Smith M, Ma A. Effect of tofu supplementation on endogenous estrogen levels and urinary incontinence in female spayed dogs: A randomized, controlled clinical study. Am J Trad Chin Vet Med 2023; 18(2):17-24. DOI: 10.59565/001c.84479

Clinical Studies

Effect of Tofu Supplementation on Endogenous Estrogen Levels and Urinary Incontinence in Female Spayed Dogs: A Randomized, Controlled Clinical Study

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ABSTRACT

The objective of this randomized controlled study was to determine whether supplementing a spayed female dog's diet with tofu can raise levels of endogenous estrogen (EE), which could be beneficial for estrogen-responsive incontinence. A total of 23 client-owned spayed female dogs diagnosed with urinary incontinence but no other known health conditions participated in the study. Enrolled subjects were randomly assigned to 2 groups: Control Group (n=10) who received normal diet with a placebo supplement or Test Group (n=13) who received tofu supplementation. Supplements were given twice daily for 14 days. Blood samples were collected on Day 0 and Day 14 to assess pre-treatment and post-treatment EE levels. Effects of tofu supplementation were assessed based on group comparison of EE level improvement. The EE level within the Control Group had a nonsignificant decrease from days 0 to 14 (56.9±22.8 → 54.6±25.3 pg/mL, p=0.54). Test Group EE level significantly increased after 14 days (37.1±17.2 → 58.5±28.9 pg/mL, p=0.013). For intergroup comparison, the change of EE levels in the Test Group was significantly greater than the Control Group (Control: -2.3±11.5 versus Test: 21.4±27.5 pg/mL, p=0.0002). The study also found that Test Group owner impression of urinary incontinence improvement (11/13) was significantly greater (p=0.0001) than that in the Control Group (0/10). The results of this study suggest the potential for developing a holistic, natural food therapy for urinary incontinence in spayed female dogs. Future prospective studies, with larger enrollment, that also consider factors such as treatment dose and duration are warranted.

Keywords: endogenous estrogen, food therapy, spayed dog, tofu, urinary incontinence

ABBREVIATIONS: ACTH: adrenocorticotropic hormone; BCS: body condition score; DES: diethylstilbestrol; EE: endogenous estrogen; NoUD: number of observed urine dribbling events per day; PPA: phenylpropanolamine; PUSMI: primary urinary sphincter mechanism incontinence; TCM: traditional Chinese medicine

Perhaps the most common clinical problems associated with gonadectomized (i.e. spayed) female dogs are urinary incontinence, dribbling at rest and cystitis.[1] Primary urinary sphincter mechanism incontinence (PUSMI), previously called hormone responsive or estrogen responsive incontinence, is primarily observed in spayed adult female dogs and occurs when lying down as opposed to walking or standing. Previous studies report 3.5-15% of spayed female dogs suffer from urinary incontinence, while Okkens et al. reports this prevalence to be as high as 1 in every 5.[2-4] Unfortunately, because treatment is lifelong and the condition worsens with age, many unmanaged clinical cases ultimately end in euthanasia, resulting in frustration and grief for both the attending veterinarian and the dog owner.

Multiple etiologies, although not entirely agreed upon, are presumed to include: conformational abnormalities in the large breed dog (urethral length and pelvic bladder position), obesity, adhesion development between the bladder and vagina/cervix after ovariohysterectomy, and the development of urethral sphincter incompetence due to estrogen deficiency following removal of the ovaries.[1] Still others propose the incontinence results from a combination of neurological, vascular and hormonal changes that collectively follow ovariohysterectomy.[1]

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Current treatment options for PUSMI consist of medical therapy with alpha adrenergic agents such as phenylpropanolamine, estrogen supplementation, urethral bulking agents, collagen injection, botulinum toxin injection and surgery in select mechanical cases.[5] Phenylpropanolamine (PPA)[a] is approved for use in dogs for treatment of PUSMI, and is thought to exert its effects by stimulating alpha-adrenergic receptors along the urethra, bladder neck and urethral sphincter.[6] The use of PPA, however, has potential side effects which can be quite serious (e.g. emesis, hypertension, weight loss proteinuria). As a result of these potential side effects in humans and pets, PPA is no longer available without a prescription for animals; and due to the increased risk of hemorrhagic stroke is not available for human use in the United States.[6-7] It has also been withdrawn from the market in a number of other countries.[8]

Estrogen supplementation represents the next most common treatment option; however, the side effects are significant and include induction of estrous, mammary gland enlargement and the formation of mammary tumors. Estriol and diethylstilbestrol are the most commonly prescribed estrogen supplements. Diethylstilbestrol (DES), which has longer-acting receptor binding affinity, has a well-documented history of bone marrow toxicity.[5] Estriol (Incurin®), on the other hand, is a naturally occurring, short-acting estrogen licensed for use in spayed female dogs with incontinence. The shorter acting receptor binding affinity of estriol does not exert the estrogenic effects of other longer acting estrogens such as DES.[5] In one study involving the use of estriol in 129 dogs with a history of acquired urinary incontinence, 83% of the dogs either became continent (61%) or had improved (22%) after only 42 days of treatment according to veterinarians' assessments. Of equal importance and in the same study, only 63% of owners classified the results of treatment as "good to excellent".[9]

With pet owners' increased interest in functional foods and more natural treatments for themselves; tofu, a staple for centuries in traditional Chinese medicine, may serve as a food therapy solution to the clinical problem of PUSMI in spayed female dogs.[10-11] Thought to first originate in the Han Dynasty some 2,000 years ago, tofu is made from soybeans. Technically speaking, soybeans as a member of the legume family, are not a grain. Due to the impact soybeans had on the development of Chinese culture, however, the Chinese consider the soybean one of the Five Sacred Grains, along with rice, wheat, barley and millet.[12-14] Soy is unique in that it contains high levels of isoflavones (genistein in particular), a type of plant estrogen (phytoestrogen) which is structurally similar to human estrogen. These naturally occurring plant chemicals exert and mimic the effects of estrogen in both humans and animals and have been extensively studied in humans.[15-21] Multiple epidemiological studies, comparing Asian and American dietary habits, confirm that Asian populations that consume more soy have a lower risk of breast and prostate cancer.[22]

Researchers have examined 24 commercially available pet foods for phytoestrogen content and found that products formulated with soybeans or soybean fractions, such as soybean meal, contained phytoestrogens in amounts comparable to those that have biological effects in other species, specifically humans.[23] There was also a report evaluating the effect of a soy-based diet on adrenocortical and thyroid gland function in adult dogs.[24] In the study, dogs were fed one of two diets, either a "high isoflavone diet" or a "low isoflavone diet", and estrogen levels were measured after adrenocorticotropic hormone (ACTH) stimulation at 0, 6, and 12 months. Significant differences in estrogen values existed between the two groups after ACTH stimulation testing at 1 year. According to the authors, findings from these studies suggest that phytoestrogens may affect endocrine function in dogs and can be associated with increases in serum concentrations of endogenous estrogens.[24]

Whether these findings are valid for tofu remains to be elucidated. The objective of this study was to investigate whether tofu supplementation would raise endogenous estrogen levels in spayed incontinent female dogs and recapitulate study findings described by Cerundolo.[24] This study hypothesized that supplementing the diet of a spayed female dog with phytoestrogen-containing tofu can increase endogenous estrogen levels.

MATERIALS AND METHODS

Animals

The study subjects consisted of spayed female canine patients diagnosed with urinary incontinence but otherwise with no known health conditions. Subjects were recruited from Natchez Trace Veterinary Services (Nashville, Tennessee, USA) with the following inclusion criteria: 1) at least 2 years of age and at least 1 year since ovariohysterectomy; 2) no apparent health issues except for urinary dribbling; and 3) a body condition score (BCS) between 3-6, according to Nestle Purina BCS system.[25] Giant breed dogs (e.g. Mastiff, Great Dane) were excluded as these breeds might have other subtle, subclinical orthopedic conditions and greater dribbling volume of larger dogs. After diagnosing urinary incontinence, the investigator asked dog caretakers if they would be willing to participate in a short clinical study on the effects of tofu added to the diet. Verbal consent was obtained prior to study enrollment.

Materials

Subjects that qualified for the study were randomly assigned to the Control Group or Test Group. The randomization was performed by flipping a coin (heads: Control Group; tails: Test Group) until each group reached its desired sample size. Subjects in the Control Group received their normal diet with placebo supplementation, whereas those in the Test Group received tofu supplementation during the trial period.

Tofu cubes[b] [firm variety, 2 ounces (oz) each] were procured from a grocery store, repackaged, and then frozen. Listed ingredients for the test subject tofu included water, soybeans, calcium sulfate and magnesium chloride. Two frozen cubes were included in each daily package dispensed to the owners (total of 14 packs per dog). They were instructed to keep the tofu frozen and give 1 cube top dressed on the dog's food twice daily. The placebo supplementation for the Control Group consisted of a frozen beef meatball (70% lean/30% fat ground beef). The meatballs were procured from a grocery store, packaged and then frozen. Two meatballs (2 oz each) were included in each package and dispensed to the owners with instructions to keep the meatballs frozen. They were instructed to give 1 meatball top dressed on the dog's food twice daily.

Treatment Procedure

After group assignment and before the trial period, each dog's body weight was recorded. Owners were counseled on the importance of offering only the prescribed diet twice daily and no other additional dietary items or prevention medicines (including heartworm and/or flea prevention). All subjects had their diet supplemented (with placebo or tofu), twice daily for 14 days, during their regular feeding times.

The biological effects of phytoestrogens on dogs are currently unknown, therefore, the amount of tofu supplement given to the Test Group subjects was derived from human studies. The threshold intake of phytoestrogens necessary to achieve biological effects in humans is reported to be 30-50 mg/day, which is equal to 0.4-0.7 mg/kg/d for a 70-kg human.[10] Tofu supplementation with an amount greater than this threshold presumably would achieve biological effects. Given that the average weight by breed for female dogs rarely exceeds 30-kg (excluding giant breed dogs), an effective threshold for this study, based on human data, would be 21 mg/day (30kg x 0.7mg/kg). To be above this threshold for canines, subjects were given 4 oz of uncooked tofu per day. This daily quantity would contain an average of 28 mg of phytoestrogens.[26] Notably, the amount of tofu given to each subject was independent of each dog's weight as this amount of supplementation should be greater than the threshold, even for the larger subjects. The amount of the placebo supplementation for Control Group subjects was equal to the tofu supplementation in the Test Group.

For each subject, blood samples were collected on Day 0 to obtain pre-treatment endogenous estrogen levels. On Day 14 when the subject returned to the clinic, a second blood sample was collected to obtain the post-treatment endogenous estrogen levels in the blood. All subject blood samples were collected before 12 PM (central standard time, USA) on Day 0 and again on Day 14. They were collected in a plain tube, allowed to clot, centrifuged to obtain a minimum of 0.3ml of serum and shipped in a plain red top tube via overnight delivery on ice. All blood samples were submitted to a veterinary endocrinology lab[c] for processing. Assays for estradiol were run once weekly by the reference laboratory[c] on submitted samples. Lab technicians responsible for measuring blood-estrogen levels were blinded to the study design and group assignments of dogs.

Outcome Measurements

Endogenous estrogen (EE) levels were measured for each subject on Day 0 (pre-treatment) and Day 14 (post-treatment) blood samples. Day 0 samples were taken to establish a baseline blood-estrogen level for each subject. The change of EE level from pre-treatment to post-treatment was calculated for each subject as the primary endpoint for testing the study hypothesis.

In addition to EE level, the study also collected the subject's urinary behavior data. This was based on owner observation, with an owner blinded to their dog's treatment group (Appendix). The owners also verbally reported to the primary investigator (MS) both volume and frequency of urine dribbling events on Day 0 and then again on Day 14. This was recorded as the daily number of observed events of urine dribbling (NoUD) on pretreatment Day 0 and on post-treatment Day 14. The change of NoUD from Day 0 to Day 14 was compared between the two groups.

Statistical Analysis

Based on the data of EE levels, the study tested the null hypothesis, H_0: the change in the Test Group population equals that in the Control Group population vs. H_A: the change in the Test Group population is greater than that in the Control Group population. To evaluate NoUD the study tested the null hypothesis, H_0: the NoUD reduction in the Test Group population is equal to that in the Control Group population vs. H_A: the NoUD reduction in the Test Group population is greater than that in the Control Group population. One-sided nonparametric (distribution-free) Wilcoxon tests (signed rank test for within-group analysis; rank sum test for between group comparisons) were employed to test these hypotheses. Significance level 0.05 was used for all tests.

The study planned to recruit 13 subjects in each of the control and tofu-supplementation groups (total n=26). With this sample size, the one-sided Wilcoxon signed rank test, based on asymptotic relative efficiency to paired t-test, would have a 95% power to reject the null hypothesis with a 0.05 significance level when a mean increase was at least as large as the standard deviation among subjects. For the rank sum test, the study could achieve approximately 78% power to reject the null hypothesis with a 0.05 significance level when the group difference was at least as large as the standard deviation among subjects. All data graphic presentations and statistical analysis were performed using commercial statistical software[d].

RESULTS

Within the study timeline, a total of 23 incontinent, spayed female dogs were enrolled in the trial. The randomization scheme resulted in 10 subjects assigned to the Control Group and the other 13 subjects to the Test Group. The majority of dog breeds in the Control Group were mixed breeds (6/10), with the remaining 4 being Shih Tzu, Pug, Boston Terrier, and German Shepherd. Similarly, most of the subjects in the Test Group were mixed breeds (8/13) with the remaining breeds including Labrador Retriever, Bulldog, Golden Retriever, Collie, and Poodle (Tables 1 and 2). Significant group differences were not found in age (p=0.914), weight (p=0.250) and BCS (p=0.333). There were no test article-associated adverse events; and all subjects completed the trial.

Blood samples for measuring EE were collected on Day 0 (Pre-treatment) and Day 14 (Post-treatment). In the Control Group, the mean±SD estrogen level on Day 0 was 56.9±22.8 pg/mL and was 54.6±25.3 pg/mL on Day 14 (Table 1, Figure 1). Half (5/10) of the control subjects had reduced EE levels while the other half were increased. The mean±SD change over subjects was – 2.3±11.5 pg/mL. This slight decrease (mean ≈ 5%) at the end of the trial compared to Day 0 was not statistically significant (p=0.695).

In the Test Group, the mean±SD estrogen level on Day 0 was 37.1±17.2 pg/mL and was 58.5±28.9 pg/mL on Day 14 (Table 2, Figure 2). All but 1 subject (12/13=92.3%) had increased EE levels. The mean±SD change was 21.4±27.5 pg/mL, which was statistically significant (p=0.013), with an average of 76% increase at the end of the trial compared to Day 0. Comparison between the 2 groups revealed the change of estrogen levels in the Test Group was significantly greater than that in the Control Group (Control: 2.3±11.5 pg/mL versus Test: 21.4±27.5 pg/mL, p=0.0002).

Table 1: Control Group signalment with pre-treatment and post-treatment estrogen levels, owner questionnaire response to urine dribbling improvement, and documentation of the number of urinary dribbling episodes (NoUD) on Day 0 and Day 14

Subject	Age	Weight* (BCS)	Breed	Pre-Tm Estrogen	Post-Tm Estrogen	EE Level Difference (% change)	Appendix Dribble Improved	NoUD Day 0 (Day 14)
1	7	31 (4)	Mix	43.1	39.1	-4 (-9%)	Never	4 (4)
2	4	19 (5)	Shih Tzu	56.1	66.5	10.4 (19%)	Never	2 (2)
3	7	33 (4)	Mix	40	34.7	-5.3 (-13%)	Never	3 (3)
4	9	47 (5)	Mix	30.9	23.4	-7.5 (-24%)	Never	4 (4)
5	11	17 (6)	Pug	40.3	27.9	-12.4 (-31%)	Never	2 (2)
6	6	4 (3)	Mix	77.8	90.1	12.3 (16%)	Never	2 (2)
7	6	37 (4)	Mix	93.6	93.7	0.1 (0%)	Never	1 (1)
8	9	14 (5)	Boston Terrier	67.8	70.9	3.1 (5%)	Never	2 (2)
9	11	67 (4)	German Shepherd	85.4	59.3	-26.1 (-31%)	Never	1 (1)
10	12	51 (4)	Mix	34	40.3	6.3 (19%)	Never	3 (3)
Mean±SD	**8.20±2.62**	**35.7±16.7 (4.40±0.84)**	—	**56.9±22.8**	**54.6±25.3**	**-2.3±11.5 (-4.9±19.8%)**	—	**2.4±1.1 (2.4±1.1)**

*=pounds; BCS=body condition score; EE=endogenous estrogen; NoUD=number of urinary accidents; Tm=treatment

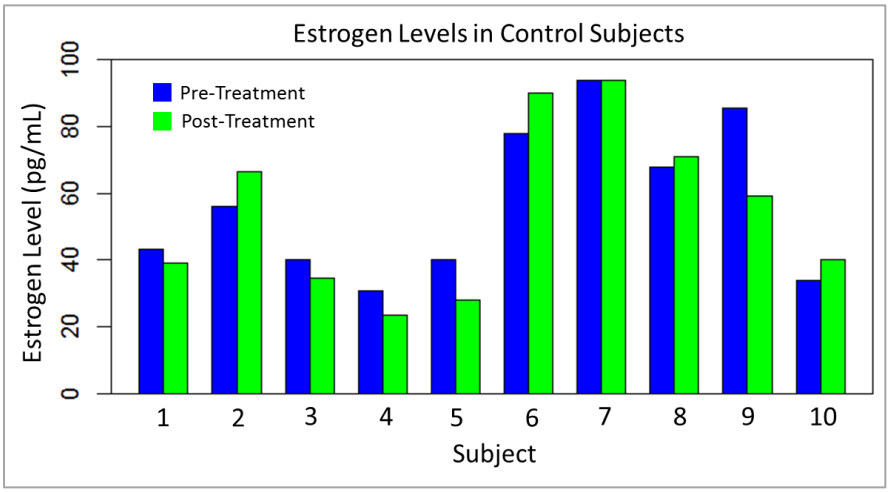

Figure 1: Pre-treatment and post-treatment estrogen levels in Control Group subjects

Table 2: Test Group signalment with pre-treatment and post-treatment estrogen levels, owner questionnaire response to urine dribbling improvement, and documentation of the number of urinary dribbling episodes (NoUD) on Day 0 and Day 14

Subject	Age	Weight* (BCS)	Breed	Pre-Tm Estrogen	Post-Tm Estrogen	EE Difference (% Change)	Appendix Dribble Improved	NoUD Day 0 (Day 14)
11	5	61 (4)	Mix	15.5	46.8	31.3 (202%)	Never	4 (4)
12	8	75 (3)	Lab	17	41	24 (141%)	Never	3 (3)
13	6	59 (4)	Bulldog	32.2	47.8	15.6 (48%)	Sometimes	3 (3)
14	11	28 (5)	Mix	39.1	71.2	32.1 (82%)	Seldom	4 (2)
15	12	42 (4)	Mix	29.9	54.5	24.6 (82%)	Seldom	2 (2)
16	6	16 (3)	Mix	24.8	39.6	14.8 (60%)	Sometimes	4 (2)
17	7	72 (6)	Golden Retriever	35.6	58.8	23.2 (65%)	Sometimes	5 (6)
18	4	27 (6)	Mix	20.7	35.7	15 (72%)	Sometimes	2 (2)
19	13	58 (5)	Collie	45.9	61.6	15.7 (34%)	Sometimes	2 (2)
20	9	22 (5)	Mix	67.5	16.9	-50.6 (-75%)	Sometimes	4 (2)
21	6	50 (6)	Mix	45.5	55	9.5 (21%)	Seldom	6 (3)
22	8	42 (5)	Poodle	70.3	124.3	54 (77%)	Sometimes	6 (6)
23	11	34 (6)	Mix	37.9	107	69.1 (182%)	Sometimes	3 (3)
Mean±SD	**8.15±2.85**	**45.1±19.1 (4.77±1.09)**	—	**37.1±17.2**	**58.5±28.9**	**21.4±27.5 (76.2±70.8%)**	—	**3.7±1.4 (3.1±1.4)**

*=pounds; BCS=body condition score; EE=endogenous estrogen; NoUD=number of urinary accidents; Tm=treatment

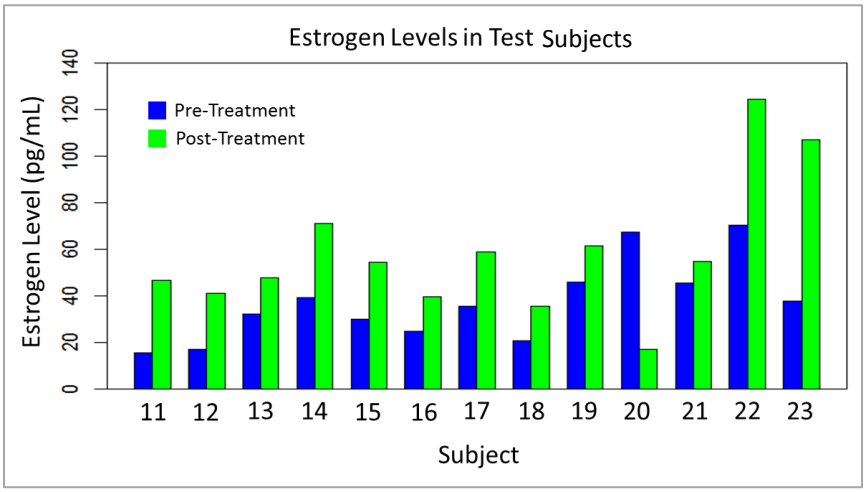

Figure 2: Pre-treatment and post-treatment estrogen levels in Test Group subjects

Owners were prompted on their questionnaire to assess general impression of improvement (never, seldom, sometimes, often, always) of observed urinary dribbling over the 14 day period. None of the owners of Control Group dogs reported improvement ("never" = 10 dogs), whereas 11 of 13 owners of Test Group dogs noted varying improvement (never = 2 dogs, seldom = 2, sometimes = 9) (Tables 1 and 2). The group difference in the owner-reported improvement rate was statistically significant (p=0.0001, Fisher's test).

The number of urine dribbling events, regardless of the volume, was recorded by dog owners at pre-treatment (Day 0) and post-treatment (Day 14) time points. In the Control Group, there was no change in the frequency of dribbling events, ranging from 1 to 4 events per day with both Day 0 and Day 14 having the same mean±SD of 2.4±1.1 (Figure 3). The test subjects on Day 0 had a mean of 3.7±1.4 dribbling events (2 to 6 range). On Day 14, the frequency of dribbling events (2-6 range) had decreased to 3.1±1.4 (Figure 4). The mean change was a decrease of 0.6±1.2. Comparing the 2 study groups, there was a 16.2% reduction in test dogs versus 0% change in the controls (p=0.06, one-sided Wilcoxon Signed Rank test).

Despite the encouraging biological trends observed in the Test Group compared to the Control Group, in urinary dribbling improvement (questionnaire) and NoUD reduction; group comparison did not attain statistical significance in this small study (p=0.08).

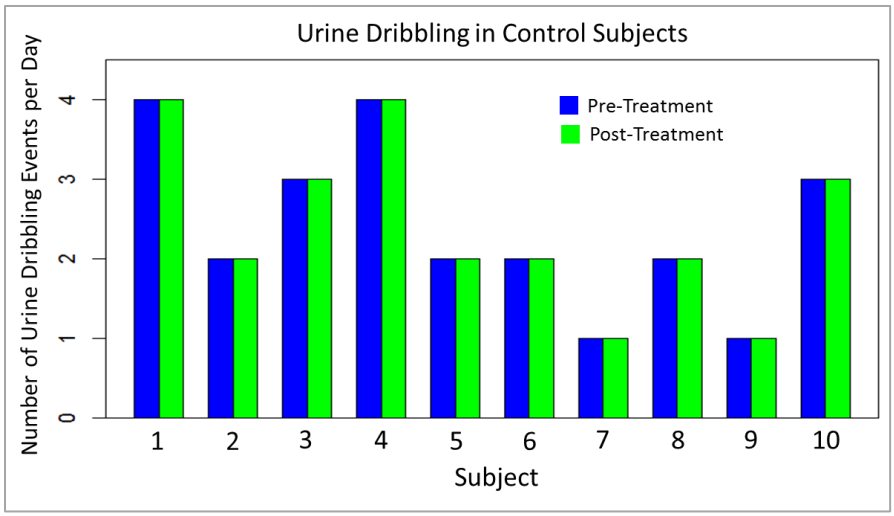

Figure 3: Urine dribbling observations during the pre-treatment and post-treatment periods from subjects in the Control Group

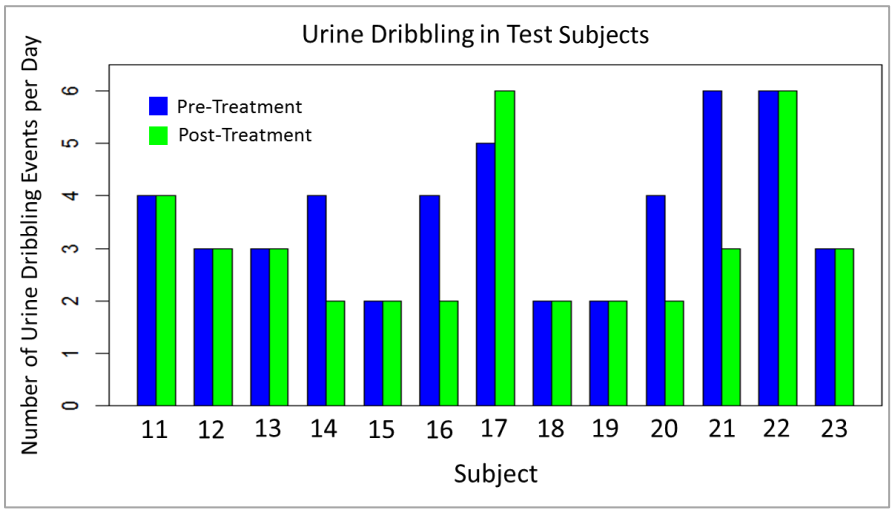

Figure 4: Urine dribbling observations during the pre-treatment and post-treatment periods from subjects in the Test Group

DISCUSSION

This randomized, controlled and owner-blinded clinical trial investigated the effects of tofu supplementation on endogenous estrogen levels of spayed dogs with urinary incontinence. Comparison between the two subject groups (control, test) revealed the change of estrogen levels in the Test Group (tofu supplemented) were significantly increased (p=0.0002) when compared to the Control Group. These findings support the study hypothesis that supplementing the diet of a spayed female dog with phytoestrogen-containing tofu can increase EE levels. Secondary outcomes assessed owner impression of urinary dribbling improvement and mean episodes of urinary accidents on Day 0 and Day 14. Study findings demonstrated that dogs receiving tofu supplementation had fewer numbers of dribbling events (16.2% reduction in test versus 0% change in controls), and owner impression of clinical improvement was greater in the tofu-supplemented dogs (p=0.0001).

Estrogen supplementation is often a recommended treatment for PUSMI. These study findings show promise in developing a natural food therapy to provide additional estrogen as a treatment for urinary incontinence in spayed female dogs. Feeding tofu is a simple and easy to implement food supplement that was readily consumed by all test dogs without adverse effects, which ultimately increased owner compliance.

An unexpected study finding was that blinded-owner assessment of subjects in the Test Group, noted greater general improvement of urinary incontinence (11 out of 13) than the proportion of subjects with documented reduction of dribbling events (4 out of 13). It appears that a small number of owners noted some overall improvement based on the observation of less dribbled urine volume but not necessarily a reduced number of events. In contrast, none of the owners of control dogs documented urinary incontinence improvement at Day 14 general impressions or number of dribbling events.

The small sample size, which was inherent in conducting research in a private practice setting, was a limiting factor. Variation in a small group of subjects (size, breeds, estrogen levels) posed challenges. Estrogen levels in the Control Group (56.9±22.8 pg/mL) were significantly higher than those in the Test Group (37.1±17.2 pg/mL) at study start, despite randomization of group assignments. Additionally, the dosing recommendation taken from human studies may not apply to dogs, particularly since dogs can be markedly different sizes versus more uniform human size. The variety in individual diets of the dogs, along with a limited treatment period were all challenges to experimental study conduct. Furthermore, the data on urine dribbling events pre-treatment was limited from an accuracy standpoint as some pet owners knew the exact number of events whereas others only recognized the dribbling and not necessarily the number of events. During the study though, the owner's awareness and count of dribbling events improved.

What was not taken into consideration in this study is the relationship of beef fat and estrogen content. The possibility of hormone implants in beef animals and the implants' effects on hormone levels has been described.[27] A follow-up study improvement might include the use of a low fat meat animal that does not normally receive hormonal implants as the food placebo for study controls.

The fewer than planned sample size (i.e. 3 controls less than planned) affected study statistical power. Limitations included 1) the power to conclude a statistical significance of EE level increase in the Control Group was reduced to 88% (from 95%); and 2) the power to conclude the Test Group had increased estrogen levels when compared to Control Group was reduced to 73% (from 78%). These study challenges are not surprising considering the small sample size, which increases the likelihood of group imbalance from randomization and the fact that canine estrogen levels are known for variability.[28] Again, larger study group size would improve the validity of study EE findings, along with measurement, in future studies, over a longer period of time (i.e. months).

In summary, 23 client-owned spayed female dogs with urinary incontinence were treated with either placebo (n=10) or a tofu-supplemented (n=13) diet for 14 days. Endogenous pre-treatment estrogen level was compared to post-treatment EE level at Day 14. Study findings demonstrated Test Group EE level was significantly increased after 14 days (37.1±17.2 to 58.5±28.9 pg/mL, p=0.013). The change of EE levels in the Test Group was significantly greater than that in the Control Group (Control: -2.3±11.5 versus Test: 21.4±27.5 pg/mL, p=0.0002). The study also found that Test Group owner impression of urinary incontinence improvement (11/13) on a questionnaire was significantly greater (p=0.0001) than that in the Control Group (0/10). The results of this study suggest the potential for developing a holistic, natural food therapy for urinary incontinence in spayed female dogs. Future prospective studies, with larger enrollment for a longer period, that also consider factors such as treatment dose (soy isoflavone dose in mg/kg of body weight) are warranted.

ACKNOWLEDGMENTS

The authors thank all the outstanding individuals who have helped and supported the work related to this project, especially Maddison Johnson, who helped administer the project, stay on track, and maintain organization. The authors also thank all pet owners who volunteered their time, effort, and faithful companion to make this project possible.

Declaration of Interest and Funding

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this paper and the authors did not receive any specific grant of funding for authorship of this paper.

FOOTNOTES

[a] Proin® (phenylpropanolamine), PRN Pharmacal/Pegasus Labs, Pensacola, FL, USA

[b] Nasoya Firm Tofu; Nasoya Foods USA, Leominster, MA, USA

[c] University of Tennessee Veterinary Endocrinology Lab, Knoxville, TN, USA

[d] R version 3.5.2. The R Foundation for Statistical Computing, Vienna Austria; http://www.R-project.org

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APPENDIX

Subject Questionnaire Form*

1. My dog consumed the specified diet at each meal.
2. My dog showed signs of gastrointestinal distress including vomiting and/or diarrhea during treatment.
3. Administering the specified diet at the recommended volume was simple, straightforward and easy.
4. My dog's incontinence improved while consuming the specified diet.
5. I prefer a natural treatment in place of a Western drug, even if the natural treatment costs more.

DOG (X)	NEVER	SELDOM	SOMETIMES	OFTEN	ALWAYS
Q1					X
Q2	X				
Q3					X
Q4	X				
Q5					X

***** Each owner answered 5 questions at study termination. The results were based on a 5-point Likert scale with a checkmark indicating the owner's response.