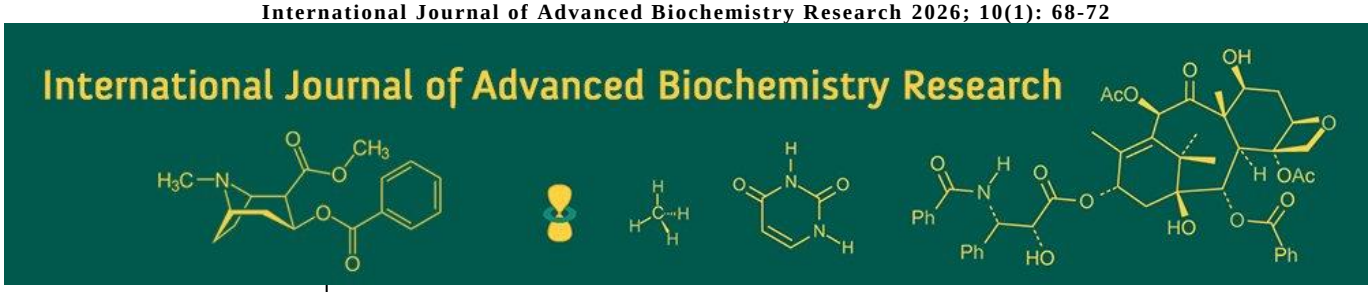

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Effect of progesterone receptor on cervical patency in canine pyometra

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Abstract
Pyometra in dogs is a life-threatening uterine condition affecting intact bitches. It most commonly occurs during the dioestrous phase of the oestrous cycle. This study focused on the role of Progesterone receptors (PR) in influencing the cervical patency in dogs diagnosed with pyometra. A total of twelve pyometric bitches—six with an open cervix and six with a closed cervix—were included, along with six healthy bitches in dioestrus serving as controls. All animals underwent ovariohysterectomy under a standardized anaesthetic protocol. Cervical tissue samples were collected and stored in RNA later for subsequent gene expression studies. The levels of PR mRNA expression were evaluated using real-time quantitative PCR (qRT-PCR). No statistically significant variation in PR expression was observed among the groups. The findings of the study indicated that PR does not play a role in the regulation of cervical patency.

Keywords: Canine pyometra, cervical patency, progesterone receptor, real time quantitative PCR

Introduction
Vermeirsch *et al.* (2000) [11] stated that because of progesterone (P_4) action, cytosol and nuclear PR were reduced and uterine cytosol PR concentration was lower in cystic endometrial hyperplasia (CEH-P) bitches than the normal ones during dioestrus which indicated a strong P_4 action and an intense PR inactivation in the uterus. Vermeirsch *et al.* (1999) [11] stated that PR expression was badly associated with the serum P_4 level, but the potency and total scores were significantly and positively correlated with the testosterone and oestradiol-17_β level. Kovacs *et al.* (2004) [5] concluded that oestradiol-17_β induced up-regulation of estrogen receptor (ER) and PR and P_4 down-regulated the ER. Ververidis *et al.* (2000) [11] reported that high serum P_4 levels helped in over-activation of uterine PR, which led to higher than usual suppression of ER and PR. In CEH-P cases, high serum oestradiol-17_β levels didn't perform during dioestrus, also when P_4 was withdrawn it activated ERs during early anoestrus. Also, they stated that in CEH-P cases, which occurred during early anoestrus, uterine cytosol ER concentration was lower than the normal.
On the contrary, Prapaiwan *et al.* (2017) [9] reported that in cyclic dogs, cervical dilation was influenced by these hormone receptors expression. Also, they stated that dilation of cervix in both pyometric and dioestrous bitches were possibly regulated by different mechanisms. They concluded that the variation of gene localization and expression of the OTR in association with ER and PR in reproductive tissues of pyometric bitches, showed different OTR localisation and expression of the ER and PRs might be involved in pathogenesis of pyometra. Also they experimented that in affected bitches, ERα localisation has been reduced in the uterus and also integrated with suppression of E_2 and/or prolonged P_4 levels for triggering uterine infection. Identically, an increased PR localization and decreased ERα expression were noticed in both segments of the cervical tissue of pyometra cases. Darko *et al.* (2018) [2] stated that uterine endometrial cells with CEH expressed a moderate reaction on PR and a strong reaction on ER. But on comparison between the sex steroid receptors expression of uteri from pyometra cases and normal dioestrus bitches, ER was expressed lesser in all the uterine layers of pyometra affected bitches. But PR was expressed higher in almost all uterine layers of pyometra cases. Based on the above findings, it was assumed that changes in ERα and progesterone receptor expression in uterus of bitch had a vital function in pyometra pathogenesis.

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 (4 °C) and transported to the laboratory and stored at-80 °C 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 (cDNA) was synthesised from isolated total RNA using RevertAid first strand cDNA synthesis kit (Thermo Scientific, Cat# K1621). Exon spanning primers were designed using online primer design software NCBI. GenBank Accession number were NM_001003074.1, and AF021873.2for amplification of PR and β-actin (Table 1). The specificity of the primers was checked using BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were custom synthesized commercially (Sigma Aldrich Chemicals Pvt. Ltd., Bangalore) and obtained in lyophilized form. . PCR was carried out to amplify 258 bp and 141bp fragments of PR and β-actin genes from canine cervix tissue. The annealing temperatures were optimised by using different temperatures ranging from 60-68 °C. 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 *PR* 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.

Quantitative Real Time PCR

The details of reaction mix and reaction protocol are given in table 2 and 3. The reagents required for real time PCR was thawed and kept ready. Illumina Eco ® qRT-PCR system was set ready with pre-programmed plate layout and thermal cycling conditions before the onset of the experiment. The exact calculations for finding relative quantification by Livak [7] 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

The qRT-PCR amplification plot and derivative melt curve of PR and β-actin are shown in Plate 1, 2 and 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.

Relative Quantification of PR gene expression between dioestrous and pyometric bitches

The expression of target gene (PR) 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, expression of PR was comparatively higher in open (14 folds) cervix pyometra than control (1 fold) group and this difference was found to be statistically significant (p value < 0.05). The expression of *PR* was comparatively higher in closed cervix pyometra (14 folds) than control group (1-fold) and this difference was found to be statistically significant (p value < 0.05). The expression of *PR* was similar in closed (1.01 folds) and open (1-fold) cervix pyometra and found to be statistically non-significant (p value > 0.05). The sequence obtained was blast using BLASTn suite available online and found 100 percent sequence similarity with *PR* gene *Canis lupus familiaris* gene. Thus, giving confirmative that the amplicon amplified for real time PCR study was specific to *PR* gene.

Progesterone receptor (*PR*) expression in uterine cervix

In the present study, expression of *PR* in closed and open cervix pyometra cases were more or less similar and non-significance was observed at five percent level. Whereas, expression of *PR* in closed cervix pyometra was 14 folds higher than the dioestrous group and this difference was found to be statistically significant (p value <0.05). Also, expression of *PR* in open cervix pyometra was 14 folds higher than dioestrous dogs and this variation was found to be statistically significant at five percent level. Hence, from the above observations it was inferred that *PR* expression was upregulated in the cervix of both open and closed cervix pyometra. The higher P_4 levels (27.36 ng/mL) in normal dioestrous dogs, recorded in the study would have down-regulated the *PR* expression in the uterine cervix which finds agreement with the findings of Amso *et al.* (1994) [1] who reported that *PR* were down-regulated by circulating serum P_4. Also, Vermeirsch *et al.* (2000) [11] stated that the *PR* expression was inversely related to the serum P_4 level. Higher expression of *PR*s (14 folds) observed in pyometric dogs could be attributed to the relatively lower P_4 levels (17.19 and 22.38 ng/mL) in open and closed pyometric dogs respectively, this would have a reduced effect on the down-regulation the *PR* expression in the uterine cervix as has been observed by Vermeirsch *et al.* (2000) [11] who stated the inhibitory effect of circulating P_4 on its receptors. The *PR* expression between open and closed pyometric dogs were similar when compared against dioestrous bitches, which signifies the effect of *PR* in the pathogenesis of CEH-pyometra.

Corroborative to the findings of increased expression of *PR* in pyometric dogs, Darko *et al.* (2018) [2] also reported a strong reaction to *PR* in the uterus of the pyometric bitches. However, the *PR* expression did not vary between open and closed indicating the non-existence of its influence on the

patency of cervix.On agreement to the present results, Tamada *et al.* (2012) [10] stated that no association was found between *PR* expression and cervical patency of pyometra bitches. Prapaiwan *et al.* (2017) [9] stated that the mechanism of cervical dilation in pyometric bitches might not be involved with the expression of *PR*.Similar study in pyometric uterus by Bosschere *et al.* (2003) [3] inferred that expression of *PR* in pyometricbitches uteri were more than that of dioestrous bitches.Also, Darko *et al.* (2018) [2] reported that the uterus with pyometra cases showed a moderate to strong reaction on *PR*, and a weak reaction on *ER*.

Kunkitti *et al.* (2011) [6] reported that *PR* concentration in the uterine cervix showed non-significance in difference between closed and open pyometric dogs regardless of oestrous stage. They also gave a different opinion regarding the expression of *PR* in pyometric bitches and stated that *PR* concentration in pyometric bitch's cervix were lower than dioestrous dogs which was contrary to our study. However, they also opined that *PR* expression didn't affect the cervical patency in pyometra affected bitches neither in anoestrus nor dioestrus. On contrary to the present findings, Volpato *et al.* (2012) [14] opined that concentrations of *PR* were comparatively higher in closed pyometric dogs when compared to open ones, however the difference in the expression of *PR* between closed and open pyometric dogs were without significance.

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

Gene	Primer	Sequences (5'-3')	Product size (bp)	Accession Number
β-actin	Forward	ATGGAATCATGCGGTATCCAC	141	AF021873.2 (Pisamai *et al.*, 2016)
	Reverse	CTTCTGCATCCTGTCAGCAA		
	Reverse	ACGGTGGATATGGTCCTTCTCT		
PR	Forward	ACGGTGGATATGGTCCTTCTCT	258	NM_001003074.1
	Reverse	ATTTTCGACCTCCAAGGACCAT		

Table 2: qRT-PCR reaction mixtures used to amplify *PR* 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)
PR 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 *PR* 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
	PR	*β-actin*				
Dioestrus	19.42±0.40	26.49±0.94	-7.07±1.02	0±1.02	1	0.014*
Open	17.61±0.66	28.50±0.42	-10.89±0.78	-3.82±0.78	14.13957	

(*Significant at p value <0.05)

Table 5: Relative quantification of *PR* 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
	PR	*β-actin*				
Dioestrus	19.42±0.40	26.49±0.94	-7.07±1.02	0±1.02	1	0.031*
Closed	16.68±1.12	27.60±0.37	-10.51±1.18	-3.84±1.18	14.37012	

(*Significant at p value <0.05)

Table 6: Relative quantification of *PR* 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
	PR	*β-actin*				
Open	17.61±0.66	28.50±0.42	-10.89±0.78	0±0.78	1	0.98 NS
Closed	16.68±1.12	27.60±0.37	-10.91±1.18	-0.02±1.18	1.016305	

(NS-Nonsignificant at p value ≥ 0.05)

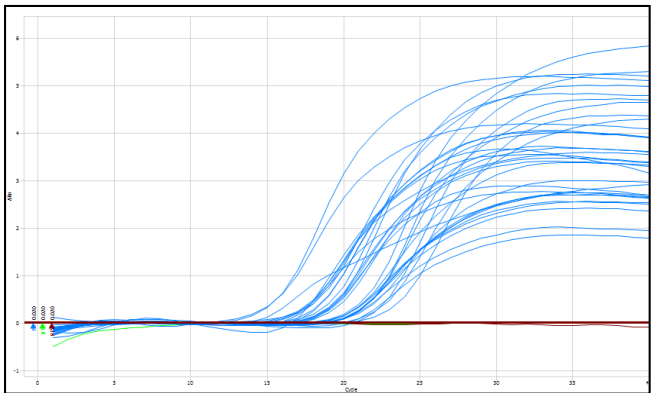

Plate 1: *PR* qRT-PCR Amplification plot

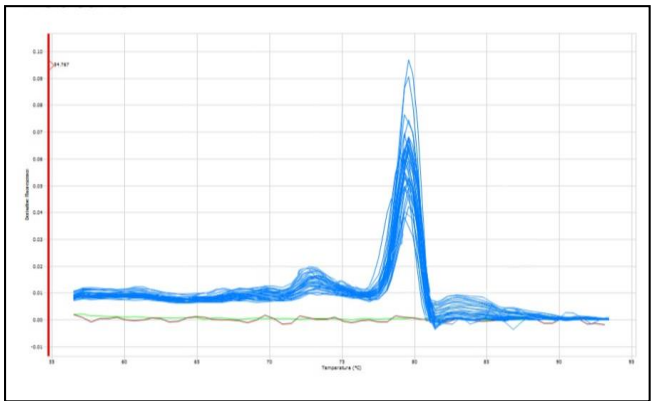

Plate 2: *PR* qRT-PCR Melt curve

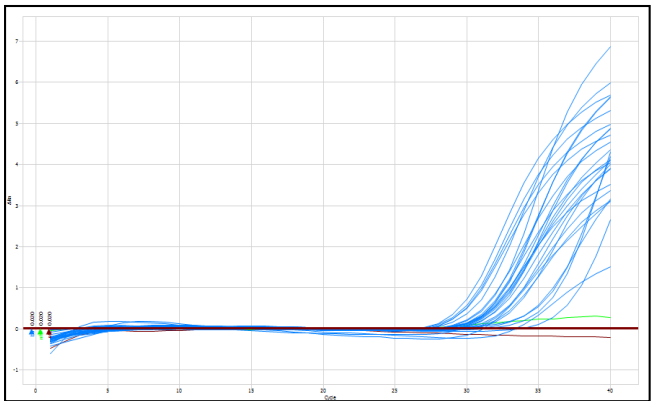

Plate 3: *β-actin* qRT-PCR Amplification Plot

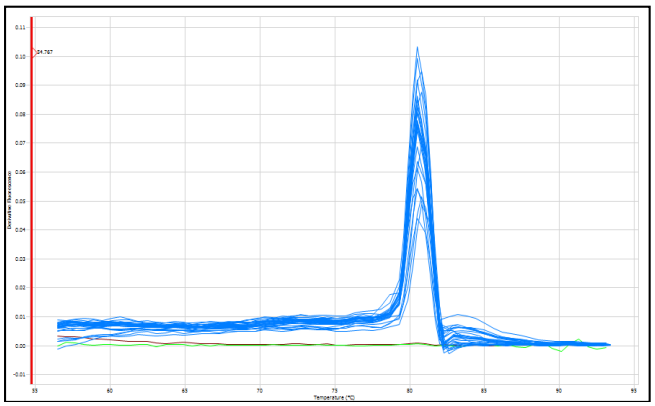

Plate 4: *β-actin* qRT-PCR melt curve

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Conclusion

In this study it is concluded that there is no association between PRs and cervical patency in pyometric bitches. Eventhough the expression of PR was high in the open pyometric bitches, statistically no significance could be noticed. Hence the cervical patency is regulated by some other factors in canine pyometra.

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