PILOT STUDY

Preliminary investigation of the influence of long-term dietary isoflavone intake on reproductive tract histology and sex steroid receptor expression in female domestic cats (*Felis catus*)

Summary

Genistein and daidzein are isoflavones which are reported to influence the reproductive system in a variety of mammalian species. This pilot study aimed to determine if dietary isoflavones could potentially influence reproductive tract histology or morphology in domestic cats, when consumed during the postnatal development period. Cats were maintained on either treatment (150 μg/g DM genistein and 150 μg/g DM daidzein, n=4) or control (isoflavone free, n=8) diets from weaning, up to 414 (± 17.2) days post-weaning. Reproductive tissues were collected during routine ovario-hysterectomy and examined for histology and sex steroid receptor expression. Findings indicate that these dietary isoflavones influenced the expression of oestrogen receptor α (ERα) and oestrogen receptor β (ERβ), and progesterone receptor in feline reproductive tissues. One cat in the treatment group developed suppurative endometritis, but no evidence of uterotrophic or histological changes were found in any other cats. The potential to alter expression of hormone receptors in the reproductive tract of domestic cats exposed to genistein and daidzein warrants further investigation.

Keywords: cat, daidzein, genistein, oestrogen, progesterone, reproduction

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Introduction

Dietary isoflavones are phenolic compounds found in soy and other legumes, which have oestrogenic and anti-oestrogenic properties (Kurzer and Xu, 1997). A diverse array of hormonal and non-hormonal effects have been observed in isoflavone-exposed rodents, pigs and humans, including oestrogen receptor binding, and morphological and histological changes (Barnes *et al* 2000; Ford *et al* 2006; McClain *et al* 2006).

Domestic cats ingest, absorb, and metabolise soy isoflavones present in commercial diets (Bell *et al*., 2006; Cave *et al*., 2007). Some feline diets expose cats to isoflavone concentrations potentially capable of eliciting physiological changes (i.e. > 2 mg/kg BW) (Bell *et al* 2006). The isoflavones, genistein and daidzein, comprise the isoflavones detected in the highest concentrations in commercially prepared cat food (Bell *et al* 2006). Thus, it is important to ascertain the reproductive consequences of this level of genistein and daidzein exposure in this species. The present study was conducted to determine the potential for genistein and daidzein to alter reproductive parameters in the domestic cat, when provided at concentrations reflective of normal dietary exposure.

Materials and Methods

Eighteen female short-haired, domestic cats (*Felis catus*) were enrolled in the study and assigned to either the control group (n = 9) or treatment group (n = 9) at weaning. However, six cats were removed from the trial (two due to failure to consume the test diet within the first week, and four due to unrelated medical conditions prior to planned tissue collection). Cats were group-housed in multi-level pens, exposed to natural day/night

cycles. At 10 weeks of age, the cats were removed from the queen's pens, and separated into treatment (mean age 72 ± 1.89 d; BW 0.87 ± 0.07 kg) and control (mean age 71 ± 2.39d; BW 0.93 ± 0.05 kg) groups. Ethical approval was obtained from the Massey University Animal Ethics Committee.

The basal diet for both groups was a moist feline diet, commercially-prepared and formulated to meet the requirements for growth in the domestic cat (AAFCO 2009). This diet was assayed to contain no detectable levels of isoflavones (Bell *et al*., 2006). The purified (99.9%) form of each isoflavone, genistein and daidzein (LC Laboratories, MA, USA), was added to the basal diet to provide a calculated dose of 300 µg total isoflavone/g DM. Samples of the control and treatment diets were assayed for isoflavone content at monthly intervals throughout the trial according to methodology described in Bell *et al*. (2006). Cats were provided water *ad libitum* during the trial, and offered enough food to provide each cat with appropriate energy intake for age (i.e. 217 kcal/kg BW/d at eight weeks old, gradually reducing to 88 kcal/kg BW/d by 40 weeks; Legrand-Defretin and Munday, 1993). Food was weighed before and after offering to each group and daily refusals were used to calculate intake per pen, which was then used to estimate intake per cat. Monthly assessments were made of individual food intake by separation of each cat into individual metabolism cages for a 24 hour period, during which time food was offered in quantities calculated to provide twice the cat's energy needs, and food intake and urinary and faecal output were recorded.

Vaginal cytology confirmed that cats were in inter-oestrous at the time of reproductive tract collection. Tissue collection was performed under halothane anaesthesia, according to standard veterinary procedures, at a mean age of 481 days (SEM 21.4) in the control

group, and 429 (SEM 62.9) in the treatment group (differences due to older cats in the treatment group being removed from the study). Surgical procedures were performed on all cats in both groups at the same point in time (across two days) and by the same surgeon. Reproductive tracts (including ovaries, uterine horns and cervix) were weighed and ovarian surfaces examined for the presence of visible follicles, corpora lutea, and corpora haemorrhagica. Reproductive organs were fixed in 10% buffered formalin before being processed for histology and immunohistochemical (IHC) analysis. Haematoxylin and eosin-stained slides were examined by a veterinary histopathologist (author; W.R.; blinded to treatment) and screened for abnormalities according to standard veterinary procedures.

An IHC assay for assessment of ERα, ERβ and progesterone receptor (PR) was developed from the method of Martin de las Mulas *et al* (2000). The ERα, ERβ and PR were identified using monoclonal mouse antibodies, IgG1 (NCL-ER-6F11, NCL-ERβ; Vision Biosystems, Victoria, Australia) and PR4-12 (Merck, Palmerston North, New Zealand), respectively) validated for use in feline tissue.

Histological sections were de-paraffinised and rehydrated by sequential immersion in xylene and graded alcohol baths. Sections were microwave-heated (high power, 750W microwave oven for 7 min) in citrate buffer (10 mM citric acid, pH 6.0). Sections were washed in phosphate-buffer solution (PBS) before non-specific binding sites in the tissue were blocked by the application of 100 µl of 10% Bovine Serum Albumin (BSA) (Roche Diagnostics, Mannheim, Germany) in ovine serum and PBS. Sections were incubated at room temperature in a moist chamber for 45 min before BSA/serum was removed and the primary antibody applied (ERα, ERβ or PR) at dilutions confirmed in preliminary

experiments to yield optimal results (1: 50 for ERα and ERβ, 1: 30 for PR). Sections were incubated overnight at room temperature before the primary antibody was removed and the tissues incubated with biotinylated goat, anti-mouse IgG (Invitrogen Life Technologies, Auckland, NZ) for 1 hour before a fluorescent marker was added (Streptavidin, Alexa Fluro 546 conjugate, Molecular Probes Inc., OR, USA, diluted 1: 20). Following a final incubation of 1 hour, slides were washed in cold tap water and counter-stained with haematoxylin.

Positive control tissues (control cat uterine tissue and human breast cancer tissue) were incubated with each of the three primary antibodies and processed according to the same methodology as test tissues. Tissues were examined by one investigator (author; KW-T) using 40 x magnification with epi-fluorescence illumination (488 nm), and 100 individual cells were analysed for fluorescence-staining intensity and extent using Java-based image processing software (ImageJ, version 1.38; Rasband 2007). The level of light staining intensity detected in negative control tissue was the threshold of background luminescence used to define positive staining in test sections.

For the statistical analysis, data that were not normally distributed were tested for differences between groups using the Mann-Whitney test. For proportional data the Fisher exact test was used to compare differences. All other parameters were tested for between-group differences using ANOVA. All statistical procedures were carried out with Minitab software (version 15, Minitab Inc., PA, USA) with confidence limits set at 95%.

Results and Discussion

Four cats consumed the treatment diet and eight cats consumed the control diet for the duration of the study. By the end of the trial, treatment cats were consuming an average of 4.88 – 5.19 mg total isoflavones/kg BW/d, providing approximately equal doses of 2.44 – 2.56 mg/kg BW/d of genistein and daidzein.

No significant differences were observed between groups in reproductive tract wet weight (Table 1), indicating no gross morphological changes following isoflavone exposure.

Table 1 here

This is in contrast with previous studies in other species in which uterine hypertrophy has been reported following exposure to isoflavones in rats (Santell *et al.* 1997; McClain *et al* 2005) and dogs (McClain *et al.* 2006). With the exception of one cat diagnosed with subacute suppurative endometritis (discussed below), no histological abnormalities were detected in ovarian or uterine tissue from any other cat, and no histological differences were detected between treatment and control groups. The number of corpora lutea, or primary, secondary, tertiary, mature or atretic follicles in cat ovaries did not differ between groups. It is possible that the lower dose provided (reflecting the higher end of the typical dietary intake range calculated by Bell *et al.*, 2006), and the use of an oral administration route, which results in low bioavailability (Cave *et al*., 2007), may explain the differences observed here compared to previous studies, whereby only dogs exposed to 500 mg/kg BW (compared to 50 mg/kg BW and 150 mg/kg BW groups) exhibited uterine hypertrophy (McClain *et al.*, 2006). Alternatively, the duration of administration in this present study exceeds previous studies, and acute responses may have been missed. This is supported by the finding that uterine hypertrophy was detected in dogs

following 13 weeks of exposure to a high genistein dose (500 mg/kg BW) but not following 52 weeks exposure (McClain *et al.*, 2006)

However, differences detected in receptor staining between groups indicate a role for dietary isoflavones in modulating reproductive physiology in domestic cats. Expression of ERα and ERβ receptors was greater in treated cats compared to control cats ($P < 0.05$), with the exception of ERα in the uterine basal endometrium and myometrium, where expression was greater in control tissues ($P < 0.05$; Table 2). No difference was detected between groups in ERβ or PR expression in the myometrium. The up-regulation of cellular expression of ERα and ERβ in the ovarian cortex, medulla and uterine endometrium observed in response to isoflavone treatment here, has been reported in other species (Jefferson *et al* 2002; Chrzan and Bradford 2007). Genistein and daidzein are capable of binding to both ERs, with preferential binding and transactivation shown for ERβ (reviewed in Rietjens et al 2013). These isoflavones act as nuclear receptor ligands to enhance interactions between oestrogen-related receptors and proline-rich nuclear receptor coactivator (PNRC) (reviewed in Ricketts *et al.*, 2005).

Table 2 here

Conversely, proportional expression of the PR was typically down-regulated in isoflavone-treated cat uterine and ovarian sections. Progesterone receptor expression was lower in treatment cats compared to control cats in ovarian cortex and uterine apical endometrium ($P <0.05$), while no difference was detectable in the ovarian medulla and myometrium; in the uterine basal endometrium PR expression was greater in treatment cats ($P < 0.05$). These sex steroid receptors are important mediators in the control of oestrogen- and progesterone-induced effects during oestrous cyclicity and pregnancy,

such that modifications at the receptor level may be reflected in aberrant physiological responses or fertility.

One cat in the treatment group was diagnosed with subacute suppurative endometritis at 267 days of age. This cat's tissue morphological data was excluded due to its diseased state. The development of suppurative endometritis in an isoflavone-treated cat was an unexpected finding. This condition is not common in cats, and is generally only reported in cats older than eight years of age (Agudelo 2005), but further research is required to determine the role that isoflavones may have played in the onset of the condition in the cat reported here.

Conclusions

Preliminary findings suggest that the isoflavones, genistein and daidzein may exert modulatory effects on the expression of sex steroid receptors in feline uterine tissue. Given the limited sample size our findings should be considered as preliminary and interpreted with caution. Future investigation should include life-time evaluation of feline fertility and fecundity, with increased sample sizes.

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Table 1 Wet weights (as grams or % body weight) of reproductive tracts obtained from domestic cats following chronic isoflavone exposure (treatment group) or control animals[1, 2].

	Control group mean (SD)		**Treatment group mean (SD)**	
	g	**% BW**	**g**	**% BW**
Entire tract wet weight	1.99 (0.41)	0.07 (0.01)	2.15 (0.45)	0.07 (0.01)
Left ovary	0.17 (0.15)	0.01 (0.00)	0.13 (0.01)	0.004 (0.00)
Right ovary	0.12 (0.2)	0.004 (0.00)	0.11 (0.03)	0.004 (0.00)

[1]Values are expressed as mean (± SD). No significant differences were detected between groups ($p > 0.05$).

[2] Data from eight control cats and three treatment cats (one treatment cat was diagnosed with acute suppurative endometritis and as such the diseased state of her reproductive tract rendered it unsuitable for inclusion in this dataset)

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Table 2 The proportion of cells staining positive for ERα, ERβ, or PR in the reproductive tract of cats exposed to dietary genistein and daidzein, compared to control cats[1].

	ERα		**ERβ**		**PR**	
	Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
Ovarian cortex	0.58 (0.44)[a]	0.79 (0.18)[b]	0.57 (0.46)[a]	0.83 (0.26)[b]	0.75 (0.49)[b]	0.66 (0.31)[a]
Ovarian medulla	0.52 (0.46)[a]	0.94 (0.05)[b]	0.79 (0.21)[a]	0.93 (0.13)[b]	0.72 (0.29)[a]	0.76 (0.42)[a]
Uterine apical endometrium	0.91 (0.13)[a]	0.95 (0.09)[b]	0.63 (0.39)[a]	0.88 (0.11)[b]	0.81 (0.30)[b]	0.64 (0.44)[a]
Uterine basal endometrium	0.91 (0.22)[b]	0.84 (0.19)[a]	0.79 (0.33)[a]	0.95 (0.11)[b]	0.76 (0.20)[a]	0.88 (0.22)[b]
Myometrium	0.60 (0.33)[b]	0.32 (0.17)[a]	0.53 (0.35)[a]	0.59 (0.22)[a]	0.64 (0.31)[a]	0.63 (0.12)[a]

[1]Standard deviation is shown in parentheses. Values with different superscripts (within row for each respective sex steroid receptor) are significantly different ($p < 0.05$).

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