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Paper

Effects in dogs with behavioural disorders of a commercial nutraceutical diet on stress and neuroendocrine parameters

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The well-being of dogs can be affected by changes in human lifestyle, eating habits and increased stressors that lead to behavioural disorders including fear, hyperactivity and anxiety, followed by negative affective moods and poor welfare. This randomised, controlled clinical evaluation involved 69 dogs, 38 males and 31 females, of different breeds, with behavioural disorders related to anxiety and chronic stress. They were fed a control diet or a nutraceutical diet (ND group) for 45 days. Neuroendocrine (serotonin, dopamine, β-endorphins, noradrenaline and cortisol) and stress (derivatives of reactive oxygen metabolites (dROMs) and biological antioxidant potential (BAP)) parameters related to behavioural disorders were evaluated at the beginning and end of the study period. Results showed a significant increase in serotonin, dopamine and β-endorphins plasma concentrations (*P<0.05, *P<0.05 and **P<0.01, respectively) and a significant decrease in noradrenaline and cortisol plasma concentrations in the ND group (*P<0.05). dROMs significantly decreased in the ND group (*P<0.05) while BAP was not affected. This study demonstrated for the first time that a specific diet significantly and positively affected neuroendocrine parameters and dROMs. These results open significant perspectives concerning the use of diet and nutraceuticals in the treatment of behavioural disorders.

Animal behaviour is the result of interplay between genotype and environment (Overall and others 2002, 2006, Sih and others 2004) and is, at the neuroendocrine level, characterised by neuromediators like dopamine and serotonin (5-HT2A and γ-aminobutyric acid reduced binding activity) or endocrine (cortisol) pathways imbalance (Paredes and Agmo 1992, Inagawa and others 2005, Peremans and others 2006, Vermeire and others 2009, 2012), Chronic anxiety status (Frank 2014, Overall and others 2006) and nutrition can significantly affect behaviour (Dodman and others 1996, DeNapoli and others 2000, Bosch and others 2007). It was, as an example, demonstrated that a diet high in tryptophan can lower territorial aggression score while a high-protein diet without tryptophan supplementation can induce a high dominance aggression score (DeNapoli and others 2000). A specific diet supplementation with amino acids, n-3 and n-6 polyunsaturated fatty acids (PUFAs) and a well-balanced amount of proteins and fibre was considered as beneficial in dogs with evident behavioural disorders (Bosch and others 2007). Similarly, several reports showed the role of such compounds in modulating animal behaviour (Kantak and others 1980, Reinstein and others 1984, 1985, Lasley and Thurmond 1985, Raleigh and others 1985, Chamberlain and others 1987, Jewell and Toll 1996, Butterwick and Markwell 1997, Chalon and others 1998, Carrie and others 2000, Moriguchi and others 2000, Koopmans and others 2005).

The benefit of nutraceuticals was demonstrated in different species. *Punica granatum* has been extensively used to treat chronic anxiety and insomnia in rats (Riaz and Khan 2014, Swarnamoni and Phulen 2014). Mild sleep disorders, but also nervous tension, have been treated with roots and rhizomes of *Valeriana officinalis* in mice (Carlini 2003, Hattesohl and others 2008, Sudati and others 2009, Wang and others 2010). On the other hand, antianxiety and antidepressant activities were observed for *Rosmarinus officinalis* (Machado and others 2009, Ulbricht and others 2010), *Tilia* species in mice (Viola and others 1994, Coleta and others 2001) and *Crataegus oxyacantha* L. in human beings and mice (Hanus and others 2004, Ernst 2007, Lakhan and Vieira 2010). L-Theanine, one of the *green tea* constituents, has been shown, in human beings, to play a role in reducing stress and decreasing the heart rate in chronic anxiety (Juneja and others 1999, Miodownik and others 2011). As to L-tryptophan, many published reports have also described the presence of anxiety, mood and depressive symptoms associated with its depletion (Delgado and others 1990, 1999). Finally,

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there are several evidences suggesting that omega-3 deficiency may be associated with mood and behavioural disorders. In dogs, all these pathologies have been consistently reported to be associated with oxidative stress (Stoll and others 1999, Owen and others 2008, Valvassori and others 2015).

The biological effects of oxidative stress are often related to the production of free radicals, rapidly reacting with other molecules and triggering the oxidation process. Free radicals, such as peroxide ion, nitrogen monoxide and hydroxyl radical, are physiologically produced in cells and released during inflammatory processes (Pasquini and others 2010). Free radicals can also be generated by drug metabolism (Wang and others 2012), following exposure to environmental pollutants (Ademiluyi and Oboh 2013) and when fear and anxiety-related behaviours are present (Dreschel 2010). Once homeostasis is compromised, a progressive oxidation of biological substrates including lipids, DNA and proteins occurs. This is followed by the production of oxidant intermediates, such as hydroperoxides, referred to as reactive oxygen metabolites. As a consequence, this cascade mechanism progressively increases the biological damage. Several other reports also pointed out the role of other factors in the production of oxidant intermediates including physical exercise, characterised by an increase in body oxygen consumption and is associated with an increase production of reactive oxygen species to a point sometime exceeding antioxidant defence mechanisms and causing major oxidative stress (Alessio and others 2000, Watson and others 2005, Ji 2008). Exercise-related oxidative stress contributes to increase muscle fatigue and muscle fibre damages, leading to reduced performances (Baskin and others 2000, Piercy and others 2000, Moller and others 2001, Hargreaves and others 2002, Kirschvink and others 2002, Berzosa and others 2011) and decreased immune defence of the organism (Nieman 1997, Sen and Packer 2000). Several recent studies have shown an improvement of overall tissue stability and protection when the animals are fed with additional antioxidants (Cherian and others 1996, Lopez-Bote and others 1997, Castellini and others 1998, Alessio and others 2000, Watson and others 2005, Ji 2008, Sechi and others 2015).

For these reasons, the evaluation of specific oxidative stress-related factors, such as derivatives of reactive oxygen metabolites (dROMs) and biological antioxidant potential (BAP), is of interest and should be performed to monitor the welfare and health of dogs under stressful conditions (Passantino and others 2014, Sechi and others 2015).

While dROMs measure the oxidant level within the blood (Gletsu-Miller and others 2009), BAP matches the total antioxidant capability of plasma and includes either exogenous (ascorbate, tocopherols, carotenoids) or endogenous components (protein, glutathione peroxidase, superoxide dismutase, catalase) involved in the overall reactive oxygen species balance (Benzie and Strain 1996).

The objectives of the present study were, in a controlled study, to evaluate for the first time the oxidative stress and neuroendocrine parameters in dogs with behavioural problems administered a specific nutraceutical-based diet.

Materials and methods

Sixty-nine dogs (31 females and 38 males) aged 3.2±0.2 years (mean±sem) of different breeds (29 crossbreds, 9 labradors, 2 German shepherds, 3 boxers, 1 Maremma sheepdog, 3 German dachshunds, 1 bull terrier, 1 Coton de Tulear, 2 Border collie, 3 Jack Russells, 2 pinschers, 1 Cirneco of Etna, 1 Maltese, 1 Pekingese, 1 pug, 1 English setter, 1 poodle, 2 Fonni's dogs, 3 American Staffordshire terriers, 1 golden retriever, 1 grey hound) were used in a randomised controlled clinical evaluation performed at the University of Sassari, Department of Veterinary Medicine, Pathology and Veterinary Clinic Section.

The dogs were randomly assigned to the control diet (CD) group (n=34) or to the nutraceutical diet (ND) group (n=35) and fed over a period of 45 days following manufacturers' instructions (Table 1).

Operative procedures and animal care were performed in compliance with the national and international regulations (Italian regulation D.L. vo 116/1992 and EU regulation 86/609/EC). The recommendations of CONSORT 2010 statement in randomised controlled trials were also consulted and considered (Bian and Shang 2011).

The diets

Both diets fulfilled the recommendations for protein, carbohydrate and fat regarding dog daily requirements (Nutritional Guidelines for Complete and Complementary Pet Food for Cats and Dogs—The European Pet Food Industry Federation). Briefly, the nutrient composition was 24 per cent of crude protein, 12 per cent of crude oils and fats, 3.7 per cent, of crude fibre, 5 per cent of crude ash, 9 per cent of moisture and a metabolised energy of 3.477 kcal/kg (or 14.6 MJ/kg). Both diets were in the form of kibbles industrially produced and had the same amount of vitamins (A, C and E), trace elements (choline chloride, zinc sulfate monohydrate and cupric chelate glycine hydrate) and amino acids (DL-methionine) (Table 2).

The ND was also characterised by the presence of cold-pressed tablets composed by 60–80 per cent of protein hydrolysed (fish and vegetable ones), 20–40 per cent of minerals used as glidants and nutraceutical substances: *P granatum*, *V officinalis*, *R officinalis*, *Tilia* species, *C oxyacantha*, green tea extract rich in L-theanine and L-tryptophan (Table 3).

Sample collection and biochemical analysis

Cephalic vein blood samples were collected from each dog before (T0) and after 45 days (T1) of diet administration. Heparinised plasma and serum samples were centrifuged at 4000×*g* for 1.5 min at 37°C and stored at −20°C up to evaluation.

Parameters evaluated

dROMs and BAP, as indicators of oxidative stress, were measured spectrophotometrically at 505 nm (Free Radical Analytical System FRAS 4, H&D s.r.l., Langhirano PR, Italy) on serum samples (Sechi and others 2015).

Concentration of dopamine, noradrenaline, serotonin, cortisol (MyBioSource, San Diego, USA; dopamine, catalogue no.

TABLE 1: Daily amount of diet suggested by the manufacturer

Daily ratio	
Weight (kg)	Amount (g)
1–10	30–180
11–20	190–300
21–35	310–455
36–50	465–595

TABLE 2: Vitamins, essential fatty acids, trace elements and amino acids amount per kilogram of complete food in nutraceutical diet and control diet

Essential fatty acids	Amount per kilogram of complete food
Omega-6	12.5 g/kg
Omega-3	16 g/kg
Vitamins	
A	18,500 UI/kg
E	120 mg/kg
C	250 mg/kg
Trace elements	
Choline chloride	1000 mg/kg
Zinc sulfate monohydrate	137 mg/kg
Cupric chelate glycine hydrate	39 mg/kg
Amino acids	
DL-Methionine	500 mg/kg

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TABLE 3: Nutraceutical substances amount per kilogram of complete food in nutraceutical diet (ND)

Nutraceutical substances	Amount per kilogram of complete food (mg/kg)
Punica granatum	457
Valeriana officinalis	260
Rosmarinus officinalis	0.44
Tilia species	635
Crataegus oxyacantha	392
l-Theanine	310
L-Tryptophan	329

MBS494632; cortisol, catalogue no. MBS703711; noradrenaline, catalogue no. MBS739721; serotonin, catalogue no. MBS283892) and β-endorphins (antibodies-online GmbH, Aachen, Germany; catalogue no. ABIN364677) were assessed by ELISA.

Statistical analysis

Data were analysed using Prism 6 (GraphPad Software, San Diego, USA). All data are presented as the means±sem and were first checked for normality using the D'Agostino-Pearson normality test. Differences in dROMs, BAP, serotonin, dopamine, noradrenaline, cortisol and β-endorphins serum concentrations between the two diets before (T0) and at the end of the evaluation period (T1) or between groups were analysed using a two-way analysis of variance followed by Sidak's multiple comparisons test. $^{*}P<0.05$ was considered significant.

Results

In Fig 1, the neurotransmitter's serum concentrations before (T0) and at the end of the evaluation period are shown for the two groups of animals (Fig 1).

In the nutraceutical treated group (ND), serotonin, dopamine and β-endorphins concentration significantly increased from 7.67±1.01 ng/ml to 23.91±7.64 ng/ml, 1.05±0.49 to 2.35±0.76 ng/ml, 70.20±23.82 ng/ml to 317.0±124.1 ng/ml, respectively, for T0 to T1 (Fig 1a–c). In the same group, noradrenaline and cortisol significantly decreased from 2.57±0.52 ng/ml to 1.36±0.15 ng/ml, or 10.44±3.38 ng/ml to 5.86±1.95 ng/ml at T0 and T1, respectively (Fig 1d, e). No significant variations for any of the evaluated parameters were observed in the CD group.

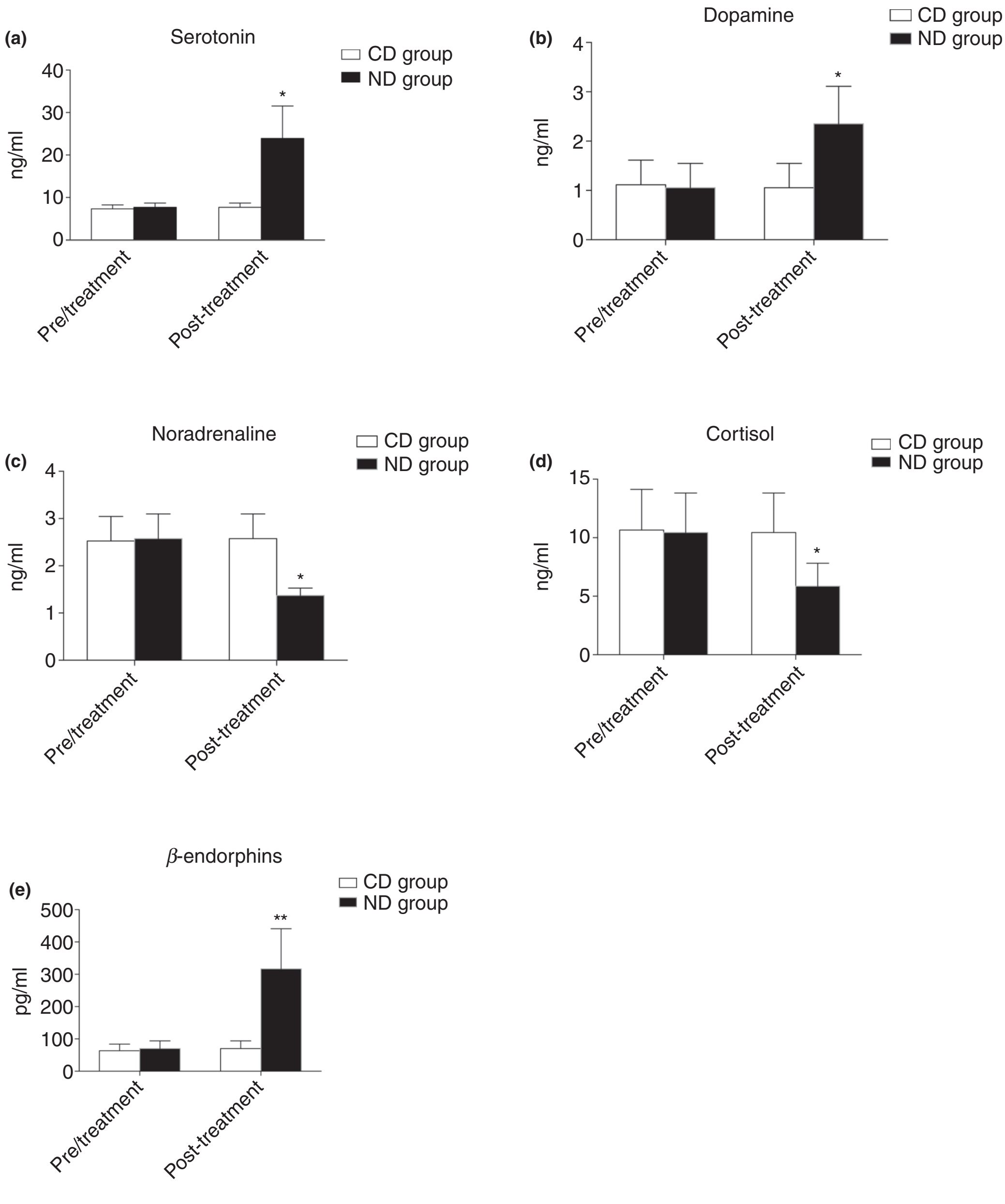

FIG 1: Graphical representation of serum neurotransmitters concentration of dogs belonging to control diet (CD) and nutraceutical diet (ND) groups before (T0) and after 45 days (T1) of diet supplementation. (a) Serotonin, (b) dopamine, (c) noradrenaline, (d) cortisol and (e) β-endorphins.

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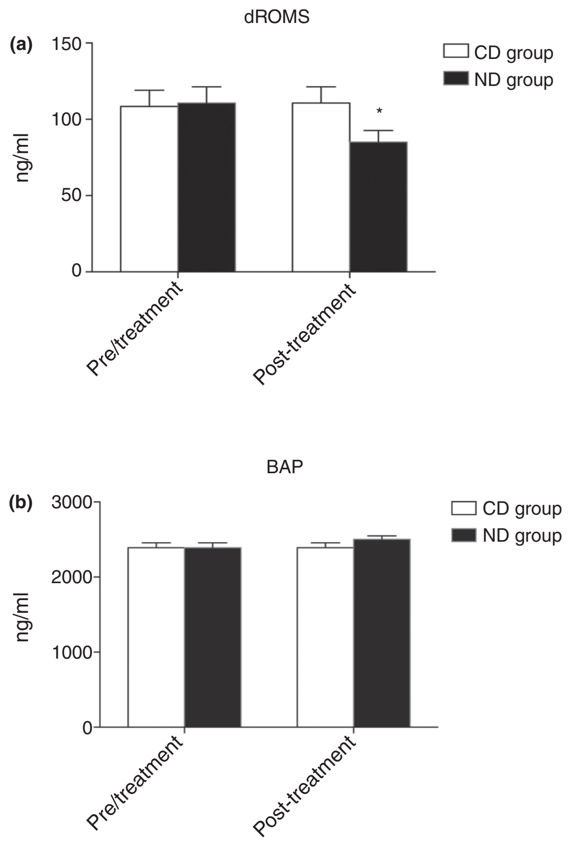

FIG 2: Graphical representation of serum oxidative stress parameters concentration of dogs. belonging to control diet (CD) and nutraceutical diet (ND) groups before (T0) and after 45 days (T1) of diet supplementation. (a) derivatives of reactive oxygen metabolites (dROMs) values and (b) biological antioxidant potential (BAP).

dROMs values presented a significant decrease from T0 to T1 (110.7±10.64 to 85.06±7.6 U. CARR) in ND group (*P<0.05) (Fig 2a).

Discussion

In this randomised, controlled evaluation assessing neuroendocrine blood parameters in dogs with behavioural disorders, the authors demonstrated the significant positive effects, obtained within a short 45-day period, of a combination of hydrolysed fish proteins, rice carbohydrates, *P granatum*, *V officinalis*, *R officinalis*, *Tilia* species, *C oxyacantha*, green tea extract and L-tryptophan and a omega-3/6 (1:0.8 ratio).

According to Bosh *et al*, normal behaviour is characterised by a stable neurotransmitter and hormone balance; however, it is easily affected by stress, anxiety or any behavioural disorders (Bosch and others 2007). Low serotonin plasma concentrations have been associated with aggressive behaviour (Reisner and others 1996, Badino and others 2004, Cakiroglu and others 2007). Similarly, impulsivity, defined as an abnormal over-reactivity to normal stimuli (Wright and others 2011), has been usually associated with reduced monoaminergic (dopamine and serotonin) circulating levels (Reisner and others 1996, Wright and others 2012). In addition, a dopamine increase was associated with satiety and reward expectation (Tobler and others 2003).

In the present work, serotonin and dopamine, used as behavioural markers, significantly increased while cortisol and norepinephrine, used as stress markers, decreased: all returning to values expected in normal animals demonstrating the positive and beneficial effects of the nutraceutical diet on overall homeostasis balance.

Different factors included in this diet may be responsible for the positive and significant observations made in the present work including L-tryptophan known to affect general mood and behaviour (Fernstrom and Wurtman 1972, Lucki 1998, Barnes and Sharp 1999, Koopmans and others 2005). Similarly, cortisol reduction is also observed after administration of L-theanine known to have beneficial clinical effects in stress and anxiety management (Miodownik and others 2011).

Diet clearly influences the overall health status in dogs like in other species (Stein and others 1994, Odore and others 2015). For instance, seasonal allergies are generally associated with the onset of skin disorders including symptoms like intense itching, dandruff or flushes and have also been linked to obvious changes in behaviour (Nuttall and others 2013). Similarly, bone meal including chicken meat and bones derived from intensive poultry farming (one of the main ingredients of dry pet food; Maine and others 2015) has been shown to induce pro-inflammatory cytokines (i.e. interferon-γ) release in vitro (Di Cerbo and others 2015, Odore and others 2015, Guidetti and others 2016). Thus, the chronic intake of contaminated food is suggested to affect overall homeostasis and possibly induce a chronic inflammatory status in healthy animals, triggering potentially behavioural changes, such as anxiety and depression (Maier and Watkins 1998), dermatological changes, such as itching and erythema (Noli and others 2015), paving the way with depressed immunity for secondary infections, that is, by *Malassezia pachydermatis* and *Candida parapsilosis* (Yurayart and others 2013).

We recently published data supporting the immune-modulatory and anti-inflammatory effect of a specific diet, which shared part of the present formula including hydrolysed fish protein, rice carbohydrates *Tilia cordata* and *P granatum*, in dogs affected by chronic otitis externa, characterised by an overall ear overgrowth of *M pachydermatis*, *Leishmania* and keratoconjunctivitis sicca, both characterised by an overall inflammatory status, respectively (Cortese and others 2015, Destefanis and others 2016, Di Cerbo and others 2016). Low concentrations of aqueous *T cordata* extract have been shown to stimulate a T lymphocyte proliferation (Anesini and others 1999), potentially neutralising the constant solicitation exerted by the food contaminants. Moreover, monoterpenes such as eugenol and geraniol have also been detected in the flowers of *T cordata* (Toker and others 2001) and, along with carvacrol, thymol and also *R officinalis*, have been demonstrated to exert antioxidant and anti-radical activities (Horvathova and others 2014).

Oxidative stress has been also suggested to contribute to the aetiology of anxiety disorders and depression becoming a consequence of either increased generation of reactive oxygen species or impaired enzymatic or non-enzymatic defence against it (Hovatta and others 2010). An overload of reactive oxygen metabolites can lead to damages of all major cellular functions and contribute to cognitive decline (Hovatta and others 2010, Sechi and others 2015).

dROMs were significantly decreased in dogs receiving the nutraceutical diet (Pasquini and others 2010). However, the antioxidant status revealed by BAP was not affected by the diet and values remained optimal throughout the whole observation period. It is speculated that the diet only affected dROMs species but not the endogenous antioxidant components, which remained in the initial optimal level.

The high and balanced content of PUFAs in the nutraceutical diet may also have played a role in the neuroendocrine changes observed in the study. Indeed, PUFA are known to exert anti-inflammatory effects (Hokari and others 2013, Attaman and others 2014) and PUFA concentrations have also been shown to modulate behaviour in aggressive dogs (Re and others 2008). Re and others in 2008 indeed observed in aggressive dogs lower docosahexaenoic acid concentrations and higher omega-6/omega-3 ratio (Re and others 2008).

Aggressive behaviour is generally seen as a major behavioural problem in dogs; however, nutrition is rarely considered as one possible contributing factor to this issue (Bosch and others 2007). In the present study, the authors demonstrated, in a randomised, control clinical study using dogs with abnormal

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behaviour, that an equilibrated nutraceutical diet was highly tolerated without any adverse effects.

In conclusion, this study demonstrated the positive effects of a nutraceutical diet on neuroendocrine parameters associated with stress, anxiety, aggression and numerous behavioural disorders. If a better understanding of dog behaviour and psychological and clinical signs associated with suffering is warranted, the authors demonstrated that the use of adapted and appropriate diet, devoid of contaminants and including specific nutraceuticals, may help ensuring a better quality of life of animals and improving behavioural disorders (Sonntag and Overall 2014). An easy and medication-free approach to behavioural issue treatment can be proposed.

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Effects in dogs with behavioural disorders of a commercial nutraceutical diet on stress and neuroendocrine parameters

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