Changes in ovarian contraction by endothelin-2/receptor system in the feline ovary

Joe Cacioppo[1], Patrick Lin[1], SangWook Oh[1,2], Arnon Gal[1], Yongbum Koo[1,3], and CheMyong Ko[1]

[1]Comparative Biosciences, College of Veterinary Medicine, University of Illinois, Urbana-Campaign, IL 61802 USA; [2]Biology Education, Chunbuk National University, Jeonju, South Korea; [3]Department of Biotechnology and Biomedical Science, Inje University, Gimhae, South Korea.

Abstract

Endothelin-2 (ET-2) is transiently expressed in the granulosa cells of mammalian periovulatory follicles immediately prior to ovulation. *Ex vivo* experiments showed that, upon treatment with ET-2, the rodent ovary rapidly contracts. When the endothelin receptor pathway is antagonized *in vivo*, ovulation is inhibited in rodents. These findings led us to postulate that ET-2–induced contraction of smooth muscles in the ovarian cortex is a final trigger for follicle rupture at the time of ovulation. Similar to humans and other mammals, feline ovaries possess a layer of contractile smooth muscle-like cells around developing follicles, known as the theca externa. In addition, feline ovaries have been documented to spontaneously contract *ex vivo*; however, the function of this spontaneous contraction and whether ET-2 induces ovulation remains to be determined. Here, we investigated the characteristics of feline ovarian cortical contraction using myography in the absence or presence of physiological doses of ET-2. Whole fresh feline ovaries were collected after spay procedures through the Junior Surgery Program of the College of Veterinary Medicine at the University of Illinois. Of 13 ovaries tested, all demonstrated a period of strong and sustained contraction when washed with a 50 nM solution of feline ET-2 peptide for 30 minutes, with an average increase in base tensile force of 2.48 ± 0.40 mN. Additionally, when washed for 20 minutes with a 140 nM solution of the dual ET receptor antagonist tezosentan contraction was reduced in a dose-dependent manner. Of these ovaries, 4 demonstrated spontaneous contractions prior to ET-2 treatment, with average amplitude of 4.08 ± 2.45 mN, duration of 22.2 ± 6.4sec, and a time of 60.9 ± 20.2sec between contractions. These contractions continued after ET-2 treatment, but contraction amplitude was reduced (1.25 ± 0.95 mN) as was the time between contractions (13.8 ± 6.5 sec) for all ovaries. There was no change in the duration of these contractions (20.13 ± 4.66 sec). Measurement of mRNA expression by polymerase chain reaction showed that feline ovaries express mRNA for ET-2, both isotypes of endothelin receptors (ET-A and ET-B), and endothelin converting enzymes 1 and 2 (ECE-1 and ECE-2). This study demonstrates that ET-2 produces a feline ovarian cortical contraction. Future work will determine the impact of inhibiting ET-2 and the endothelin receptor pathways *in vivo* on follicle rupture in the feline ovary.

Introduction

We have previously shown that ET-2 was exclusively and transiently expressed in the granulosa cells (GC) of preovulatory ovaries immediately prior to ovulatory follicle rupture (1) via a hypoxia-driven pathway (2). When treated *in vitro*, ET-2 induced an immediate and sustained contraction in the preovulatory ovary via an endothelin receptor A (ET_A)-mediated pathway (3). These findings led us to hypothesize that ET-2 induces follicular rupture by contracting periovulatory follicles as a last moment-trigger of ovulation. As ET-2 expression and follicle rupture occur almost simultaneously (4), we suggest that the shortening of theca externa cells drives the oocyte, cumulus complex, and antral fluid from the ovary into the periovarian space and infundibulum. We hypothesize that ET-2 plays critical roles for ovulation via follicular contraction. In this study, we tested a portion of the hypothesis by treating whole feline ovaries with ET-2 and ET-receptor antagonists and quantifying their tensile response.

Working Hypothesis

ET-2 induces follicular contraction in periovulatory follicles at the time of ovulation in the cat

Aim of this Study

To test if ET-2 induces contraction in the feline ovary at physiological concentrations; to quantify changes in feline ovary tension in response to ET-2.

Materials and Methods

1. Ovarian Collection: Feline ovaries were collected through the Jr. Surgery Program at the University of Illinois from October through January. Ovaries were either fixed in a formaldehyde solution, snap-frozen in liquid nitrogen, or transferred alive in a 4̊C Phosphate Saline Solution (PSS) to the lab. Special thank you to Heather Soder for her support and assistance.

2. Histological & Immunohistochemical Analysis: Fixed ovaries were sectioned at 7µm and stained with Hematoxylin and Eosin or anti-αSMA antibody for smooth muscle detection.

3. RNA Extraction and Endothelin Gene Expression: Eight frozen ovaries were homogenized by mortar and pestle and RNA was extracted with TRIzol® and chloroform. Following purification, RNA was used as a template for cDNA synthesis in a 1:1 ratio through reverse transcription. DNA was then used as template for detection and semi-quantitative PCR of genes involved in the Endothelin system.

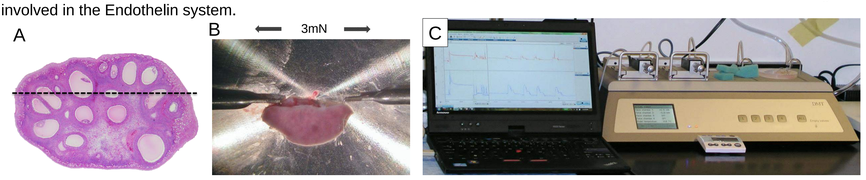


Figure 1. Measurement of ovarian contraction. A sectioned cat ovary (A) was placed onto two 4mm pins (B) of the *DMT* (*Danish Myo Technologies*) myograph machine (C), which is composed of two chambers with a force transducer to sense tissue tension, a heated base to optimize tissue temperature, and gas and vacuum lines to supply tissues with oxygen and remove solutions. Electrical signals are sent to an amplifier and then to the LabChart™ software on a connected computer.

4. Ovary Mounting: The bursa was removed from each ovary under dissection microscope. The ovary was bisected longitudinally by scalpel blade to separate a portion of the cortex and medulla away from the hilus and large ovarian arteries (Figure 1). The ovary was then mounted onto two 4mm pins of a myograph machine; the pins were punctured through the internal face of the sectioned ovary. Tissues were maintained under PSS buffer at 38̊C with a continuous supply of oxygen and CO_2.

5. Tension Analysis: After equilibration, ovaries were stretched to a passive tension of 3mN. A 'wake-up' protocol (6) was used to return muscle cells to an active state through intermittent washes with heated PSS buffer and a 60mM Potassium solution. ET-2 peptide was added in increasing amounts up to a physiologically relevant level of 50nM to determine the optimal solution concentration that would evoke a contractile response. Similarly, Tezosentan, which blocks both ET_A and ET_B receptors, was added step-wise by orders of magnitude to generate an ideal dose to use for ET-2 antagonization, determined to be 140nM. To quantify feline contraction in response to these predetermined doses, a baseline tension measurement was first made under PSS buffer alone, and after the potassium solution was added. Next, the ovary was maintained in a 50nM solution of ET-2 peptide in PSS and observed for 30 min. Lastly, tissues were maintained in a 140nM solution of the dual receptor antagonist Tezosentan and PSS buffer, and changes in tension were again measured. Thirteen ovaries were used in tensile analysis (n=13).

Cats possess a layer of Contractile Cells around Antral Follicles

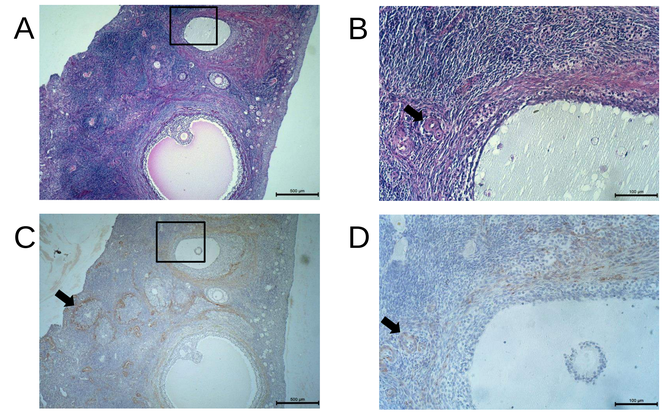
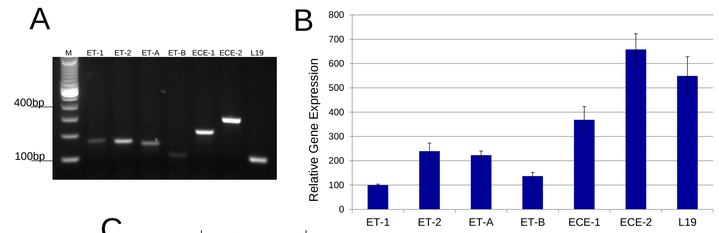


Figure 1. Representative histological sectioning of a feline ovary. Sectioned at 7µm, seen at 4X (left) and 20x (right). A, B: Hematoxylin and Eosin staining. C, D: Immunohistochemistry for α-Smooth Muscle Actin with Hematoxylin counterstain. Note staining of contractile cells of the theca layer around antral and preantral, but not primordial, follicles; and the more intense smooth muscle staining around ovarian vessels (arrows).

Detection of Genes of the Endothelin Contraction System



C

Gene	Primer Name	Sequence 5' - 3'
Edn1	FelisET1_F	TCT TGG GCG CAG AGC TCA GCA C
	FelisET1_R	GGA GTG TTG ACC CAG ATG ATG TCC A
Edn2	FelisET2_F	CAA GGG CCA GGT GGC TGC TG
	FelisET2_R	CGG GAG TGT TCA CCC AGA TGA TGT C
EdnRA	FelisETA_F	TAC CAA GAT GTG AAA GAC TGG TGG CTC
	FelisETA_R	GTT TAA GGT GTT CAC TGA GGG CAA TTC
EdnRB	FelisETB_F	CTT GCC GAG GAC TGG CCC TTT G
	FelisETB_R	CTT AGA GCA CAT AGA CTC AAC ACA G
ECE1	FelisECE1_F	CGC CCC CAG TGT GCC TAA GTG A
	FelisECE1_R	AGG TGC TTG ATG ATG GCT TGG TTG TG
ECE2	FelisECE2_F	TGT GGG AAC AGT GCC CTG AGC T
	FelisECE2_R	ACT CAG CAC CTG GTC CAC AGT G
L19	FelisL19_F	GCT CAG GCT TCA GAA GAG GCT TGC
	FelisL19R	GTT GGC ATT GGC GAT TTC ATT TGT CTC

Figure 2. Relative expression of feline genes of the Endothelin system. A,B: Semi-quantitative PCR of cDNA generated from RNA extracted from whole feline ovaries (arbitrary units). C: Primer sequences used for gene expression analysis

Feline Ovarian Contraction in Response to ET-2 and Tezosentan Addition

Figure 4.

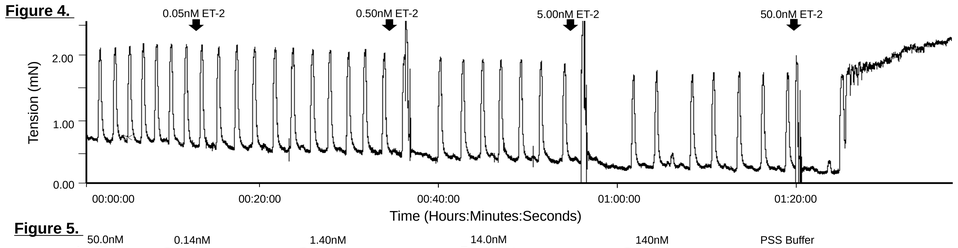


Figure 5.

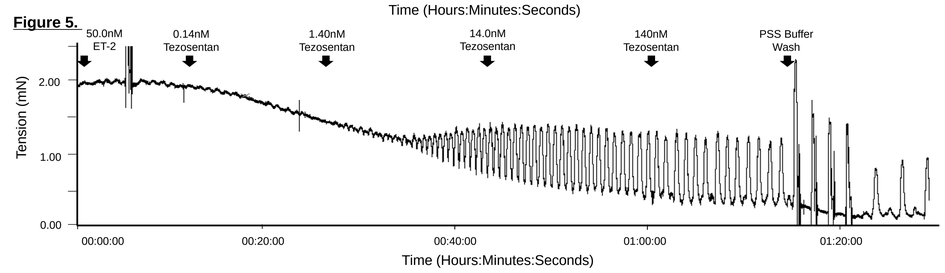


Figure 6.

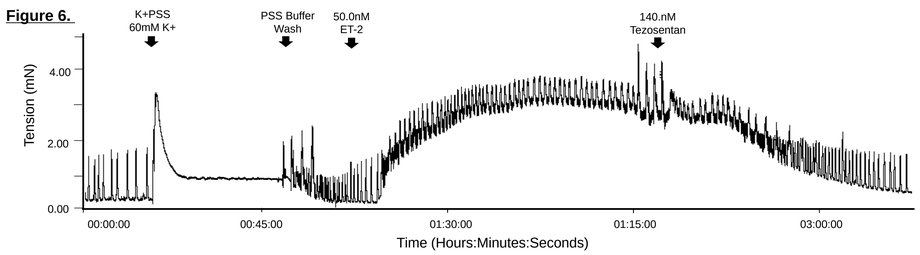
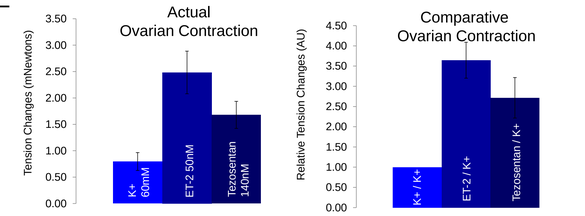


Figure 4. ET-2 Peptide Titration (Top). Feline ovary response in mNewtons tension vs. time to titrated doses of Endothelin-2 peptide (0.05 – 50.0nM). The final dose, 50.0nM of ET-2, is the physiologically relevant concentration found in human antral follicles. Note the strong and sustained contraction it evokes in the ovarian tissue. Arrows indicate the timing of different doses (9.8mN = 1.0g).

Figure 5. Tezosentan Titration (Center). Feline ovary response in mNewtons tension vs. time to titrated doses (0.14nM – 140nM) of the dual Endothelin-receptor antagonist Tezosentan following ET-2 peptide stimulation. There is a dose-dependent decrease in ovarian tension in response to Tezosentan, as well as a return to spontaneous contractions in ovaries that were previously contracting.

Figure 6. Feline Ovarian Contraction in Response to Potassium Solution, Endothelin-2 Peptide, and Tezosentan Administration (Below). A representative feline ovary response to a 60mM K+PSS solution, a 50nM ET-2 solution, and a 140nM Tezosentan solution. This schema was used to measure responses to the three treatments in each ovary. The average responses of ovaries are displayed below in mN (left), and in arbitrary units after normalization to the K+ response from each individual ovary (right). Changes in tension were significantly different from each other and from baseline measurements ($p<0.05$). Spontaneous contractions were present in several ovaries (4/13) as pictured; all contractions ceased during K+PSS treatment; a general trend was seen for contractions of lower amplitude and shorter duration that occurred more frequently during ET-2 treatment, though these parameters were not statistically significant ($p>0.05$, data not shown).



n=13

	K+	ET-2	Tezosentan	(AU)	K+/K+	ET-2/K+	Tezosentan /K+
Average (mN)	0.80	2.48	1.68		1.00	3.65	2.72
Std. Error	0.17	0.40	0.26		N/A	0.44	0.50

Conclusion and Future Directions

- **"Follicular Contraction"**: To determine if ovarian contraction seen in feline and murine species is the result of follicular / thecal contraction or vasoconstriction.
- Whole F1 follicles will be collected from laying hens just prior to ovulation. Sections of the theca layer opposite the stigma will be isolated and measured for contractile response to potassium, Endothelin-2 peptide, and Tezosentan in a similar manner to this study. Histology, RNA quantification, and immunohistochemistry will also be performed.

- **"Generation of a smooth muscle cell-specific ET_A knockout mouse (smETaKO)"**
- Smooth Muscle Actin-iCre mice will be crossed with Endothelin Receptor A $^{flox/flox}$ mice. smETaKO and wildtype mice will be treated for superovulation at 60 days of age, and the numbers of ovulated oocytes will be counted at hCG 24h, and compared between the two groups. The ovaries will be collected and examined by H&E staining for counting the number of CLs and unruptured follicles. Ovaries of 2-month old smETaKO and wildtype mice will be histologically examined to see if the SMA-specific *ednRA* deletion also affects CL formation and follicle recruitment.

References

1. **Ko C, Gieske MC, Al-Alem L, Hahn Y, Su W, Gong MC, Iglarz M, Koo Y** 2006 Endothelin-2 in ovarian follicle rupture. Endocrinology 147:1770-1779.
2. **Na G, Bridges PJ, Koo Y, Ko C** 2008 Role of hypoxia in the regulation of periovulatory EDN2 expression in the mouse. Canadian journal of physiology and pharmacology 86:310-319.
3. **Bridges PJ, Jo M, Al Alem L, Na G, Su W, Gong MC, Jeoung M, Ko C** 2010 Production and binding of endothelin-2 (EDN2) in the rat ovary: endothelin receptor subtype A (EDNRA)-mediated contraction. Reproduction, fertility, and development 22:780-787.
4. **Stocco C, Telleria C, Gibori G** 2007 The molecular control of corpus luteum formation, function, and regression. Endocrine reviews 28:117-149.
5. **Klipper E, Levit A, Mastich Y, Berisha B, Schams D, Meidan R** 2010 Induction of endothelin-2 expression by luteinizing hormone and hypoxia: possible role in bovine corpus luteum formation. Endocrinology 151:1914-1922.
6. **Park J, Whitesall S, D'Alecy L, Shu L, Shayman J** 2008 The vascular dysfunction in the α-galactosidase A knockout mouse is an endothelial cell, plasma membrane-based defect. Clinical and Experimental Pharmacology and Physiology. 35: 1156-1163.

This work was supported by the NIH (R01HD052694 to C.K.); presentation supported by the Alliance for Contraception in Cats and Dogs.