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L-Tryptophan supplementation increases serotonin blood levels in dogs fed a dissociated carbohydrate-based diet

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Abstract: The aim of the present study was to investigate the effect on blood serotonin (5-HT) concentrations of the addition of L-Tryptophan (L-TRP) to a dissociated diet, in the meal consisting only of the carbohydrate component. Six sheltered dogs (5 males and 1 neutered females), weighing between 15 and 30 kg and living in the shelter for more than six months, were recruited. The dogs were fed two daily meals (at 8.00 A.M. and 4.00 P.M.). The morning meal was composed of puffed rice with the addiction of1mg/kg of L-TRP, whereas the evening meal was composed of the commercial diet previously eaten. Blood was collected the day before the first administration of the diet (T0) and the 30th day of the treatment (T1). A further blood sample was collected 14 days after the discontinuation of the diet (T2). To prevent possible influence of circadian rhythm, all three samples were collected at 4.00 P.M., just before the second meal. The results were compared using the Wilcoxon test ($p < 0.05$). The statistical analysis revealed a significant difference between the blood concentrations of TRP at T1 and those determined at T2 ($p = 0.028$). No statistical differences between T0 and T1, and between T0 and T2 was found. Significant difference between the blood 5-HT concentration at T1 and T0 ($p = 0.028$), and between T1 and T2 ($p = 0.028$) were found. On the contrary,no statistical difference between T0 and T2 was found.

The data obtained, albeit carried out on a small number of animals showed that the blood levels of TRP could be correlated with those of 5-HT. Indeed, the determination of blood 5-HT concentrations seems to provide a faithful image of the cerebral metabolism of this amine, as increases in circulating TRP concentrations is followed by significant increases in 5-HT.

Key Words: dogs, serotonin, shelter, tryptophan.

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Introduction

The dog has shared its life with humans for thousands of years (Pörti & Jung, 2017; Jung & Pörti, 2018) and it is able to develop an attachment bond towards the owner, proven by numerous studies (Carlone et al., 2019; Riggio et al., 2021 a, b, c; Mariti et al., 2013 a, b; Mariti et al., 2014; Mariti et al., 2017; Mariti et al., 2018; Mariti et al., 2020). In recent years, increasing attention has been paid to prevent and treat the behavioral problems of this animal that can jeopardize its welfare and damage the relationship with humans. Despite the intra and interspecific social behavior of the dog seem to be regulated by the oxytocinergic system (Ogi et al., 2020; Ogi et al., 2021), many behavioral problems seem to be related to the serotoninergic pathways in the brain, characterized by serotonin (5-HT) deficiency (Sachs et al., 2015; Rosado et al., 2010 a, b).

Recently, a growing interest has been directed towards the possibility of modifying the levels of 5-HT in the brain, by acting on the amino acid composition of the diet. In fact, 5-HT derives from tryptophan (TRP) introduced with the diet and serotoninergic neurons are influenced by brain TRP concentration (Fernstrom et al., 1990; Sharp et al., 1992). TRP passes through the blood-brain barrier transported by a carrier for which it competes with other large neutral amino acids (LNAAs), i.e. leucine, isoleucine, valine, tyrosine and phenylalanine (Oldendorf & Szabo, 1976). In the brain, TRP hydroxylase, that hydroxylates TRP to 5-hydroxytryptophan, is about half saturated with the amino acid (Carlsson, 1978). For this reason, an TRP increase in the brain could double the 5-HT synthesis (Carlsson, 1978). Actually, protein meals fail to raise brain TRP concentrations because of the contemporary increase of both serum TRP and LNAA concentrations by proportionally similar amounts, resulting in no net change in competition for the carrier (Crandall & Fernstrom, 1980). In previous research it has been demonstrated that the ingestion of a large

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carbohydrate, protein-free meal by rats, rapidly raises brain TRP concentrations and stimulates 5-HT synthesis (Crandall & Fernstrom, 1980). This positive effects on TRP are realized via insulin secretion, which depresses the plasma concentrations of LNAAs, increasing their uptake into muscle. TRP is preserved by this uptake for its bond with albumin (Fernstrom & Wurtman, 1972; Lotspeich, 1949) with an increase of its bioavailability. Our recent study has, in fact, demonstrated that, in dog, a carbohydrate-based diet led to a decrease in LNAAs levels and consequently led to a significant higher TRP/LNAAs ratios 6 h after the provision of carbohydrates (Gazzano et al., 2018). However, our further studies showed that the dissociated diet is not able to raise the levels of serotonin in the blood, even though it alters the relationship between TRP and LNAAs (Gazzano et al., 2019) and that there is no correlation between TRP and 5-HT blood levels (Riggio et al., 2021).

This result could be explained by the low TRP amounts in the carbohydrate-only meal that dogs fed in the morning.

To clarify this aspect, the aim of the present study was to investigate the effect, on blood 5-HT concentrations of the addition of L-TRP to a dissociated diet, in the meal consisting only of the carbohydrate component.

Animals, materials and methods

The present study was approved by the Ethical Committee of the University of Pisa, Italy (protocol n° 38/2016) in accordance with Directive 2010/63/EU. Six shelter dogs (5 males and 1 neutered females), weighing between 15 and 30 kg and living in the shelter for more than six months, were recruited. The animals were fed two meals (at 8.00 A.M. and 4.00 P.M.) for 30 days with a commercial diet to standardize their metabolic status before the administration of the experimental diet. After this period, the dogs were fed two daily meals (at 8.00 A.M. and 4.00 P.M.). The morning meal was a carbohydrate-based one (puffed rice), integrated with 1mg/kg of L-TRP, whereas the evening meal was composed by the commercial diet previously eaten during the adaptation period. Ingredients and diet analytical constituents are reported in Table 1.

Table 1: Ingredients and diet analytical constituents (as fed basis).

		Commercial kibble	Puffed rice	Diet
Moisture	%	10.0	5.5	7.9
CP	%	28.0	7.5	18.8
FAT	%	21.0	1.0	13.4
CF	%	2.6	2.5	2.5
NSC	%	31.9	83.0	53.0
ASH	%	6.5	0.5	3.8
EM	Kcal/kg	3800	3250	3690

Blood was collected the day before the first administration of the diet (T0) and at the 30th day of the treatment (T1). A further blood sample was collected 14 days after the discontinuation of the diet (T2). Blood samples (4 ml) were at 4.00 P.M., just before the second meal, and left to coagulate at room temperature for 60 minutes, then centrifuged in ALC 4237R Refrigerated Centrifuge at 7000 rpm for 20' to 4°C to obtain the serum. The serum was divided into 200 µl aliquots and frozen until the time of analysis. The extraction and quantification of 5-HT and TRP in serum samples were performed following an HPLC method, as previously described in the literature (Bearcroft et al., 1995; Atkinson et al., 2006) and based on fluorimetric detection. This method was slightly modified as follows: 200 µl $HClO_4$ 4% v/v containing 2 mM EDTA were added to 200 µl of serum or standard solution to precipitate proteins; the extract was mixed and centrifuged at 13000 rpm in micro centrifuge (ALC micro CENTRIFUGETTE ® 4214) for 3 minutes. 50 µl of supernatant were taken with MICROLITER™ Syringes #705 and 20 µl injected into HPLC for analysis. HPLC analyses were performed using a RP Gemini C18 column (250 mm x 4.6 mm, 5 um) (Phenomenex, Torrance, CA, USA) and a Jasco HPLC apparatus (Jasco Corporation, Ishikawa-Machi Hachioji-Shi, Tokyo, Japan) equipped with 2 gradient pumps (PU-1580), a mixer unit (HG-2080-03) and a fluorescence detector (FP-920). The mobile phase consisted of methanol (CH_3OH) and ammonium acetate (CH_3COONH_4) 100 mM (20:250 v/v), pH 4.5, degassed and filtered with 0.2 µm diameter filters and eluted at a flow rate of 0.800 ml/mi. Fluorescence detector was set at 290 nm excitation wavelength and 337 nm emission wavelength. Data was acquired using Jasco Borwin 1.5.0 software (Jasco Corporation, Ishikawa-machi Hachioji-shi, Tokyo, Japan). The interface between chromatography instruments and a PC based data acquisition is the JMBS electronic interface box HERCULE 2000 VI.0. Serotonin creatinine sulfate monohydrate and L-tryptophan (TRP) were purchased from Sigma-Aldrich Inc. (Saint Louis, MO, USA). Stock solution (10 mM) of 5-HT and stock solution (100 mM) of TRP were prepared in 10 ml $HClO_4$ 10%, divided in aliquots of 1 ml and stored at -20°C. Diluted standard solutions in $HClO_4$ 4% were prepared daily and employed to identify chromatographic peaks and to calculate calibration curves. Data were statistically analyzed applying Wilcoxon test, by using SPSS® STATISTICS 17.0.

Results

In the table 2 and 3 are reported the concentrations, at different times, of TRP and 5-HT, in the investigated dogs.

Table 2: Blood TRP concentrations (µM/ml) in six shelter dogs at different times: the day before the first administration of the diet (T0), the 30th day of the treatment (T1) and 14 days after the discontinuation of the diet (T2).

Dog number	T0	T1	T2
1	32.1	24.2	16.9
2	19.3	35.0	22.0
3	24.8	26.6	24.6
4	11.3	43.7	14.4
5	21.2	66.6	22.0
6	19.9	16.6	10.0
Mean ± S.D.	21.43±6.85	35.45±17.88	18.32±5.53

Table 3: Blood 5-HT concentrations (µM/ml) in six shelter dogs at different times: the day before the first administration of the diet (T0), the 30th day of the treatment (T1) and 14 days after the discontinuation of the diet (T2).

Dog number	T0	T1	T2
1	1.7	2.8	2.2
2	1.5	1.9	1.4
3	1.3	1.4	0.7
4	0.8	1.0	0.7
5	0.6	2.2	1.8
6	1.6	2.0	1.8
Mean ± S.D.	1.02±0.53	1.88±0.65	1.36±0.54

In the figures 1 and 2 are reported the concentration of blood TRP and 5-HT of the investigated dogs.

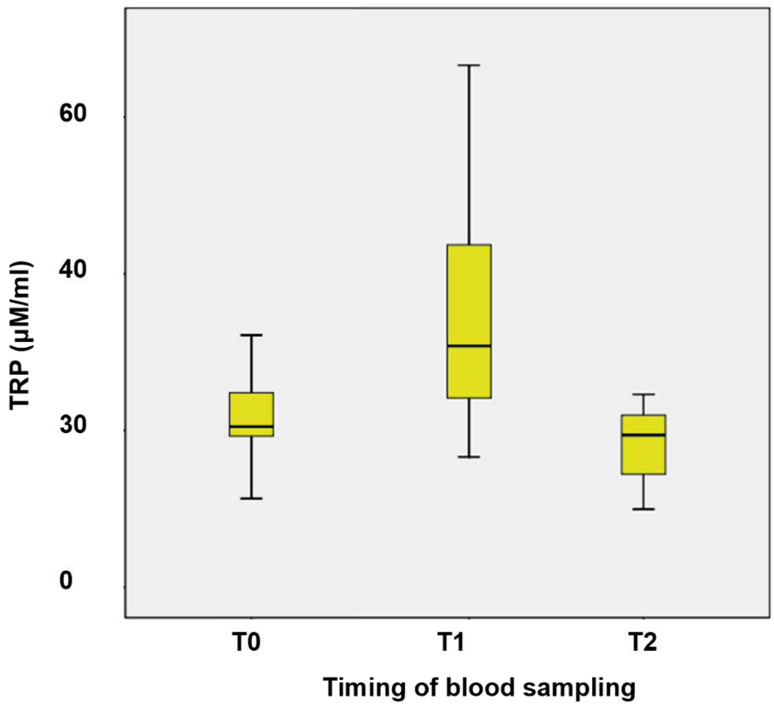

Figure 1: Blood TRP concentration in six shelter dogs at different times: the day before the first administration of the diet (T0), the 30th day of the treatment (T1) and 14 days after the discontinuation of the diet (T2).

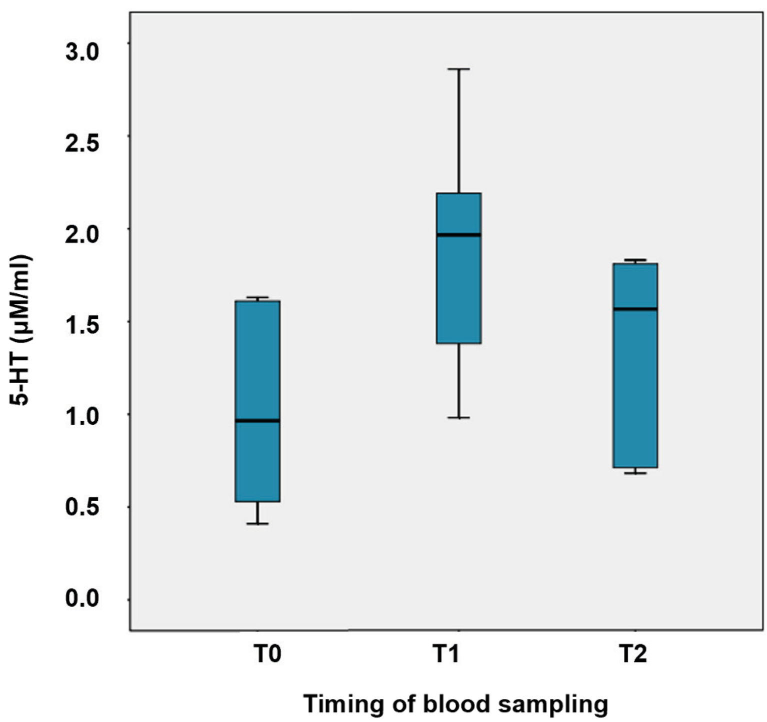

Figure 2: 5-HT blood concentration in experimental dogs at different times: the day before the first administration of the diet (T0), the 30th day of the treatment (T1) and 14 days after the discontinuation of the diet (T2).

The results were compared using the Wilcoxon test ($p < 0.05$). The statistical analysis revealed a significant difference between the blood concentrations of TRP at T1 (mean ± standard deviation (SD) = 32.77 ± 6.61 μM/ml) and those determined at T2 (mean ± SD = 18.32 ± 5.53 μM/ml; Z = -2.201; p = 0.028). No statistical differences between T0 and T1, and between T0 and T2 were found.

As regards blood 5-HT concentrations, statistical analysis revealed a significant difference between the concentration at T1 (mean ± SD = 1.88 ± 0.65 μM/ml) and the concentration at T0 (mean ± SD = 1.02 ± 0.53 μM/ml; Z= -2.201; p=0.028). Moreover, a significant difference between the concentration at T1 and T2 (mean ± SD = 1.36 ± 0.54 μM/ml; Z= -2.201; p = 0.028) was found. No statistical differences between values at T0 and T2 were found.

Discussion

The behavioral problems are the most common reported reasons of abandoning or relinquishing dogs to the shelter: aggression, phobias and separation problems are the principal cause (Patronek et al., 1996). For some of these behavioral problems the correlation with low intracerebral 5-HT levels is known (Amat et al., 2016). Current pharmacological therapies for phobia and aggression are in fact based on the administration of drugs that inhibit the re-uptake of 5-HT, thus increasing its activity. However, due to the reduction of the effectiveness of these drugs over time, more and more attention is paid to other methods that can increase the intracerebral levels of 5-HT.

Since 5-HT has its precursor in the TRP which is taken with the diet and since the conversion of TRP into 5-HT occurs only partially, due to the reduced amount of TRP that overcome the blood-brain barrier, a manipulation of the diet, to favor a greater bioavailability, could be a useful method to increase the synthesis of 5-HT and well accepted even by people who exclude the use of psychotropic drugs in the therapy of behavioral pathologies of their animal.

The results achieved in the rat (Crandall & Fernstrom, 1980) have shown the efficacy of this method, but the data obtained so far in the dog have not provided the same clear demonstration (De Napoli et al., 2000; Dodman et al., 1996). Although it has been verified the possibility of increasing the bioavailability of TRP, through a dissociated diet, consisting of two meals, one of which only based on carbohydrates, physiological data fail to demonstrate a real efficacy of a similar diet (Gazzano et al., 2019).

The results of this study, even if carried out on a small sample of animals, seems to show that there is a sort of correlation between blood levels of TRP and 5-HT. In fact, the animals fed a dissociated diet, supplemented with 1 mg/kg of L-TRP, showed a significant increase of TRP at T1 in respect to T2, and a significant increase in the levels of 5-HT at T1 vs T0 and vs T2.

The TRP levels at T0 are not statistically different from those found at T1 and this is probably due to the presence of TRP in the commercial diet the dogs are fed with. However, the presence of TRP in the diet seem to not produce elevated levels of 5-HT, possibly due to competition between TRP and LNAAs.

Based on these results we can deduce that, if there is an increase in the production of 5-HT following the integration of the diet with TRP, this could be detectable by evaluating the concentrations of amine in the blood. This cannot be the final proof that, in the brain, a greater synthesis of 5-HT occurs, an event widely demonstrated, but with much more invasive experimental methods, in the rat (Fernstrom et al., 1990). However, 5-HT synthetized in the brain can overcome the blood-brain barrier, as demonstrated by Nakatani et al. (2008): 5-HT transporters located on the brain endothelial cells may act as the efflux transport system for the 5-HT that crosses from the brain into the circulating blood. This fact could permit to observe a rise of 5-HT concentrations in the blood after TRP integration in the diet.

We must also bear in mind that 95% of 5-HT is produced in the intestine by chromaffin cells that largely release it into the bloodstream. This could be at the basis of the variability in 5-HT blood concentrations that was found in different subjects, although blood samples were always collected at the same time of day, to avoid the effects of a possible circadian rhythm.

Another interfering factor is the photoperiod which can cause an increase in 5-HT production. Bright light is, in fact, a standard treatment for human seasonal depression, but some studies also suggest that it is an effective treatment for nonseasonal depression (Golden et al., 2005) and also reduces depressed mood in women with premenstrual dysphoric disorder (Lam et al. 1999), and in pregnant women suffering from depression (Epperson, 2004).

In conclusion, the data obtained with this research, albeit carried out on a small number of animals, showed that the blood levels of TRP could be correlated with those of 5-HT. Indeed, the determination of 5-HT concentrations in blood serum seems to provide a faithful image of the cerebral metabolism of this amine, as increases in circulating TRP concentrations is followed by significant increases in 5-HT.

Further studies, which analyze the behavior of dogs fed a dissociated diet supplemented with TRP, may provide evidence relating to the efficacy of this method in improving the clinical conditions of dogs suffering from behavioral pathologies that benefit from the increase in brain values of 5-HT.

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L'integrazione di L-triptofano aumenta i livelli di serotonina nel sangue di cani alimentati con una dieta dissociata a base di carboidrati

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Sintesi

Lo scopo del presente studio è stato quello di indagare l'effetto sulle concentrazioni di serotonina (5-HT) nel sangue dell'aggiunta di L-triptofano (L-TRP) ad una dieta dissociata, in un pasto costituito solo dalla componente di carboidrati. Sono stati reclutati sei cani (5 maschi e 1 femmina sterilizzata), di peso compreso tra 15 e 30 kg e che vivevano in canile da più di sei mesi. I cani sono stati nutriti con due pasti giornalieri (alle 8:00 e alle 16:00). Il pasto mattutino era composto da riso soffiato con l'aggiunta di 1 mg/kg di L-TRP, mentre il pasto serale era composto dalla dieta commerciale precedentemente consumata. Il sangue è stato raccolto il giorno precedente alla prima somministrazione della dieta (T0) e al 30° giorno del trattamento (T1). Un ulteriore campione di sangue è stato raccolto 14 giorni dopo l'interruzione della dieta (T2). Per prevenire possibili influenze del ritmo circadiano, tutti e tre i campioni sono stati raccolti alle 16:00, appena prima del secondo pasto.

I risultati sono stati confrontati utilizzando il test di Wilcoxon ($p < 0{,}05$). L'analisi statistica ha rivelato una differenza significativa tra le concentrazioni ematiche di TRP a T1 e quelle determinate a T2 ($p = 0{,}028$). Non sono state riscontrate differenze statistiche tra T0 e T1 e tra T0 e T2.

Sono state trovate differenze significative tra la concentrazione di 5-HT nel sangue a T1 e T0 ($p = 0{,}028$) e tra T1 e T2 ($p = 0{,}028$). Al contrario, non è stata trovata alcuna differenza statistica tra T0 e T2.

I dati ottenuti, sebbene effettuati su un numero ristretto di animali, hanno mostrato che i livelli ematici di TRP potrebbero essere correlati con quelli di 5-HT. In effetti, la determinazione delle concentrazioni ematiche di 5-HT sembra fornire un'immagine fedele del metabolismo cerebrale di questa ammina, poiché l'aumento delle concentrazioni di TRP circolante è seguito da aumenti significativi di 5-HT.