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Serum serotonin (5-HT) in dogs (*Canis familiaris*): Preanalytical factors and analytical procedure for use of reference values in behavioral medicine

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ABSTRACT

The potential role of serotonin (5-HT) as a marker for identifying sensitivity to clinical diseases or behavioral disorders and for monitoring treatment emphasizes the need for reference values of serum 5-HT in dogs. Preanalytical factors such as patient variables, time of sample collection, specimen handling, storage time, and temperature can potentially cause variations to laboratory results. The aim of the present study was to evaluate canine serum 5-HT concentration measured by enzyme-linked immunosorbent assay and to propose standardized preanalytical conditions. Blood samples were collected from the cephalic vein, between 9.00 AM and 11.00 AM, from 120 healthy fasted dogs; the samples were then stored into anticoagulant-free tubes and centrifuged. The serum was stocked at −20°C and analyzed by enzyme-linked immunosorbent assay within 3 months from collection. Because the results were not normally distributed in the reference population, the 2.5 and 97.5 percentiles were calculated to characterize the 95% reference intervals (201-650 ng/mL, 361 median). For statistical analysis, the results were organized into 3 different groups based on the age of the dogs: 1-2 years old ($n = 35$), 3-7 years ($n = 43$), and 8-12 years ($n = 42$). 5-HT levels were higher in 3-7 years group than in other age groups, but no significant age-related dissimilarities were found (Kruskal-Wallis test, $P = 0.08$). The results of the present study contributed to establish reference intervals for serum 5-HT in dogs which could support both clinical researchers and veterinary behaviorists in designing studies and monitoring the effects of medications. Furthermore, we provide recommendations to decrease 5-HT measurement variation depending on preanalytical factors, to improve reliability and validity of serum measurements.

Introduction

The monoamine neurotransmitter serotonin (5-HT) plays a modulatory role in almost every physiological function. It is involved in neuronal excitability regulation and affects mood, cognition, and behavior (Smythies, 2005). The 5-HT system is phylogenetically the oldest and most expansive among all mammalian' central nervous system neurotransmitter networks (Jacobs & Fornal, 1995), although only about 1%-2% of the total amount of 5-HT lies in the serotonergic neurons in the brain (Gershon and Tack, 2007). Most 5-HT is located in the gastrointestinal tract, where it works as an enteric neurotransmitter, affecting intestinal smooth muscle neural modulation. It may act either directly on mesenteric vessels' smooth muscle or through enteric nerves, influencing gastrointestinal blood flow (Ormsbee and Fondacaro, 1985). Moreover, 5-HT can be detected in several non-neuronal tissues of the cardiovascular and renal systems, as well as in blood (Hoyer et al., 2002). Approximately, 95% of the 5-HT in blood is carried in platelet dense granules (Anderson et al., 1987); serum contains the secretion products of activated platelets, including their dense granules. Thanks to the use of improved methods of serotonin measurement, it has been found that 5-HT content in cerebrospinal fluid, usually determined in central nervous system applications, is well correlated with blood 5-HT levels (Audhya et al., 2012). Although serum and plasma specimens have been considered equivalent for many assays, consistent differences

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in 5-HT concentrations have also been reported (León et al., 2012, Cremer et al., 2015). Because most blood serotonin is stored in platelets and the release reaction of serotonin occurs easily, platelets and plasma measurements are complicated by artifacts caused by improper sample conservation and/or preparation (Veregut et al., 2012). Serum 5-HT is considered an accessible model for the investigation of the central serotonin system (Karakulova et al., 2013). Considering the simplicity of the methodology, sampling serum is the most suitable method for the measurement of circulating serotonin in dogs (León et al., 2012). In the veterinary literature, serum 5-HT levels compared with controls were significantly high in dogs with degenerative mitral valve disease (Arndt et al., 2009) and significantly lower in dogs with naturally acquired myxomatous mitral valve disease (Ljungval et al., 2013).

Nevertheless, what makes the serotonergic system particularly fascinating for scientists is its involvement, not fully understood, in a vast range of emotions and behaviors. Cerebrospinal fluid concentration of 5-hydroxyindoleacetic acid, the principal end product of 5-HT (Some and Helander, 2002) or the administration of 5-HT precursor tryptophan, tend to be positively correlated to prosocial behaviors in rhesus monkeys and in humans, respectively (Higley et al., 1996; Moskowitz, et al. 2001), and tend to be negatively correlated to antisocial behaviors such as aggression and social isolation. In humans, an association between serotonin transporter promoter gene polymorphism and violent behavior has been reported (Retz et al., 2004). There is evidence for a modulatory role of the serotonergic system in behavioral traits in dogs as well because behavioral problems such as aggression or anxiety have been associated with low serum serotonin levels compared with controls (Rosado et al., 2010, León et al., 2012, Amat et al., 2013, Sechi et al., 2017). Furthermore, lower serum 5-HT levels have been related to a lower frequency of sociable behavior toward humans and to one case of repetitive circling in pen in shelter dogs (Alberghina et al., 2017).

Some cases of pet behavior problems require treatment with psychoactive medications (Simpson and Papich, 2003). Nevertheless, patients affected by diseases such as hypothyroidism, diabetes mellitus, renal failure, and hyperlipidemia show increased sensitivity to 5-HT levels and might be at increased risk of developing serotonin toxicity because of combination therapy (Mohammad-Zadeh et al., 2008). Serotonin syndrome and its spectrum of symptoms are a product of the overactivation of both the central and peripheral 5-HT receptors as a result of high levels of 5-HT (Volpi-Abadie et al., 2013). The mechanisms are inhibition of 5-HT uptake, decreased 5-HT metabolism, increased 5-HT synthesis, increased 5-HT release, and activation of serotonergic receptors (Volpi-Abadie et al., 2013). Serotonin toxicity has been documented in laboratory animals as well as companion animals (Gwaltney-Brant et al., 2000) and it represents an increasingly common toxic syndrome in veterinary medicine (Hopkins et al., 2017). Unfortunately, there are no established guidelines for the prevention of serotonin syndrome or specific laboratory tests to diagnose it (Ables and Nagubilli, 2010). In humans, serum 5-HT concentrations do not correlate with the severity of this syndrome (Boyer and Shannon, 2005), but no data are available for dogs. To establish reference values, methods of analysis and storage conditions should be defined. To the best of the authors' knowledge, there has been no other recent study investigating serum 5-HT in dogs with other methods than enzyme-linked immunosorbent assay (ELISA), but its measurements had not been sufficiently standardized in dogs. Measurement of a biologic analyte by ELISA requires a means for distinguishing the bound fraction (antigen-antibody complex) from the unbound fraction (free antigen or free antibody depending on the type of ELISA). Competitive ELISA requires only a small amount of antibody with high sensitivity. A small amount of analyte is capable of producing a large signal (because the bound fraction is measured) (Slagle and Ghosn, 1996). According to the manufacturer's instructions reported on the ELISA kit, 5-HT is stable in serum up to 6 months at −20°C. It is important to be aware of the possibility of using −20°C freezers to store samples without affecting 5-HT results, as many investigators and clinicians may not have access to −80°C storage. The aim of the present study was to standardize preanalytical factors and use ELISA to investigate the concentration of serum 5-HT in dogs, to establish reference intervals, identify potential variations related to age, and help veterinary clinicians and behaviorists identify pathological conditions related to high 5-HT serum levels.

Materials and methods

Subjects

The sample included 120 medium-large sized dogs (N = 56 males and 64 females) aging from 1 to 12 years (Table 1). The subjects were chosen from two animal shelters (N = 98) and from owned dogs (N = 22) in Sicily, Italy. The study took place during the period 2014-2018. Some preliminary results included in this study have been published (Alberghina et al., 2014, 2017). All dogs were fasted ≥12h to avoid feeding/fasting influence. Dogs that appeared ill, aggressive (growling, barking, showing canines), excessively timid (avoiding eye contact, tucking the tail between their hindlimbs, or cowering at the back of the cage), or cachexic were excluded from the study. Protocols of animal husbandry and experimentation were reviewed and approved in accordance with the standards recommended by the Guide for the Care and Use of Laboratory Animals and Directive 86/609CEE.

Blood was drawn from the cephalic vein to check the health status of each dog. The samples were scheduled to be collected between 9.00 AM and 11.00 AM. All dogs had received routine health care (e.g., antiparasitic treatment, blood sample examination) and had been declared healthy by shelter and private veterinarians.

Serum serotonin assessment

The samples were collected into anticoagulant-free tubes and centrifuged (1,785 g for 10 minutes). The serum aliquots obtained from each sample were transported to the laboratory in a cooler with an ice block within 1 hour of being drawn. Serum for 5-HT analysis was stored frozen at −20°C. Samples were analyzed within 3 months of collection. A commercial competitive ELISA (5-HT ELISA$^{\text{Fast Track}}$, LDN GmbH & Co. KG) kit was used to measure 5-HT levels in the serum following the manufacturer's instructions. In the used kit, the antigen is bound to the solid phase of the microtiter plate. Standards and test samples are added to the wells, along with conjugated-serotonin antigen and an antibody specific to 5-HT. After incubation, the excess reagents are washed away. Substrate is added, and after a short incubation, the enzyme reaction is stopped and the yellow color generated. The intensity of the yellow coloration is inversely proportional to the amount of 5-HT captured in the plate. As indicated in the manufacturer's description, this kit

Table 1
Distribution of sample group (n = 120)

Sex	Gonadectomized/entire	Age (years)	Breed/crossbreed	Shelter/owned
64 F	39/25	1-2 (n = 35)	25/95	98/22
56 M	35/21	3-7 (n = 43)		
		8-12 (n = 42)		

is used for the quantitative ultrasensitive determination of 5-HT in any species and various biological samples. Absorbance level of each sample was measured using a microtiter plate reader (EZ Read 400 ELISA; Biochrom, Cambridge, UK) at 450 nm, with concentration calculated according to a standard curve. Intra- and inter-coefficient of variability were 4.5% and 8%, respectively. The amount of 5-HT in the sample was estimated with the calibration curve and expressed in ng/mL. All samples were analyzed by the same individual (D.A.).

Statistical analysis

The Shapiro–Wilk's test was used to assess normality. Outliers were identified according to Grubb's test. Mann-Whitney test was used to compare 5-HT levels between males and females, between shelter and house dogs, or between different shelters. For statistical analysis, dogs were divided into 3 age classes. We have decided to include, considering the medium-large size of the dogs, in the first-age-class youngs from 1 to 2 years of age, n = 35, in the second-class adults (3-7 years, n = 43), and in the third-class elderly dogs (8-12 years, n = 42). The nonparametric Kruskal-Wallis test was used to investigate the overall differences in serum 5-HT concentration among age groups. Data were analyzed using the software STATISTICA 7.5 (StastSoft Inc., Tulsa, OK).

Results

Table 2 shows mean values for 5-HT concentrations as well as a comparison with the values reported in veterinary literature. Significant *P* value (Shapiro–Wilk's test: W = 95, *P* = 0.02) was found for 5-HT, indicating that these results were not normally distributed in the reference population. Four outliers were detected and were excluded from the calculation of reference intervals. Therefore, the 2.5 and 97.5 percentiles were calculated to characterize the 95% reference intervals (George et al. 2010). The comparison among the groups did not show significant differences in age (Kruskal-Wallis test, *P* = 0.08). The age pattern is illustrated in Figure 1: levels tend to increase from 1 to 2 years of age to 3-7 years of age and decrease with aging (8-12 years group). No sex difference was found, but females and gonadectomized dogs showed slightly higher 5-HT levels than males and entire dogs, respectively. No statistical differences were found between different shelters and between house or shelter dogs, but house dogs showed lower 5-HT levels than shelter dogs.

Discussion

The results of the present study demonstrate that serum 5-HT levels are not significantly influenced by sex, age, and environment conditions. In a previous preliminary study, 5-HT levels had been found to be significantly higher in shelter dogs than in house dogs (Alberghina et al., 2014). This finding had been justified by

Table 2
Reference intervals for serum 5-HT (ng/mL) as determined by ELISA in clinically healthy dogs and comparison with values reported in veterinary literature (number of subjects in brackets)

Mean ± SD	Percentile			Values reported in literature
	2.5	50	97.5	
382.9 ± 105.0 (n.120)	201	361	650	
	147	252	391	Höglund et al., 2018 (n.483)
852.77 ± 449.8		891		Amat et al., 2013 (n. 20)
		508		Arndt et al., 2009 (n. 36)
32.5				Cakiroğlu et al., 2007 (n.18)

ELISA, enzyme-linked immunosorbent assay.

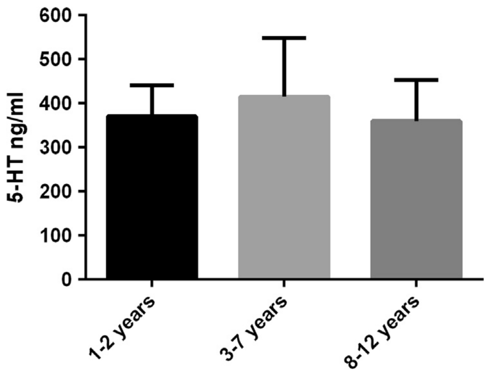

Figure 1. Canine serum 5-HT levels (ng/mL) in three groups based on age.

greater social interactions and olfactory presence of cospecifics, but after increasing the number of subjects, no significant difference between shelter and house dogs was found. 5-HT levels were higher in the 3-7 years group than in the other groups. As illustrated in Table 2, the median values found were somewhat different from those reported by previous studies (Arndt et al., 2009; Höglund et al., 2018). However, the difference between our findings and other researchers' (Arndt et al., 2009) could be largely due to the fact that blood samples in the previous study were obtained by patients who had not been fasted. Meal ingestion influenced serotonin levels in human serum (Kwon et al., 2018) and in equine plasma (Alberghina et al., 2011). Furthermore, diurnal fluctuations in 5-HT levels have been found in humans (Kwon et al., 2018), as well as in other mammalian species (Piccione et al., 2005; Kirsz & Zieba, 2012), so time of day is a factor that should be considered in studies evaluating serotonin levels. The lower median reported by Höglund et al. (2018) may reflect difference in storage conditions: the storage time was long (about 5 years) and samples were thawed and refrozen. Much larger differences were apparent between the values in control dogs reported by Cakiroğlu et al. (2007) and Amat et al., (2013) who, respectively, reported serum 5-HT values of 32.5 ng/mL (N = 18) and 852.77 ng/mL (N = 20). Because Cakiroğlu et al. (2007) did not specify the type of blood collection containers used in the preanalytical procedure, the values reported may be related to plasma, not serum, as suggested in the text. The quality and reliability of laboratory test results begin with the selection and preparation of the patient, continue with the collection and handling of the specimen, and terminate with the analytical report (Meyer et al., 1992). Reliable information about fasting of the patients, time of blood collection, method of analysis, and storage conditions could help researchers and veterinary practitioners handle and store samples appropriately, to have good sample quality for evaluable diagnosis. To the authors' knowledge, this has not been carried out for serum 5-HT in any previous study in the field of veterinary medicine. Although the use of standardization of preanalytical procedures and analytical method for measuring serum 5-HT in dogs has been suggested in this study, further investigation is necessary.

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Conflict of interest

The authors declare no conflict of interest.

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