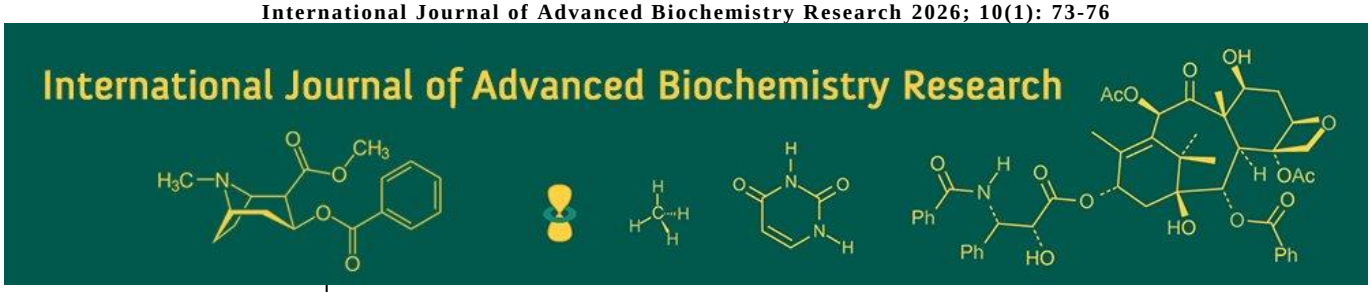

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Influence of oestrogen receptor-*α* gene expression on cervical patency in pyometric bitches

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Abstract
Canine pyometra is a common, serious and potentially life-threatening reproductive disorder affecting intact female dogs, usually during the dioestrus phase of the oestrous cycle. The study focused on the factors concerned with cervical patency in pyometric dogs. Twelve clinical cases confirmed of pyometra, open (Group I, n = 6), closed (Group II, n = 6) and six healthy dogs in dioestrous phase (Group III) were utilized for the study. All bitches were subjected to ovariohysterectomy (OHE) under standard anaesthetic protocol. Sections of the cervix were collected and stored in RNA later for gene expression studies. Levels of mRNA expressions for estrogen receptor-*α* (ER-*α*) was determined by real time quantitative PCR reaction (qRT-PCR). No significant difference in the expression of ER-*α* between the groups could be noticed. The study concluded that ER-*α* wasn't involved in controlling the cervical patency.

Keywords: Canine pyometra, cervical patency, oestrogen receptor-*α*, qRT-PCR

Introduction
Chastain and Ganjam (1986) [2] stated that endometrial gland proliferation was induced by the oestrogen (E_2) and the rise in serum progesterone (P_4) level down regulated the ER in normal dogs. Oestrogen supported the proliferation of endometrial glands and P_4 which aided in secretory activity. Hence concurrent prolonged action of both E_2 and P_4 resulted in the proliferative cystic changes which finally resulted in cystic endometrial hyperplasia (CEH). Hardy *et al.* (1994) [5] reported that expression of ER was high in CEH-P cases. Cock *et al.* (1997) [1] concluded that the abnormalities in the ER played a crucial role in progress of pyometra in dog and weren't only factor responsible for the disease. Kovacs *et al.* (2004) [8] reported that increased concentrations of E_2 led to increased *PR* and ER expression, while decrease in concentrations of these receptors was related to increased plasma concentrations of P_4. Reduced ER expression may contribute to cervical closure, resulting in retention of uterine contents and increased systemic illness (De Bosschere *et al.*, 2003) [4]. Understanding the role of ER expression in cervical patency may help to explain the pathophysiology of pyometra and aid in better diagnosis and therapeutic decision-making.

Materials and Methods
Female dogs of various breeds showing clinical signs of pyometra were selected for this study. Six healthy female dogs in the dioestrous stage were included as the control group (Group III, n = 6). Pyometra was confirmed using trans-abdominal ultrasonography. Dogs diagnosed with pyometra were classified into open cervix (Group I, n = 6) and closed cervix (Group II, n = 6) based on clinical signs. All these bitches were subjected to OHE under standard anaesthetic protocols. After OHE, part of the cervix was collected and stored in the RNA later for gene expression studies. The samples were kept in ice box (4ºC) and transported to the laboratory and stored at-80ºC in deep freezer. After thawing, 30 mg of the collected tissue was removed from RNA later (Origin) and sectioned into small pieces using scissors and immediately processed for RNA isolation. The total RNA isolation was carried out using RNeasy fibrous tissue mini kit (Qiagen) as per manufacturer's instructions. Quality and integrity of isolated total RNA was checked by agarose gel (one percent W/V) electrophoresis. The concentration and quality of RNA was checked by NanoDrop (Thermo Scientific, NanoDrop TM 2000 Spectrophotometer, USA) method.

Complementary DNA was synthesised from isolated total RNA using RevertAid first strand cDNA synthesis kit (Thermo Scientific, Cat# K1621). For cDNA synthesis, 500 ng of RNA from each sample was reverse transcribed. Exon spanning primers were designed using online primer design software NCBI. GenBank Accession number were NM_001286958.1 and AF021873.2 for amplification of ER-*α* and *β-actin* (Table 1). The specificity of the primers was checked using BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). PCR was carried out to amplify 345 bp, and 141bp fragments of ER-*α,* and *β-actin* genes from canine cervix tissue. The amplified products were checked by two percent agarose gel electrophoresis.

Relative quantification using real time (qRT-PCR)

Quantitative real time PCR was used to quantify mRNA in both relative and absolute terms. In the present study, this technique was used to find relative expression of genes ER-*α* in canine cervical tissue using *β-actin* as a reference gene. The relative quantification of gene expression was carried out using Illumina Eco ® qRT-PCR system using SYBR green chemistry. The details of reaction mix and reaction protocol are given in table 2 and 3.

The exact calculations for finding relative quantification by Livak (2001) [10] method given below:

ΔC_T = Average C_T (Target gene)-Average C_T (References gene)

$\Delta\Delta C_T = \Delta C_T$ (Test Sample)-ΔC_T (References Sample)

Relative Quantification (RQ) = $2^{-\Delta\Delta C_T}$

Statistical analysis

Independent t-test was used for the statistical analysis of relative expression of ER-*α,* genes in cervix of pyometric and normal dioestrous bitches in three groups under study. The data were analysed with one way ANOVA using SPSS software version 24.0 (IBM Corp. 2016) [6].

Results and discussion

RNA samples having optical density ratio (OD) 260/280 above 1.8 were used for this study. Average value of OD 260/280 and 260/230 ratio for the cervical tissue samples were 2.06±0.09 and 2.05±0.18 respectively. Good quality total RNA was isolated from eighteen cervical tissue samples. Clear bands for 28S and 18S were obtained by 0.8 percent agarose gel electrophoresis of RNA. Total RNA isolated was converted into cDNA using random hexamer primer method.

Real time quantitative PCR of ER-α in cervical tissue

The composition of PCR, temperature and number of cycles were optimized for the efficient amplification of ER-*α* and *β-actin* in Illumina PCR machine. The qRT-PCR amplification plot and derivative melt curve of *β-actin* and ER-*α* are shown in plate 1, 2, 3 & 4 respectively. Melt curve analysis showed a single peak for each gene confirming absence of any non-specific product or primer dimer during qRT-PCR. The sequences obtained were subjected to NCBI BLASTn software alignment and was found to have 100 percent sequence similarity with ER-*α* gene of *Canis lupus familiaris* gene. Thus, giving confirmative that the amplicon amplified for real time PCR study was specific to ER-*α* gene.

Relative Quantification of ER-α gene expression between dioestrous and pyometricbitches

The expression of target gene (ER-*α*) and reference gene (*β-actin*) was compared to calculate ΔC_T (table 4, 5 & 6) and the ΔC_T of the control and experimental groups were compared to calculate $\Delta\Delta C_T$. A simple formula, $2^{-\Delta\Delta Ct}$ is used to calculate the relative fold gene expression of the samples. In the present study, there was no statistically significant difference in the expression of ER-*α* in control (1-fold) and open (1.05-fold) cervix pyometra at five percent level. Expression of ER-*α* was similar in control (1 fold) and closed (1.04 fold) cervix pyometra and found to be statistically non-significant (p value > 0.05). Expression of ER-*α* was similar in open (1 fold) and closed (0.99 fold) cervix pyometra and found to be statistically non-significant (p value > 0.05).

Oestrogen receptor (ER-*α*) **expression in uterine cervix**

In the present study, ER-*α* in the uterine cervix of control, open and closed cervix pyometra had no significant difference in expression signifying the non-existence of its influence in controlling the cervical patency. Corroborative to the findings of current study, indicating similarity in expression of ER-*α* in the cervix of dioestrous, closed, open cervix pyometric dogs, Tamada *et al.* (2012) [13] reported that there was no correlation observed between ER-*α* expression in cervix and its patency in closed and open cervix pyometric dogs. Similar study reports by Kunkitti *et al.* (2011) [9] also reported that ER-*α* concentration in the uterine cervix showed non-significance between closed and open cervix pyometric dogs. They also reported that quantification and identification of ER-*α* in the cervix and uterus of open and closed cervix pyometra groups did not showed any difference. They concluded that ER-*α* expression didn't affect the cervical patency in pyometra affected bitches neither in anoestrous nor dioestrous. Volpato *et al.* (2012) [15] concluded that ER-*α* and *β* concentration was same in closed and open cervix pyometric dogs and ruled out involvement of these receptors in controlling cervical patency. Kovac *et al.* (2004) [8] reported that E_2 induced up-regulation of ER, but in the present study, though the E_2 concentration was more, the high circulating P_4 levels in pyometric and dioestrous bitches, would have down-regulated the ER expression in the uterine cervix. Similar observations were made by Johnston *et al.* (1985) [7] who reported that P_4 suppressed ER synthesis and activation. Similar observations on the down regulations of ER in the uterus of dogs was also reported by Darko *et al.* (2018) [3] who observed a weak reaction on ER in dogs with pyometra during the dioestrous phase, signifying the down-regulatory effect of P_4 on ER. Also, Ververidis *et al.* (2004) [14] had mentioned that eventhough serum oestradiol-17_β levels were high it couldn't activate uterine ER, unless circulating P_4 level was withdrawn.

Table 1: Primers for canine *ER-α* and *β-Actin* genes

Gene	Primer	Sequences (5'-3')	Product size(bp)	Accession Number
β-actin	Forward	ATGGAATCATGCGGTATCCAC	141	AF021873.2 (Pisamai *et al.*, 2016)
	Reverse	CTTCTGCATCCTGTCAGCAA		
ER-α	Forward	AGGGTACCAGGCTTTGTTGATT	345	NM_001286958.1
	Reverse	ACGGTGGATATGGTCCTTCTCT		
	Reverse	TCCAGGCACACCTCATTTCC		

Table 2; Real time quantitative PCR reaction mixtures used to amplify *ER-α* and *β-actin* genes

Sl. No.	Components	Volume (µL)
1.	Maxima SYBR Green qPCR Master Mix (2X)	6.25
2.	Forward primer	0.5
3.	Reverse primer	0.5
4.	Template (cDNA from 500ng of RNA)	1
5.	Nuclease free water	4.25
	Total	12.5

Table 3: Thermal cycling conditions performed for each gene of interest

Gene	Initial denaturation		40 cycles					
	Temperature (°C)	Time (min)	Denaturation		Annealing		Extension	
			Temperature (°C)	Time (sec)	Temperature (°C)	Time (sec)	Temperature (°C)	Time (sec)
ER and *β-actin*	95	5	95	30	60	30	72	5
Fluorescence signal were recorded during extension stage of each cycle								

Table 4: Relative quantification of *ER-α* expression between dioestrous and closed pyometra bitches

Group	Mean C_T±SE		ΔC_T	$\Delta\Delta C_T$	Fold change ($2^{-\Delta\Delta C_T}$)	p value
	ER-α	*β-actin*				
Dioestrus	16.04±1.46	26.49±0.37	-10.44±1.51	0±1.51	1	0.97 NS
Closed	17.08±1.12	27.605±0.94	-10.51±1.47	-0.07±1.47	1.04	

(NS-Nonsignificant at p value ≥ 0.05)

Table 5: Relative quantification of *ER-α* expression between dioestrous and open pyometra bitches

Group	Mean C_T±SE		ΔC_T	$\Delta\Delta C_T$	Fold change ($2^{-\Delta\Delta C_T}$)	p value
	ER-α	*β-actin*				
Dioestrus	16.04±1.12	26.49±0.94	-10.44±1.47	0±1.47	1	0.97 NS
Open	17.98±0.58	28.51±0.42	-10.52±0.72	-0.07±0.72	1.05	

(NS-Nonsignificant at p value ≥ 0.05)

Table 6: Relative quantification of *ER-α* expression between open and closed pyometra bitches

Group	Mean C_T±SE		ΔC_T	$\Delta\Delta C_T$	Fold change ($2^{-\Delta\Delta C_T}$)	p value
	ER-α	*β-actin*				
Open	17.98±0.58	28.51±0.42	-10.52±0.72	0±0.72	1	0.99 NS
Closed	17.08±1.46	27.61±0.37	-10.51±1.51	0.00±1.51	0.99	

(NS-Nonsignificant at p value ≥ 0.05)

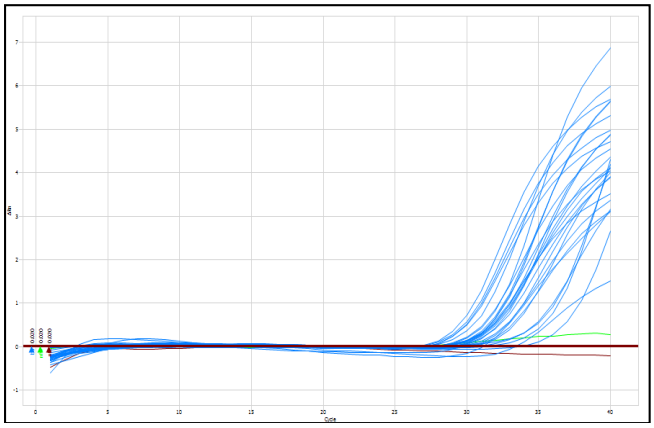

Plate 1: *β-actin* qRT-PCR Amplification Plot

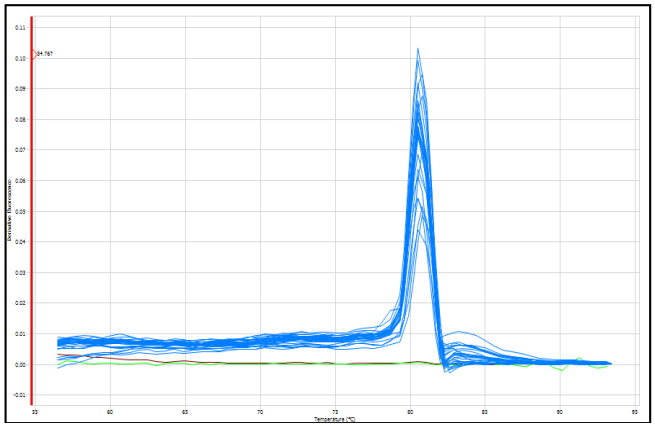

Plate 2: *β-actin* qRT-PCR Melt curve

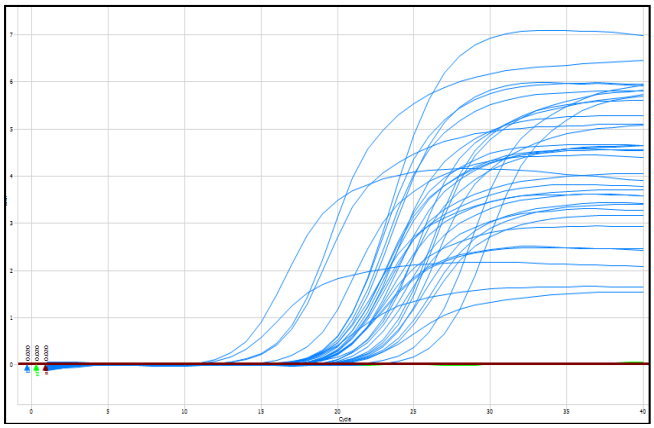

Plate 3: *ER-α* qRT-PCR Amplification plot

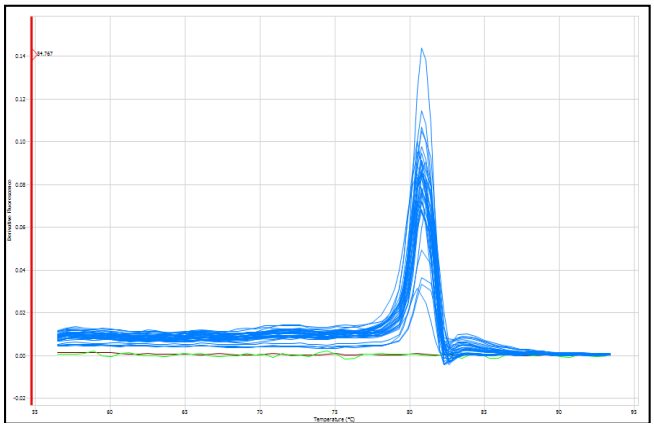

Plate 4: *ER-α* qRT-PCR Melt curve

Conclusion

In agreement to the present results, Prapaiwan *et al.* (2017) [12] stated that the mechanism of cervix dilation in pyometra bitches might not be involved with the expression of ER . In the present study, ER-*α* in the uterine cervix of control, open and closed cervix pyometra had no significant difference in expression signifying the non-existence of its influence in controlling the cervical patency.

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