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RESEARCH ARTICLE

Is Bradykinin 1-5 a Reliable Biomarker for Pain in Dogs?

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Animal pain and suffering have long been evaluated in subjective terms, due to the inherent challenge of quantifying the signs. Correspondingly, there is a dearth in objective indices and related tools through which to more rigorously assess these parameters. Such resources would be integral for legal purposes, as in the pursuit of cases of suspected animal mistreatment. In response, it is necessary to identify, examine and if possible, quantify parameters that are produced by painful or stressful stimuli to gauge animal mistreatment objectively. In that spirit, and with a focus on dogs, the present study sought to evaluate the viability of bradykinin (BK 1-5) as a biomarker for pain, in canine plasma. This study took place in central Spain, with a primary goal to determine whether BK 1-5 level by itself could be used as a biomarker for dogs with pain. In total, 26 dogs that were either patients of a veterinary clinic or admitted to a local shelter with known history were sampled for this study. The dogs were categorized into one of three distinct groups, namely those without apparent mistreatment, diagnosed with a 'painful' illness and receiving treatment against it, or allegedly having been mistreated or neglected (e.g., hoarding, physical abuse). Although plasma volume issues precluded detection in individual samples, BK 1-5 was detected in pooled/group samples (n = 3), at concentrations of: 2.26 fmol/ml (Gp1), 2.66 fmol/ml (Gp 2), and 1.72 fmol/ml (Gp 3). The role of sex and age of each dog and the types of individual medication (where applicable) was also considered relative to observed sources of variation in the measurements. Lessons learned during this exploratory study are considered, including the opportunistic identification of other potentially promising biomarkers. Recommendations for further work and exploration are also offered.

KEYWORDS*:* BK 1-5; dog; mistreatment; plasma

Concept of Pain

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage". While the definition acknowledges that there need not be tissue damage as such for pain to be experienced, the pairing of intangible sensory and emotional components may intrinsically skew an assessor towards this comparably 'discernible' parameter. A biophysiological model of pain is required which specifies numerous causal factors that go well beyond tissue pathologies (Williams and Craig 2016).

There are two types of pain — acute and chronic. Acute pain is by its nature always nociceptive - manifesting through the chemical, mechanical, or thermic stimulation of certain nociceptors. Chronic pain is persistent and may remain present for an extended period of time post-injury, even once an injury has ostensibly healed. It is usually refractory to treatment, and generally associated with significant psychological symptoms (Mach 2006).

Many nociceptors are capable of modifying the activity of afferent fibers (i.e., acting as chemoreceptors). This implies a sensitization to mediators (bradykinin, cytosine, eicosanoids), neurotransmitters (serotonin, noradrenaline), histamine, lactic acid, diverse peptides (substance P, opioids), potassium (K+), and hydrogen (H+) ions, as well as certain substances such as prostaglandins and leukotrienes. Additionally, a painful stimulus can depolarize the nerve cell membrane (Raja 1984, Levine 1987).

In the case of chronic pain, be it inflammatory or neuropathic, the periphery continues to send nociceptive information to the dorsal horn of the spinal medulla. The

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neuron emits vesicles from the soma, releasing both Substance P and the peptide that is associated to the gene of calcitonin (CGRP). Once these two substances have been liberated to the periphery, they attach to receptors in different cells involved in the inflammatory process, such as neutrophils, mast cells, and basophils. This attachment causes the liberation of a series of pro-inflammatory substances (cytosine, bradykinin, and histamine) (Goicoechea and Martín 2006).

In short, there is a vast array of literature on pain-related substances and associated biomarkers. Outside of laboratory work, and measurement of parameters and substances related to pain response in animals, assessments and indices such as the 'Grimace Scale' have been created based on consistently observed behavioral and physical responses of animals in painful situations. Based on facial expressions, Grimace Scales for 10 different species (mouse, rat, rabbit, cat, horse, cow, pig, sheep/lamb, ferret, seal) were recently developed (Mogil et al. 2020). This expansion to a variety of species reflects the complimentary role of this type of assessment with biomarker measurement, while the recent publication (2020) shows this is still an emerging and evolving approach. Our study, and its emphasis, serves to further demonstrate that there remains much to learn on the topic of pain in animals, and means by which to accurately and impartially assess it.

Bradykinin as a Mediator of Pain: Biosynthesis and Pharmacology.

Bradykinin is a nonapeptide (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) with low molecular weight (1,060.21 g/mol, $C_{50}H_{73}N_{15}O_{11}$) (Sala, 2013). It is considered one of the most recognizable substances within nociceptive stimulation, and one of the most relevant in reference to tissue damage and pain (Brierley et al. 2012).

Satake et al. (1972) points out that bradykinin has the capability to cause vasodilation, increase vascular permeability, cause hypotension, accumulate polymorphonuclear leukocytes, and to cause pain. For example, bradykinin levels were measured at triple or quadruple from normal/baseline during oral surgery, from double to triple in patients with rheumatoid arthritis, and double in rats with inflammations provoked by carrageenan (Hargreaves et al., 1988). Satake et al. (1972) demonstrated the change in levels of bradykinin in plasma and lymph after inducing acute pancreatitis in dogs.

Bradykinin can be formed in two ways; via the tissue enzyme kallikrein (Margolius 1998) and from low molecular weight kininogen (Jacobson and Kriz 1967). Figure 1 shows these two general pathways. The simpler of the two has only two components— an enzyme tissue kallikrein (Margolious et al. 1998) and a plasma substrate, low-molecular-weight kininogen (LK) (Jacobson and Kritz 1967, Mueller-Esterl et al. 1985). Tissue kallikrein is secreted by many cells throughout the body, however, certain tissues produce particularly large quantities. These include glandular tissues (salivary glands, sweat glands, and pancreatic exocrine glands) and the lung, kidney, intestine, and brain. Kallikrein is processed intracellularly from the precursor, prokallikrein; however, the enzyme responsible for this conversion has not been identified. Tissue kallikrein is secreted and digests LK to yield a 10-amino-acid peptide, lysyl-bradykinin (kallidin), with the sequence lysarg-pro-pro-gly-phe-ser-pro-phe-arg. A plasma aminopeptidase cleaves the N-terminal lys from it, and the 9-amino-acid peptide bradykinin results (Kaplan et al. 2002).

The second pathway for bradykinin formation is far more complex and is part of the initiating mechanism by which the intrinsic coagulation pathway is activated (Kaplan et al. 1998). Factor XII is the initiating protein that binds to certain negatively charged macromolecular surfaces and autoactivates (autodigests) to form factor XIIa (Silverberg et al. 1980, Tankersley and Finlayson 1984). There are two plasma substrates of factor XIIa, prekallikrein (Mandle and Kaplan 1997), and factor XI (Kurachi and Davie 1977, Bouma and Griffin 1997). Each of these circulates as a complex with high-molecular-weight kininogen (HK) (Mandle et al. 1976, Thompson et al. 1977). These complexes also attach to initiating surfaces, with the major attachment sites on two of the domains of HK, thereby placing both prekallikrein and factor XI in optimal conformation for cleavage to kallikrein (plasma kallikrein) and factor XIa, respectively. It is important to note that plasma kallikrein and tissue kallikrein are separate gene products and have little amino acid sequence homology, although they have related functions (ie, cleavage of kininogens). Tissue kallikrein prefers LK but is capable of cleaving HK, whereas plasma kallikrein cleaves HK exclusively. The two kininogens have an identical amino acid sequence starting at the N-terminus and continuing to 12 amino acids beyond the bradykinin moiety (Mueller-Esterl et al. 1985) but differ in C-terminal domains because of alternative splicing (Kitamura et al. 1985, Takagaki et al. 1985).

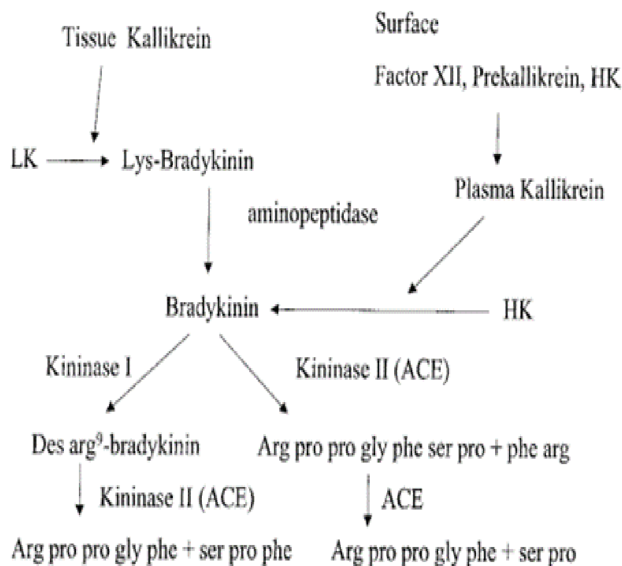

FIGURE 1-- Pathways for formation and degradation of bradykinin. Taken from Kaplan et al. (2002).

BK 1-5 as a biomarker

Bradykinin is rapidly degraded by enzymes such as carboxypeptidase N (kininase I), the angiotensin convert enzyme I (ACE or kininase II), neutral endopeptidase, enkephalinase (or CALLA antigen), and aminopeptidase P, which generates peptides such as Des- [Arg9] -BK, Des- [Phe8-Arg9] -BK (BK1-7), Arg-Pro-Pro (BK1-3), and Arg-Pro-Pro- Gly-Phe (braxidin 1-5, BK1-5) Bhoola et al. 1992).

In terms of persistence, bradykinin is only present in blood for a matter of seconds. Table 1 reflects both this relatively short duration, and lifespan differences of bradykinin in several species.

TABLE 1-- Persistence of bradykinin in blood of various organisms

Species	**Lifespan (seconds)**
Dog	13 ± 1[a] 17 – 30[b]
Cat	17 – 30[b]
Rat	10 ± 1[a]
Rabbit	30 ± 1[a]
Human	49 ± 2[a] 17 – 30[b]

a. Décarie et al. (1996)
b. Bhoola et al. (1992), Cyr et al. (2001)

In contrast, the lifespan of the bradykinin metabolite BK 1-5 has been estimated at approximately 86 and 101 minutes in human blood (Murphey et al. 2000). Using high-resolution liquid chromatography, it is possible to isolate bradykinin and its fragments for the purpose of detection (Murphey et al. 2000, Bujack-Gizycka et al. 2011). To achieve even more specificity, liquid chromatography is accompanied by mass spectrometry along with electrospray. This method has been used effectively to quantify the concentration of BK 1-5 in human blood (Murphey et al. 2001).

From a study group of 18 female beagles weighing between 8 to 13 kilograms, Edgerton et al. (2009) determined levels of BK 1-5 of 13 ± 2 fmol/ml (before the treatment) and 14 ± 5 fmol/ml (65 minutes after the treatment), while those of bradykinin have been determined in 11 ± 2 fmol/ml and in 7 ± 1 fmol/ml. These values were obtained from arterial blood by using the previously described method by Murphey et al. (2001).

Three main factors influenced our selection of BK 1-5 for assessment of viability as a prospective pain biomarker in dogs: a considerably greater lifespan than bradykinin, prior established (measurable) presence in canine plasma, and the availability of documented/validated methodology to determine plasma levels. As will be pointed out further, the blood concentration of bradykinin can be disrupted by several parameters, including sex and age, among others. However, another goal of this project was to determine the influence of such parameters on bradykinin blood concentration.

Materials and Methods

This study was conducted in Spain between August 2017 and July 2019. Authorization to proceed was requested from the department of Animal Protection of the Council for the Environment, Local Administration, and Territorial Government of the Community of Madrid, in relation to the Royal Decree 53/2013 of the 1st of February, which states the basic applicable norms for the protection of animals used as experimental subjects and for other scientific purposes. Based on the information provided during the request, the departmental representative was satisfied that animal protection would be maintained as per the norms outlined in the decree, and so no further authorization was required (issue record: 10/149528.9/18).

The plasma samples were drawn from canine patients at a single veterinary clinic and from selected individuals being housed at a rescue center/shelter. Three groups were established. Group 1 consisted of dogs without apparent mistreatment (n=10). Group 2 consisted of dogs being treated for pain from a diagnosed illness (n=6). Group 3 consisted of dogs that had allegedly been mistreated but were not being treated for pain (n=10).

Groups 1 and 2 were populated by canine patients from the Tres Olivos veterinary clinic (Madrid) and

sampled by clinic personnel once signed consent was given by their owners. Dogs from Group 1 and 2 were selected by the veterinarian in charge of the clinic on the basis of their owners showing receptiveness to participate in our study. In tandem, this created a valuable opportunity for the veterinarian to draw blood for the annual leishmania test offered to clients as part of a preventive initiative by the clinic. The previous veterinary history was not considered for dogs from Group 1. Individuals in Group 3 were being housed in a center for animal protection where abandoned and stray animals are cared for. More specifically, three of the ten dogs comprising this group were reportedly abused (beaten). The remaining seven were taken in as 'hoarding' cases found in circumstances of deficient hygiene and poor sanitation. Animal hoarders are categorized as a special class of animal abusers whose actions derive from a complex and poorly understood mental condition (Sinclair et al. 2007). The hoarding case dogs were included in our study because, as we previously described from the IASP definition, even absent overt injuries such as tissue damage, it is still possible for animals to experience pain – in this case at least from the extenuating circumstances of being in poor condition. In these particular hoarding cases, no final judicial sentence was issued to any of the owners regarding the treatment of these animals. These 10 dogs were sampled by the Universidad Complutense de Madrid's Sanitary Veterinary Vigilance Center (VISAVET), under a former agreement signed with the Center for Animal Protection. Tables 2, 3 and 4 summarize the individual parameters of each dog in the study according to the group they were assigned.

TABLE 2-- Group 1 dog parameters, assigned to the category of 'without apparent mistreatment'. (Sampling: Madrid, dates 03/07/2018, 06/07/2018 and 13/01/2019).

Breed	*Sex*	*Age (months)*	*Weight (kg)*
Mixed Breed	♂	20	5.7
Yorkshire	♂	96	5.3
Shih Tzu	♂	15	5.7
Mixed Breed	♂	60	10
Andalusian Bodeguero	♀	60	8.6
Mixed Breed	♀	72	36
Mixed Breed	♀	36	24
Podenco	♀	84	20
Mixed Breed	♂	84	7.8
Catalan Sheepdog	♀	48	17.5

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TABLE 3. Group 2 dog parameters, assigned to the category of 'suffer pain from illness but are treated against it'. (Sampling: Madrid, dates 03/07/2018, 06/07/2018 and 13/01/2019).

Breed	*Sex*	*Age (months)*	*Weight (kg)*	*Clinical condition*	*Treatment*
Labrador Retriever	♂	72	35.5	Pain in left elbow	Meloxicam
American Staffordshire Terrier	♂	96	35	Hip joint pain	Meloxicam
Mixed Breed	♂	168	15.7	Gallstones	Meloxicam (0.1 mg/kg/24h)
Labrador Retriever	♀	60	33	Good	Meloxicam (0.1 mg/kg/24h)
Mixed Breed	♂	156	11.5	Cardiopathy	Acupuncture
Yorkshire	♀	144	0.350	Bilateral knee pain	Firocoxib

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Table 4. Group 3 dog parameters, assigned as 'have presumably suffered mistreatment'. (Sampling: Madrid, dates 13/02/2019, 03/04/2019, 24/04/2019, 16/05/2019 y 17/05/2019).

Breed	*Sex*	*Age (months)*	*Weight (kg)*	*Clinical Condition*	*Observations*
Pitbull	♂	3	Not available	Aged scars on the latter back and the head. Slightly thin and dirty.	Along with other dogs, poor hygiene and sanitary condition.
Galgo	♂	5	Not available	Extremely thin and dirty. Bare spots across the body (possibly scabies).	Along with other dogs, poor hygiene and sanitary condition.
Galgo	♀	5	Not available	Thin and dirty. Bare spots across the body (possibly scabies).	Along with other dogs, poor hygiene and sanitary condition.
Galgo	♀	12	Not available	Thin and dirty.	Along with other dogs, poor hygiene and sanitary condition.
Mixed Breed	♀	72	10	'Apparently healthy' during examination.	Suffered from kicking and violent turning. Former owner has been reported by police.
Mixed Breed	♂	12	18-20	Very fearful during examination. Recent wound on the tip of the left ear, old wound in the right thigh above the back of the knee.	The owner panhandled accompanied by the dog. The present authorities witnessed owner hitting the animal.
Yorkshire	♀	60	3.5	'Apparently healthy' during examination.	Found with forty-five (45) cats and 2 dogs cohabitating in 50 square meters.
Mixed Breed	♀	66	15-16	'Apparently healthy' during examination.	Found with forty-five (45) cats and 2 dogs cohabit 50 square meters. Owner suffers from Diogenes/Noah syndrome.
Mixed Breed	♀	108	23	Multiple wounds, pyoderma, and slightly thin.	The animal has been allegedly mistreated by its owner, who has been witnessed hitting her.
Mixed Breed	♂	12	24	Cachexia, diarrhea with fresh blood, congestive mucous membranes, erythema on the inside of the ears and around the eyes. Dorsal alopecia in the right ear. Small flesh wounds in the inner thigh.	The owner's roommates denounce constant mistreatment of the animal, such as starving or not giving water to the animal and even hitting it.

Sampling consisted of extracting 1 ml of blood from the cephalic vein, unless anatomical or physiological circumstances (e.g. in the case of small dogs, less than 5kg) impeded it. In these cases, the jugular vein was used instead. The drawn blood was carefully ejected into a tube already containing 4 ml EDTA (ethylenediaminetetracetic acid) which had been refrigerated in a portable freezer. This was topped up with 3 ml of ethanol drawn from a separate syringe. Tubes were then returned to the portable freezer, which maintained an approximate temperature of between 0 and 8ºC.

Approximately 1.5 hours elapsed between the first extraction and arrival to the laboratory, where samples were placed in a refrigerated centrifuge (4ºC) at 1500 g for 25 minutes. The serum was then extracted and two aliquots were gathered from each sample - the first having a volume of 1 ml, the second containing the remaining volume of the plasma residue - in glass vials with metallic screw caps. Finally, the aliquots were subjected to supercooling to a temperature of -80º C.

Samples were analyzed in a stepwise fashion. BK 1-5 was measured in each plasma sample taken from members of Group 3 (Table 5) via liquid chromatography (Phenomenex Gemini 5u C18 110 A 150 x 2 mm) in combination with mass spectrometry with electrospray ionization. A chemically pure standard solution of unmarked BK 1-5 was used, along with a series of Nexus ABS Elut Extraction Cartridges (sorbent polymer 3 ml/60 mg) and Spin-X filters (nylon membrane, 0.22 mm filter), following the process stated by Murphey et. al (2001). The procedure was undertaken with the support of Universidad Complutense de Madrid's Center for the Development of Research on Mass Spectrometry (School of Chemistry). Table 5 highlights the analytical

Table 5. Analytical parameters for BK 1-5 samples from Group 3

(*) mass /charge number of ions

Standard solution	*Quantifier (m/z)**	*Productions (m/z)*	*CE (V)*
BK 1-5 (unmarked)	28.2	120.20	-23
BK 1-5 (unmarked)	287.2	69.95	-41
BK 1-5 (unmarked)	287.2	166.10	-12

4. Results and Discussion.

BK 1-5 was not detected in any of the 1 ml aliquots, possibly due to the peptide being absent from the sample, or to insufficient volume. Indeed, the method referenced for this study (Murphey et al. 2001) used 5 ml samples drawn from humans. To determine whether low volume was impeding detection, all the remaining aliquots were pooled for each group, resulting in three samples totaling 7.25, 8.5 and 8.75 ml, respectively. Table 6 shows the pooled sample results.

Table 6. Analysis of serum samples pooled by group.

Parameter	*Group 1*	*Group 2*	*Group 3*
Sample volume (ml)	7.25	8.5	8.75
BK 1-5 concentration in plasma (fmol/ml)	2.26	2.66	1.72
Average age (months)	55-56	116	35 -36
Median age (months)	60	120	12 (+ 15 days)
Sex	4♂ 6♀	4♂ 2♀	5♂ 5♀
Medication/treatment against pain?	No	Yes	No
Weight range (kg)	5.3 – 36	0.35 – 35.5	Min. 3.5

This study examined whether BK 1-5 levels can be measured using the described methodology and whether those results can be used to determine or confirm that a dog is in pain. Even though the sampling method itself may induce pain, dogs with a pre-existing painful condition are hypothesized to still have/exhibit BK 1-5 levels higher than the control subjects. For this reason, no analgesia was used for aid in sample collection.

This study examined whether differences can be found among dogs living in painful situations, hoarding included, versus clinically normal dogs. If BK 1-5 level is higher in dogs that seem to suffer pain than in dogs that apparently do not have pain, further research into its use in a forensic setting is warranted.

The amended direction we had to take for this experiment, namely pooling the individual samples, removed the possibility of assessing individual levels. However, pooling samples did allow for the recognition of variation in the concentrations of BK 1-5 in the groups of animals that were subject to mistreatment, contrasted with those that ostensibly had not. As such, we could still meet the main purpose of this study— to determine whether BK 1-5 blood levels are higher when pain arises.

Edgerton et al. (2009) reported BK 1-5 concentrations of 13 ± 2 fmol/ml and 14 ± 5 fmol/ml in the arterial blood of female beagles weighing between 8 and 13 kg. Unfortunately, the volume of blood drawn for their study was not reported. Drawing the full 5 ml volume of blood reported by Murphy et al. (2001) from a dog could endanger the animal's health or simply be infeasible, in the case of small-sized individuals. Since the sample volume we drew for this work was five times less, the same reduction factor was followed with the ethanol added (reduced from 15 ml). Thus, the study volumes are five times smaller than those in the reference study methods.

In addition to the loss of individual value determination that results from pooling samples, bradykinin levels in blood could be disrupted by other biological parameters.

To determine the levels of concentration of bradykinin, and interpret them, it is important to note that certain types of tissue have destructive effects over peptides. In cats, a loss of bradykinin ranging between 75% and 83% has been reported after movement through the lungs (Ferreira and Vane 1967). Bumpus et al. (1964) marked bradykinin with tritium and demonstrated the

presence of the marker in the kidneys, liver, and urine. In venous blood, with a sample group of 18 individuals (12 men and 6 women ranging from 53 to 86 years of age), the determined amount of bradykinin concentration averaged at 2.6 fmol/mol (0.23-8.20) (Cugno et al. 2000).

Drawing from the pronouncements made by Ferreira and Vane (1967) to the concentrations of BK 1-5 in arterial blood determined by Edgerton et al. (2009), we sought to calculate reference concentration values of BK 1-5 for results we obtained -- in venous blood. Specifically, we used as a reference the destruction percentages of bradykinin in the lungs as reported by Ferreira and Vane (1967) and the concentrations of bradykinin in arterial blood from Edgerton et al. (2009); 9 – 19 fmol/ml (see levels and ratios before treatment in the reference). From this, after applying the percentage of bradykinin loss after the passing of blood through the lungs we estimated values of 1.53 and 2.25 fmol/ml (applying 75% 'destructive percentage') and 3.23 and 4.75 fmol/ml (applying 83% 'destructive percentage'). The estimated value range (1.53 fmol/ml and 4.75 fmol/ml) coincides well with the concentrations of BK 1-5 determined by the present study, which range between 1.72 fmol/ml (Group 3) and 2.66 fmol/ml (Group 2).

Our results can be further considered in tandem with the potential destructive effects of age, NSAIDs treatment and sex.

Regarding age, the levels of kinins in serum increase progressively in F344 rats, with an increase of 400% in individuals in their middle age, and of 600% in rats of advanced age (63 ± 16 nmol/L in adults, 242 ± 43 nmol/L in middle aged rats, and 398 ± 102 nmol/L in advanced age) (Pérez et al. 2005). In this publication, the ages of rats are defined as 6 months (adults), 15 months (middle age), and 24 months (advanced age).

A priori, dogs in Group 3 were expected to present the highest concentration of BK 1-5, due to having 'suffered' the most. Dogs in Group 3 have an average age from 35 to 36 months. The reduced concentration levels of BK 1-5 in Group 3 may have been caused by the average age (between 35 and 36 months), despite the fact that this group would have been expected to have the highest concentration of BK 1-5. This could also explain why the highest levels of BK 1-5 were found in Group 2, with an older average age of 116 months.

It should be noted that Group 2 is comprised of dogs currently under NSAID treatment (all except the cardiopathy case, instead being treated with acupuncture). The administered NSAIDs were either meloxicam (which belongs to the group of oxicam NSAIDs) or firocoxib (which is a part of the COX-2 group). NSAIDs hinder cyclooxygenases (both constitutive and induced), which leads to the inhibition of the synthesis of prostaglandins. In painful situations (including with onset of inflammation and fever), prostaglandins sensitize nerve endings to the irritating action of mediators such as bradykinin and histamine. By lowering levels of prostaglandins, NSAIDs indirectly restore the normal thresholds of nociceptors, and through their anti-inflammatory action, reduce the presence of the aforementioned mediators. Currently, it is considered that most NSAIDs also possess an analgesic effect that acts over medullar synapsis and can even inhibit the prostaglandins in the central nervous system (Olguín 2005).

NSAIDs inhibit the synthesis of bradykinin either directly or indirectly, which led us to expect that the lowest levels of BK 1-5 would be seen in Group 2. The concentration levels of NSAIDs in the blood of the individuals were unknown during the moment of extraction, so we cannot infer anything about their effect on the levels of bradykinin and BK 1-5 in the samples. Regarding the inclusion of the individual cardiopathy case (Table 3), wherein this dog was treated with acupuncture rather than an NSAID, we note that in this case NSAID treatment would not have been appropriate, whereas the attending veterinarian is qualified by a recognized Chinese institution. However, since the rest of the dogs in this group were treated with NSAIDs, the inclusion of this individual's blood sample may have introduced heterogeneity and variation. We acknowledge this as a potential, albeit unintentional, flaw in this study.

Blood bradykinin levels could also be influenced by the sex of the animal. For example, levels of immunoreactive kallikrein and mRNA kallikrein decrease after ovarioectomy**,** yet these alterations are usually repaired through the administration of estrogen (Maeddu et al. 1991). The regulation of the kininogen gene through estrogen treatment has been demonstrated in rat livers (Chen et al. 1992). Estrogens may improve the activity of kinins due to the enzymatic action of kallikrin over kininogen after affecting the activity

of kininase II (Proudler et al. 1995).

Group 1 had 4 males and 6 females, Group 2 had 4 males and 2 females, and Group 3 had 5 males and 5 females. However, it is impossible to determine how much the sex factor influenced our results here. Note that we did not consider (or report on) any of the dogs being intact or neutered since we did not uncover any research examining whether this could have an effect. However, we also recognize that, as such, this parameter could be playing an as yet undocumented role.

This study has sought to identify a biomarker of pain that could be applicable to the largest number of cases of mistreatment. In Group 3, only 4 dogs were physically witnessed to be victims of physical violence, while the rest were found in a state of poor hygiene. This latter fact may have influenced that these individuals as a group did not present BK 1-5 levels that were above those of the two other groups in the study.

Conclusions

Our study reflects an ongoing need to gather further information on the topic of pain in dogs (and other animals), to help develop accurate and impartial tools, methodologies and biomarkers. The findings of this study suggest that the method described by Murphey et al. is not fit for the detection of BK 1-5 in 1 ml samples of dog plasma. Because of this, the test samples had to be mixed by group, which caused a loss in traceability. Since the volume of the dog plasma samples increased in this group mix, this allowed for the detection of BK 1-5. The pooled, consolidated data does not allow to establish if BK 1-5 is an indicator of pain or suffering under the conditions of this experiment. It may be necessary to refine the technical procedure to treat lower sample volumes or to apply more sensitive techniques —for instance, ELISA— which has already been used by Edgerton et al. (2009) to detect bradykinin.

The main premises of this study should be taken further but using groups that are more homogenous, in the first instance, in terms of the age and size of the subjects, since this will enable sample collection of larger volumes for individual study. Other factors for standardization are injury/symptomology, treatment/medication and, potentially, spay-neuter status. Follow up studies should include dogs presenting with more overt and 'obviously painful' types of injuries (e.g., laceration, bone breakage and fracture, gunshot wound). Taken together, these parameters might help further elucidate the validity of BK 1-5 as an indicator of canine/animal mistreatment. Going further, future study could also involve comparison of the concentration of BK 1-5 in plasma samples of dogs who have been victims of mistreatment in at least two points in time: one as close as possible to the moment of mistreatment, and secondly, after their recovery.

On the other hand, we also cannot exclude that other parameters and/or biomarkers might simply be more viable. For example, unpublished work (Melnichuk 2020) examined various potential suitable biomarkers for pain in dogs, including BK 1-5 and identified Substance P as showing even greater promise.

In order to determine the presence of pain in dogs, we recommend combining different parameter levels and other methodologies such as the Grimace Scale - which we acknowledge requires specialized training. There is still ample room and scope for researching and refining assessments of pain in animals, in order to provide scientific arguments to courts instead of interpretations based on cultural or biased views. This in turn necessitates knowing the type and nature of 'tangible' mistreatment in question (e.g., battering, starvation) and factoring in mental mistreatment - which may not cause 'conventional' pain and instead requires that another biomarker be assessed.

Fundamentally, we strongly recommend further work in this area to provide tools to more objectively assess physical and mental pain in dogs and promote animal welfare at judicial level.

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Disclosures

The authors declare no conflict of interest

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