. Our

results might partly be distorted by the low precision of the PGE2 assay. According to the producer, the inter-assay and intra-assay precision of the immunoassay is quantified by about 10% coefficient of variation. Furthermore, the assay basically depends on the optical density (O.D.) of a product; the PGE2 is plate is read at 405 nm. Curcumin and its degradation products have a weak absorption band in the 400 nm region, so it might add to some distortion. If the curcumin contribution to the optical density is mistakenly seen as caused by PGE2, its levels will be underestimated, as there is an inverse relation between O.D. and PGE2 in the sample. The low PGE2 levels in the MX and CX samples might raise this suspicion, but considering the levels measured in the curcumin and Meriva® blanks, a significant contribution is not likely.

As an adaption to our experiment PGE2 measurement might be performed using High Performance Liquid Chromatography (HPLC) (Hsu *et al.*, 2008; Koeberle *et al.*, 2008). Sample preparation is however elaborate and the costs for this type of analysis are relatively high.

Besides PGE2, we also measured IL-6 in our quest for the effects of curcumin in simulated inflammation in canine blood. A canine IL-6 immunoassay was used to quantify these levels in the samples of 4 dogs. However, the variety of reaction patterns seen in PGE2 was also encountered here. Therefore, a summative and summarizing diagram, as used before, is shown for the IL-6 in these dogs as well (*figure 11*).

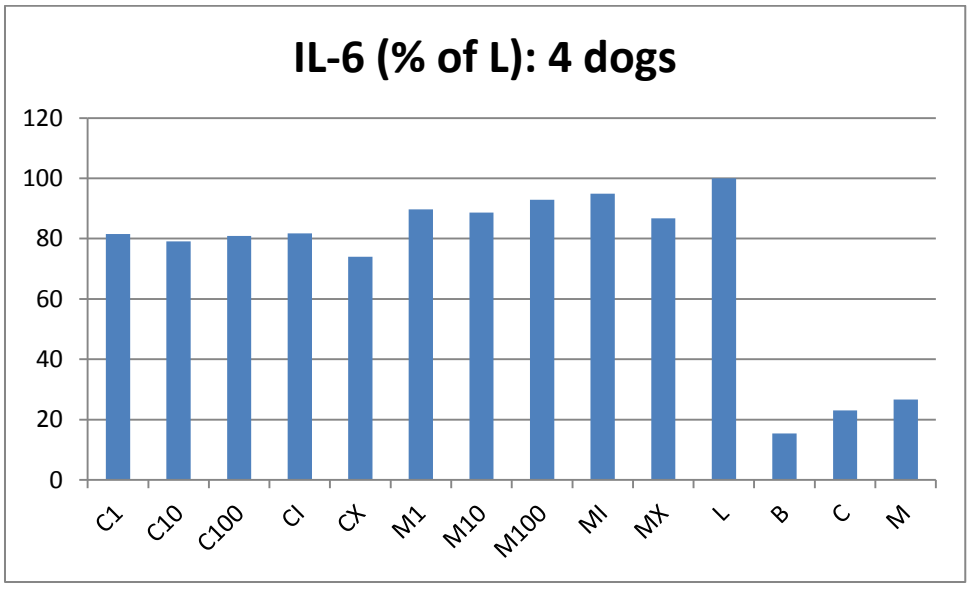

Figure 11: Summative diagram summarizing the effect of curcumin on IL-6 production, as found in the samples of 4 dogs.

In this diagram we see that LPS stimulated whole blood produced significantly more IL-6 than blanks containing no LPS. IL-6 levels in the L samples ranged from 200 to 1200 pg/mL. The amounts in the Meriva® and curcumin containing samples is not much lower than in the LPS control. Thus, in our experiment curcumin seems to have no major effect on IL-6 levels. Other studies have actually found a significant effect of curcumin on IL-6 production.

The trial by Guimaraes *et al.* (2012) has been mentioned before: in this study an oral gavage of curcumin was used as a possible protector to the effects of LPS on oral tissue. In unstimulated tissue, no IL-6 could be measured, but stimulation with LPS released about 35 pg/mL of IL-6. A curcumin dose of 30 mg/kg lowered this to less than 2 pg/mL; typically the higher curcumin dose of 100 mg/kg decreased IL-6 only to 30 pg/mL.

The veterinary formulation of Meriva®, Curcuvet®, was used in a study by Farinacci *et al.* (2009). It was administered for 15 days to 7 adult mares and 5 foals affected by osteoarthtritis and osteochondrosis respectively. In mares, the IL-6 was decreased, whereas in foals it significantly increased during the treatment.

An increase of IL-6 levels through curcumin was also found by Murphy *et al.* (2011). The effects of curcumin on intestinal inflammation were tested in the ApcMin/þ mouse, a model of colon cancer. They were given a placebo or a curcumin (2%) diet from 4 to 18 weeks of age. Curcumin decreased total intestinal polyps by 75%. Both mRNA expression of IL-6 and its protein expression was increased significantly.

Shah *et al.* (2010) did not see a significant effect of 20 µM curcumin on the IL-6 secretion in LPS-stimulated peripheral blood mononuclear cells.

In the curcumin study of Villegas *et al.* (2011) no significant on IL-6 levels could be demonstrated either. Mice were fed standard diet or a curcumin (0.6%) diet, and were exposed to dextran sodium sulfate to induce colitis. The curcumin mice showed less signs of colonic disease, but IL-6 was as much elevated as in the standard diet mice.

The fact that no significant effect of curcumin on IL-6 levels could be found in our experiment either might be due to small sample size (4 dogs). Intra-assay and interassay precision of the IL-6 assay is about 5% according to the producer, so somewhat better than for the PGE2 assay. The IL-6 plate is read at 450 nm, so the curcumin adsorption might influence the IL-6 assay as well. If the curcumin contribution to the optical density is falsely seen as IL-6, levels will be overestimated. Our results however do not show evidence of such an inclination in the samples with higher curcumin concentrations.

Conclusion

Our experiment suggests that curcumin and Meriva® in a 10 µM concentration can attenuate inflammation by decreasing PGE2 production, as demonstrated in a model of whole canine blood stimulated with LPS. IL-6 levels seem not to be influenced by curcumin or Meriva®. Considering the small sample size of our study and the intrinsic biological variation in our model, it should however be noted that no firm conclusions can be drawn from our results. Former research is ambiguous about the effect of curcumin on both PGE2 and IL-6, but curcumin has shown to be a promising cure for several diseases and ailments. There is strong suggestion that human IBD is one of the diseases that might be alleviated by curcumin, and hopefully this goes for canine IBD as well. Our experiment was set up as a small step towards a possible clinical trial testing

the effects of curcumin on canine IBD. Future research will hopefully facilitate the use of this age-old medicine in the veterinary clinic!

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