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A possible correlation between diet, serum oxytetracycline concentration, and onset of reproductive disturbances in bitches: clinical observations and preliminary results

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Abstract: Based on the increase of food-related disturbances in animals and the recent findings concerning the toxicity of oxytetracycline (OTC) presence within pet food, we compared the effectiveness of two commercially available diets in 36 bitches suffering from reproductive disturbances. We ran a randomized clinical evaluation over a 90-day period. Bitches were randomly assigned to receive a control diet (CD) or a nutraceutical diet (ND) and were assessed for the presence of OTC in their sera before and at the end of the study. Sixteen of 18 bitches in the ND group went into heat within an average of 35.5 ± 3.1 days, and 15 of them became pregnant. In the CD group, 12 of 18 bitches went into heat within an average of 54.1 ± 3.2 days, and 10 of them became pregnant. A significant decrease in mean OTC serum concentration was observed in bitches belonging to the CD and ND group ($P < 0.01$). The overall amelioration, in particular in the ND group, provides new insights for future studies involving the modification of diet in order to restore the normal physiology of bitches suffering from reproductive disturbances.

Key words: Bitches, reproductive disturbances, control diet, nutraceutical diet, oxytetracycline

1. Introduction

In dogs and cats, food intolerances are very common and can affect the skin, ears, eyes, and gastrointestinal, nervous, urinary, respiratory, and reproductive systems [1–4]. Although skin and intestinal symptoms are the most commonly reported, the reproductive system may also be affected, with consequent decrease in male and female fertility [2,5,6].

A correlation between food consumption and reproductive performance has been long established in farm animals and pets [7–9]. Gestation and lactation are critical phases of the reproductive cycle, since additional energy requirements have a pivotal role in fetal growth and milk production [10]. Therefore, a well-balanced nutraceutical diet enriched with maca (*Lepidium meyenii*), beta-carotene, folic acid, L-carnitine, vitamin E, and zinc could become of primary importance in terms of health and reproduction in pets.

Lepidium meyenii, known as maca, is an Andean plant of the family Brassicaceae containing several active substances including macaridine, macamides, macaene, glucosinolates, and alkaloids [11]. It has been used for

centuries to ameliorate sexual functions in men and animal models as well as for behavioral and estradiol-17 beta, progesterone, and testosterone serum concentration improvement [12,13]. For example, in rats, maca can enhance the serum concentration of luteinizing hormone during proestrus acting in a dose-dependent manner [14].

In mammals, several vitamins play a critical role in favoring reproductive functions. In particular, retinoids seem to improve reproductive performance by exerting an antioxidant effect acting on the pituitary gland. In cycling goats, dietary supplementation with beta-carotene improved the follicular development and ovulation rate [15]. Moreover, in bitches an addition of vitamins during the estrus cycle could enhance the plasma progesterone levels between 12 and 26 days from the beginning of the administration [16]. Vitamin E can also positively affect the reproductive system in males and bitches [17]. Vitamin E also showed a protective effect by reducing stillbirth and perinatal death in dairy cows [18]. Furthermore, in mice, supplementation with vitamin E significantly decreased nicotine-induced oocyte apoptosis [19]. It is worth noting that folic acid, L-carnitine, and zinc are often associated

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with other substances in order to achieve a synergic effect on infertility reduction in humans and animals [20–23]. The systemic administration of a combination of vitamin E and folic acid counteracted oxidative damage and apoptosis in the uterine cells of a streptozotocin-induced rat model [24], while several studies revealed the protective effect of folic acid on teratogenesis [25–27]. L-Carnitine seemed to be effective in preventing meiotic oocyte damage, significantly reducing reactive species of oxygen and apoptosis in porcine oocytes [28]. Finally, dietary supplementation with omega-3 fatty acid improved reproductive outcomes by significantly increasing the number of embryos and reducing the incidence of stillbirth [29]. This was supported by evidence that omega-3 fatty acid from fish oil improved folliculogenesis in dairy cattle [30].

The physiological canine estrus cycle is characterized by four recurring phases: proestrus, estrus, diestrus, and anestrus, with an average interestrus of 6–7 months ranging of 5 to 12 months depending on breed and environment. Dog breed significantly influences the first heat onset. It is therefore intriguing that small breeds can reach puberty between 6 and 10 months of age, while large breeds usually do so between 18 and 24 months. Bitches usually show an ovarian cycle throughout the year, with subtle peaks during the late winter and early spring [31]. Perturbations of the estrus cycle and especially the interestrus interval are quite common. This is defined as prolonged or secondary anestrus when the period existing between an estrual expression and the following one exceeds 18 months, while primary estrus is defined as the absence of a detectable heat cycle over 24 months of age. In clinical practice it is fundamental to distinguish primary anestrus and silent heat cycles. Failed or inadequate estrus detection is, in fact, one of the most frequent causes of infertility. In the case of silent heat cycles, typical evidence of estrus (swelling, vulvar discharge, behavioral modification) is rather limited or absent. Clinical diagnosis of two different anestrus periods requires time and specific analysis, e.g., hormone dosage, which owners often decided not to evaluate. Infertility in bitches is defined as the absence of pregnancy after mating or artificial insemination. Real infertility is difficult to diagnose in veterinary clinical practice and therefore most bitches are classified as generally subfertile or hypofertile subjects [32]. The present clinical evaluation was based on standardized protocols used in canine reproduction and its aim was to evaluate the effect of a specific nutraceutical diet on the reproductive performance of 36 bitches presenting evident reproductive disturbances and possibly correlate them with serum concentration of a contaminant, oxytetracycline (OTC), frequently detected in pet food [33–36]. We previously observed the usefulness

of a nutraceutical diet enriched with *Lepidium meyenii*, *Tribulus terrestris*, L-carnitine, zinc, beta-carotene, vitamin E, folic acid, and an omega-6:omega-3 ratio of 4:1 in improving motility percentage, semen volume and concentration, and total number of sperm per ejaculation in 28 male dogs suffering from infertility associated with hypospermia [37]. Moreover, literature reports evidenced an increased excretion of conjugated estrogens in the feces of men and women treated with several antibiotics, including OTC, thus reducing the hormone concentrations in circulation. Therefore, besides a decreased hydrolysis of estrogen conjugates by β -glucuronidase, we hypothesized that the alteration of reproductive functions of bitches might also be due to a state of chronic inflammation caused by the presence of harmful substances and contaminants (including OTC) within the body, introduced by routine commercial pet food consumption [33–35,38].

2. Materials and methods

We designed and performed a randomized clinical evaluation of 36 bitches using two commercially available diets based on a standardized mixture of fish and rice that differed in the presence of nutraceutical substances. The nutraceutical diet (ND) contained maca (*Lepidium meyenii*), beta-carotene, folic acid, L-carnitine, vitamin E, zinc, and an omega-6:omega-3 ratio of 4:1, while the control diet (CD) contained only zinc, vitamin E, and an omega-6:omega-3 ratio of 1:1.

Thirty-six client-owned bitches of different breeds (3 Great Danes, 6 Cavalier King Charles Spaniels, 4 Bernese mountain dogs, 5 Bordeaux mastiffs, 3 Boxers, 4 Australian shepherd dogs, 7 Golden retrievers, and 4 German shepherd dogs) suffering from evident reproductive disturbances including irregular cycles with prolonged anestrus, primary anestrus, irregular or silent estrus, and small litter size were randomly and equally assigned to receive either the ND or CD over a 90-day period according to the manufacturer's indications reported in the Table.

The ND group had a mean age \pm standard error of the mean (SEM) of 3.81 ± 0.2 years and a mean weight \pm SEM

Table. Daily rations of ND and CD recommended by manufacturers.

Body weight (kg)	Diet amount per day (g)
1–10	30–180
11–20	190–300
21–35	310–460
36–50	470–600
51–70	610–770

of 37.6 ± 5.3 kg. The CD group had a mean age \pm SEM of 3.72 ± 0.3 years and mean weight \pm SEM of 35.0 ± 3.4 kg.

According to the seasonality of the canine ovarian cycle, most of the animals evaluated were recruited during wintertime in order to begin the administration of the diets 30 days before the estimated heat date.

2.1. The diets

Both diets were commercially available in the form of kibbles and therefore completely fulfilled the recommendations reported in the Nutritional Guidelines of the European Pet Food Industry Federation.¹ The analytical constituents of both diets were crude protein 26%, crude fats 12.5%, moisture 8%, crude ash 7.3%, crude fibers 2.6%, zinc, vitamins, and an omega-6:omega-3 ratio of 4:1 for ND and 1:1 for CD, with metabolized energy of 3498 kcal/kg.¹ The ND was further enriched with maca (*Lepidium meyenii*) root extract (0.0865%), L-carnitine (0.035%), beta-carotene (0.0020%), and folic acid (0.00017%), embedded at a percentage of 20%–40% in cold pressed, heart-shaped tablets composed of 60%–80% hydrolyzed proteins (fish and vegetable). The metabolic energy requirement was calculated using the following formula: $110 \text{ kcal ME} \times \text{kg BW}^{0.75}$ (BW = body weight).

2.2. Clinical evaluation

All subjects underwent a thorough check-up with anamnestic data, particularly reproductive data, collected from the owner. When reproductive problems were reported, deep samples were taken by the veterinarian with a sleeved swab to test for vaginal mycoplasma and urea plasma. Four out of 54 subjects tested positive with a pathogen load of $>10,000$ colony forming units/mL and were given antibiotic therapy specific for the identified pathogen.

Exclusion criteria were the presence of correlated systemic diseases (hypothyroidism, hyperadrenocorticism), physical and genetic anomalies included intersex and hermaphroditism, and presence of ovary cysts determined by means of hormone dosage and abdominal echography. Inclusion criteria were the presence of symptoms only ascribable to reproduction disturbances after ruling out any concomitant infectious pathologies as a secondary cause.

The subjects included in the evaluation started the diet after the initial examination and after completion of any antibiotic treatment. Data on time to reach estrus after the beginning of the diet, mating, pregnancy, and number of puppies born were recorded from the beginning of the evaluation.

Estrus cycles were cytologically monitored and progesterone (P4) dosage was evaluated every 2 days ¹<http://www.fedialf.org/>

after the beginning of swelling [39]. Subjects were mated when vaginal cytology evidenced an amount of superficial keratinized cells of $>70\%$ and a serum P4 level of >10 ng/mL. Subjects that reached estrus were mated or not as per the owner's decision.

A score of 0 was assigned to bitches not undergoing heat and/or not becoming pregnant, while a score of 1 was assigned to bitches undergoing heat and/or becoming pregnant.

This clinical evaluation was performed in compliance with national Italian and international regulations (Italian Regulation D.L.vo 116/1992 and European Union Regulation 86/609/EC) for procedures and animal care [40]. Moreover, recommendations of the CONSORT 2010 statement pertaining to randomized controlled trials were consulted [41]. Informed owner consent was obtained for each dog.

2.3. OTC ELISA assay

Before and at the end of the 90-day period of diet supplementation, 4 mL of blood was drawn from each bitch and analyzed by means of an OTC-specific ELISA kit for pets (Cat. # DE-100430, Genemed Synthesis, Inc., San Antonio, USA).

2.4. Statistical analysis

Data were analyzed using GraphPad Prism 7 software (GraphPad Software, Inc., La Jolla, CA, USA). All data are presented as mean \pm SEM and were first checked for normality using the D'Agostino–Pearson normality test. Differences in serum OTC concentration, pregnancy, and heat rate before and at the end of the evaluation period were analyzed using two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons test. $P < 0.05$ was considered significant.

3. Results

Bitches enrolled in the evaluation suffered from different reproductive disturbances. In the ND group, 2 subjects suffered from primary anestrus, 8 from prolonged anestrus, 3 from irregular or silent heat, 3 from subfertility, and 2 from infertility. In the CD group, 2 suffered from primary anestrus, 7 from prolonged anestrus, 4 from irregular or silent heat, 2 from subfertility, and 3 from infertility.

In the ND group, 16 of 18 bitches (88.8%) went into heat between 10 and 64 days after the beginning of the diet ($P < 0.05$), with an average of 35.5 ± 3.1 days, while only 2 out of 18 (11.2%) bitches did not go into heat. All of 16 bitches were mated and 15 became pregnant (83.3%, $P < 0.05$) (Figure 1), with litters ranging from 5 to 12 puppies and an average of 7.8 puppies per mother (data not shown).

In the CD group, 12 of 18 bitches (66.7%) went into heat in 40 to 80 days after the beginning of the diet ($P <$

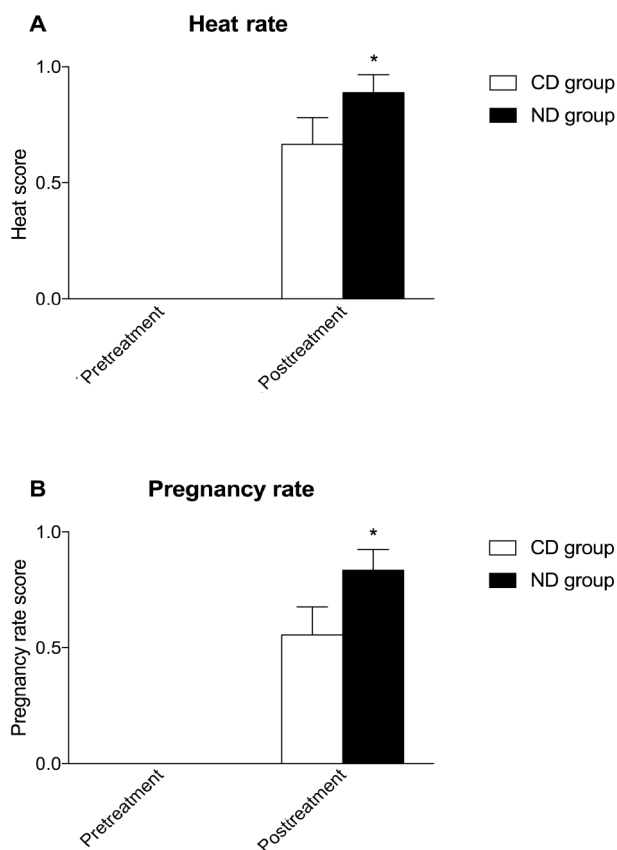


Figure 1. Schematic representation of dogs belonging to CD and ND groups that (A) went into heat and (B) became pregnant after diet supplementation. *: $P < 0.05$.

0.05), with an average of 54.1 ± 3.2 days, while 6 of 18 bitches (33.3%) did not go into heat. All 12 bitches were mated and 10 (55.5%) became pregnant ($P < 0.05$), with litters ranging from 4 to 7 puppies and an average of 5.4 puppies per mother.

In order to possibly correlate the presence of OTC with the onset of reproductive disturbances we compared the sera of bitches from the CD and ND groups with a pool of 6 bitches without any kind of reproductive disturbance (untreated dogs). As shown in Figure 2, a significant decrease in mean OTC serum concentration was observed in bitches belonging to the CD and ND groups with respect to untreated dogs, from an initial value of 105.5 ± 1.7 ng/mL to 97.5 ± 1.6 ng/mL and from 108.3 ± 1.9 ng/mL to 88.7 ± 2.9 ng/mL, respectively ($P < 0.001$), after 90 days of diet supplementation.

Interestingly, similar OTC concentrations were also found in the sperm fractions of 9 male dogs of different

breeds (mean age \pm SEM: 6.22 ± 0.8 years; mean weight \pm SEM: 33.01 ± 1.27 kg) brought to the Veterinary Hospital of the University of Sassari for the first time for infertility issues (Figure 3).

4. Discussion

We found that ND, a diet based on nutraceutical substances such as maca (*Lepidium meyenii*), beta-carotene, folic acid, L-carnitine, vitamin E, and zinc, markedly improved the reproductive performance of bitches with evident reproductive disturbances. However, similar but less marked results were also achieved with the CD diet, where the only nutraceutical substances were vitamin E and zinc. A similar trend was also observed for OTC serum concentration. We hypothesized that the alteration of reproductive functions, both in males and bitches, may be due to a state of chronic inflammation caused by the presence of harmful substances and contaminants

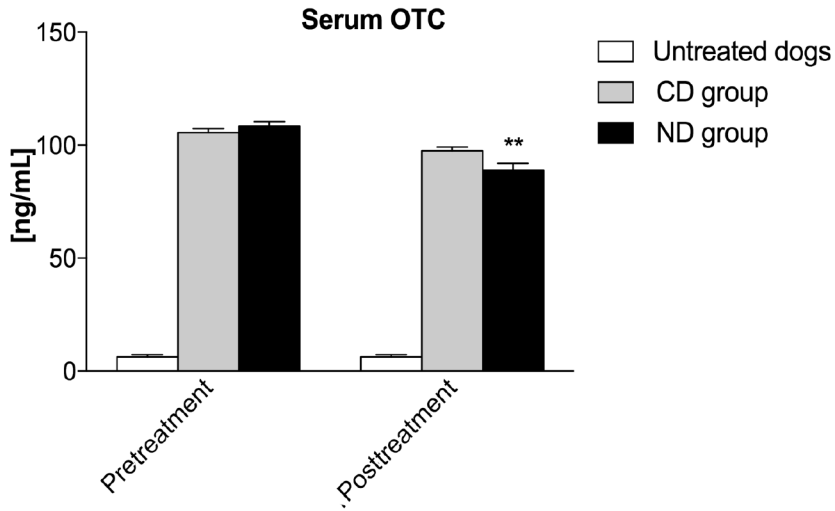


Figure 2. Schematic representation of mean OTC serum concentration of bitches belonging to CD and ND groups before and after 90 days of diets supplementation, and bitches without reproductive disturbances (untreated dogs). **: $P < 0.01$.

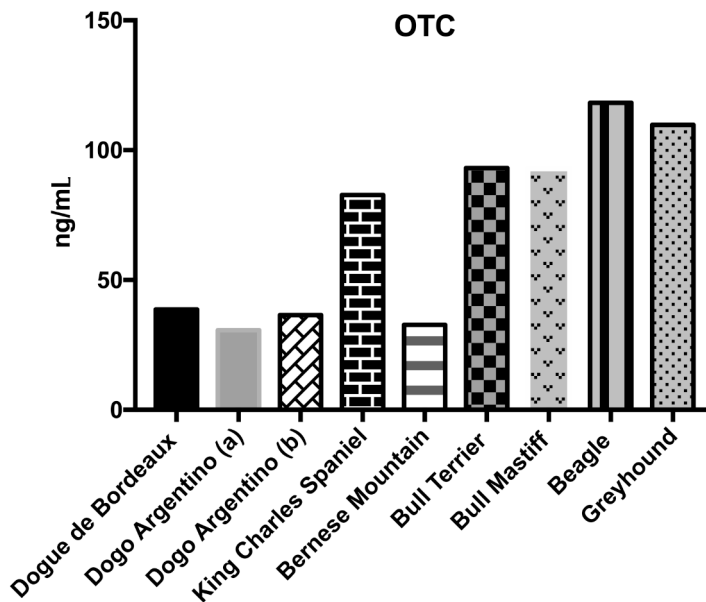


Figure 3. Schematic representation of OTC concentrations within spermatic fractions of male dogs suffering from infertility issues.

(including OTC) within the body, which can be introduced by ordinary commercial pet food consumption [33–35,37]. Moreover, several studies showed the effects of antibiotics (tetracyclines, sulfa drugs, penicillins, aminoglycosides,

and nitrofurans) and also heavy metals (cadmium, lead, and mercury) on the reproductive system, inducing estrous cycle disruption, impaired embryo implantation, impendent follicular development, breast and endometrial

cancer, endometriosis, spontaneous abortions, sperm motility, and viability reduction [41–46]. It is also worth noting that previous in vitro evaluations highlighted the cytotoxic, proapoptotic, and proinflammatory effect of chicken bone treated with OTC according to appropriate withdrawal times [37,47,48] as well as the ability to induce DNA damages (activation of ATM and p53, phosphorylation of H2AX, and modifications of histone H3 methylation of lysine K4 in the chromatin) of the liquid form of OTC [49].

Therefore, it is reasonable to speculate that the overall inflammatory state could interfere with normal physiological mechanisms at different levels, altering hormonal axes and/or causing anatomical lesions, and that a well-balanced diet, free of contaminants, could relieve the chronic inflammation in the medium-long term, thus explaining why some of the bitches went into estrus more than a month after starting the diet.

This provides a plausible explanation for the data resulting from the present evaluation. We also hypothesize that the marked difference in OTC serum concentrations between the two diets might be due to the presence of the nutraceutical substances in the ND, stimulating bone metabolism and consequently OTC release from skeletal bones. We have already demonstrated the in vitro antiinflammatory activity of some nutraceutical substances present within a commercially available diet [50–52]. In particular, a significant reduction in interferon- γ production by human and canine lymphocytes incubated for 10–12 h with liquid OTC was observed [50]. In addition, a clinical study clearly correlated the consumption of maca with a decrease in interleukin-6, an inflammatory marker of the onset of negative health-related events [53,54]. Moreover, methanol maca leaf extract reduced

lactate dehydrogenase activity and increased superoxide dismutase in PC12 cells exposed to 6-hydroxydopamine [55]. Conversely, β -carotene supplementation reduced interleukin-8 production along with normalization of light ferritin, ferroportin, and transferrin in Caco-2 cells challenged with interleukin-1 β (IL-1 β) to mimic the iron-induced inflammation that characterizes anemia [56]. IL-1 β and tumor necrosis factor- α were conversely significantly reduced by folic acid in human promyelomonocytic cells under hypoxic conditions [57]. Folic acid also decreased the concentrations of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and C-reactive protein, indicators of inflammation, in patients with primary arterial hypertension [58]. C-reactive protein and serum amyloid A were also significantly reduced in hemodialysis patients supplemented with L-carnitine [59].

In conclusion, our data support the hypothesis that the ND was able to restore the physiological state of the subjects suffering from reproductive disturbances. The ND showed a pronounced stimulatory effect on the reproductive cycles of bitches with respect to the CD. This product is therefore not only intended to improve the clinical condition of animals suffering from reproductive disturbances but also to enhance their state of health and wellbeing.

Conflict of interest statement

This research was performed in collaboration with a scientist from the Department of Research and Development, SANYpet S.p.A., Padua, Italy, as indicated in the author affiliations according to the scientific and ethical principles of the scientific community. No financial funding was obtained from SANYpet for this research study other than the provision of the ND and CD. SANYpet did not have any involvement in the study design, data analysis and interpretation, or writing of the manuscript.

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