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RESEARCH ARTICLE

Expression Profile of Prostaglandin Enzymes in Cystic Endometrial Hyperplasia in Dogs: The Results of a Hypothesis in Clinical Trial

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Abstract: The expressions of prostaglandin synthesis enzymes and estrogen, progesterone receptors in canine cystic endometrial hyperplasia (CEH) were reported in this manuscript. Uterine tissue samples were collected from bitches with CEH (n=5), CEH-P (Cystic endometrial hyperplasia-Pyometra) (n=5) and healthy-negative control group, CG (n=5). Immunohistochemistry was carried out for the estrogen (ER) and progesterone receptor (PR) detection. Shock-frozen samples were utilized in mRNA extraction and Real-Time PCR was performed. Gene expression of $PTGS_2/COX_2$, PTGES, PTGER4, PGFS, PTGFR and PGR were detected higher in the CEH group compared with CG. The PGFS and PTGFR (FP) mRNA expressions were significantly increased in CEH compared with other groups. Expression of progesterone receptor mRNA (PGR) was highest in CEH and statistically different from the CEH-P group ($P<0.05$). No PR immunostaining was observed. ER staining had been detected in endometrial glands, endometrial stoma and myometrium, however hyperplasic glands in propria mucosa had lower or no ER scores. Based on the results of this study, the high levels of prostaglandin enzymes and low ER scores in CEH could be a preliminary step for the next stages of severe differentiation of endometrium.

Keywords: *Canine, Cystic endometrial hyperplasia, Estrogen receptor, Prostaglandin*

Köpeklerde Kistik Endometriyal Hiperplazide Prostaglandin Enzimlerinin Ekspresyon Profili: Klinik Deneylerde Bir Hipotezin Sonuçları

Öz: Bu çalışmada köpek kistik endometriyal hiperplazi (KEH)'de prostaglandin sentez enzimleri, östrojen ve progesterone reseptörlerinin ekspresyonları rapor edilmiştir. Uterus örnekleri KEH (n=5) ve KEH-P (Kistik endomteriyal hiperplazi- pyometra) (n=5)'lı köpeklerden ve sağlıklı negatif kontrol köpeklerinden CG (n=5) toplanmıştır. Östrojen (ER) ve progesterone (PR) reseptörü belirlenmesi için immunohistokimya kullanılmıştır. Şok dondurulan doku örnekleri mRNA ekstraksiyonunda kullanılmış ve bu örneklerden Real Time PCR yapılmıştır. $PTGS_2/COX_2$, PTGES, PTGER4, PGFS, PTGFR ve PGR gen ekspresyonu KEH'de, kontrol grubuna göre yüksek bulunmuştur. PGFS ve PTGFR (FP) mRNA ekspresyonları KEH grubunda diğer gruplara göre istatistiksel olarak farklı bulunmuştur. Progesterone reseptor mRNA (PGR) ekspresyonu KEH'de en yüksek ve KEH-P grubuna göre farklı bulunmuştur ($P<0.05$). PR immun boyamada belirlenememiştir. ER boyanmaları ise endometriyal bezler, endometriyal stroma ve miyometriyumda gözlenirken, proprio mukozadaki hiperplastik bezlerde ya çok az boyanmış ya da hiç boyanma gözlenmemiştir. Bu çalışmanın sonuçlarına dayanarak, CEH'deki yüksek prostaglandin enzim seviyeleri ve düşük ER skorları, endometriumun şiddetli farklılaşmasının sonraki aşamaları için öncü bir adım olabilir.

Anahtar Sözcükler: *Köpek, Kistik endometriyal hiperplazi, Östrojen reseptör, Prostaglandin*

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Introduction

Cystic endometrial hyperplasia (CEH), is an important uterine pathology of non-castrated female dogs. The degenerative changes within the uterus could be life-threatening when CEH is accompanied by opportunistic pathogens [1]. The development of CEH has been considered the initial phase of pyometra and these morphological changes could be mostly diagnosed with pyometra [2]. Repeated endocrinological alterations, bacteriologic toxic factors (mainly *E. coli),* and the application of medroxy-progesterone acetate and tamoxifen which could be the consequence of altered receptor expressions in the uterus, are the underlying causes of the disease [1,3-5]. However, CEH develops in all older bitches some of which could progress to pyometra. As CEH does not inevitably progress to pyometra, CEH and pyometra could originate independently from each other [2,3].

The substantial changes resulting in CEH are widely accepted as the association of estrogen/progesterone imbalance on endometrium during estrus cycles in bitches [1]. However, various applications during diestrus have been reported to cause CEH such as intrauterine inoculation of *Escherichia coli* and intraluminal insertion of a wire [6,7]. Either way, the initiation of cellular changes is induced by infectious agents and/or foreign bodies in the uterus in diestrus. On the other hand, alterations in steroid hormone concentrations during estrus cycle could influence uterine immune surveillance in bitches [8,9]. The control of this process is achieved by prostaglandins [10,11]. Because, irreversible tissue differentiation is accepted that they are acting as wounds that fail to heal [12]. Apart from this information, a direct relationship between the levels of PG synthesis and the incidence of malignancy has been reported in human and animal models [13].

Cyclooxygenase (COX_2-PTGS2) is a rate-limiting enzyme that mediates the conversion of arachidonic acid to prostaglandins [14]. PGE_2 is an important COX protein product that is synthesized by specific synthases (PGESs). COX_2 and PGE_2 contribute not only to physiological processes but also to inflammation and oncogenesis [14-16]. PGE_2 exerts its cellular effects by interacting with EP receptors (EP1, EP2, EP3, or EP4), which are coupled to G proteins [17]. The interaction between PGE_2 and its EP receptors plays important roles in differentiation and progression in cells by complex regulation of signaling transduction [18]. Based on the close relationship between chronic inflammation and endometrial cancers, the central inflammatory pathway involved in carcinogenesis is known as cyclooxygenase 2-prostaglandin E_2-prostaglandin E_2 receptors (COX-2-PGE_2-EPs) [19].

In the light of the detailed information on the relation between malignancy and prostaglandin synthesis in humans [19], the possible transcription pattern of genes encoding prostaglandin synthesis in cystic endometrial hyperplasia has been studied. Endometrial hyperplasia is a precursor lesion for endometrial carcinoma in women [20]. Although cystic endometrial hyperplasia is widely not accepted as a malignancy of bitches, the non-infected CEH cases were evaluated in terms of prostaglandin synthesis. The hypothesis of this study is based on the determination of levels of genes encoding prostaglandin enzymes in cystic endometrial hyperplasia in the dog. The high levels of prostaglandins in non-infected CEH cases could be a precursor indicator for severe differentiation of cells. Although endometrial adenocarcinoma is a rare tumor in bitches and queens [21], a case of adenocarcinoma with cystic endometrial hyperplasia-pyometra complex in a dog and a cat were reported [22,23]. Owing to the information about the diagnosis of cystic endometrial hyperplasia, pyometra, and uterine neoplasia in a bitch, the results of the hypothesis of this study could provide new information on canine uterine disorders.

Material and Methods

Ethical Statement

Approval from the ethics committee of the Bursa Uludag University to use the animals was obtained (Approval no: 2019-09/01).

Animals

Fifteen bitches presented to the Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine in Bursa, were used in this study. All dogs had been brought to the clinic, either for purpose of spaying or with a history of pyometra. The clinic, ultrasound examinations were carried out and blood samples were collected for hematological and biochemical analysis before operations. Ovariohysterectomy was performed in bitches aged 2-10 years. They were allocated into groups after a histopathological examination of the uterus and diagnosis of canine CEH-P and CEH by two different pathologists. The classification of groups has been proposed according to Dow's morphology criteria, histological lesion and De Bosschere's histomorphological classification criteria *(Table 1)*.

No pus and/or mucus was observed either in macroscopic or histopathological examination of groups CG ad CEH. On the other hand, no signs of inflammation, including neutrophil leukocytes were observed during the histopathological examinations in these groups.

The patients were also evaluated clinically by means of the type of pyometra. The animals with open cervix pyometra were included in the study. Based on the information on last estrus of patients, obtained in anamnesis, all animals in three groups were in late diestrus [26]. Since

Table 1. Classification of groups

Groups	Dow's CEH Types Classification *	Endometrial Hyperplasia Index**	De Bosschere's Histomorphological Classification***
Cystic Endometrial Hyperplasia-Pyometra (CEH-P) Group (n=5; with mean age of 5.4±2.3 years)	Type IV Severe clinical signs of abdominal distention, damage in other abdominal organs with abnormal blood hematology and biochemistry	Grade 3 Severe hyperplastic and cystic changes with increased endometrial thickness of <2 times normal	Pyometra (hyperplastic)
Cystic Endometrial Hyperplasia (CEH) Group (n=5; with a mean age of 8.8±0.83)	Type I presenting signs of CEH without inflammatory process and no clinical signs	Grade 2 Hyperplastic and cystic change with an increased endometrial thickness of <2 times normal	Severe CEH
Control Group (n=5; with mean age of 3.0±0.70)	-	Grade 0 No uterine abnormalities and hyperplastic changes	Normal

Type of CEH classification according to Dow[24]; ** endometrial hyperplasia index according to Munson et al.[25] and * histomorphological classification according to De Bosschere et al.[3]*

the animals in the CEH-P group were processed in late diestrus (52.00±15.68 days), the scheduled operations were submitted for the bitches in CEH and CG groups in the second stage of diestrus (49.80±16.22; 45.00±7.90 days, resp), as described by Veiga et al.[26]. The results of exfoliative vaginal cytology and the existence of CLs in CG and CEH groups were also recorded. CEH-P was diagnosed after the determination of leukocytosis, increased ALT, AST, urea and creatinine concentrations, including abnormal clinical signs such as inappetence, depression, polyuria and polydipsia. The fluid filled uterus was observed by transabdominal ultrasonography before operations.

Surgical Procedures

The bitches were premedicated with the administration of intramuscular xylazine HCl (1 mg/kg) (Alfazyn® 2%; Egevet/Turkey) and for induction 10 mg/kg ketamine HCl (Alfamin®, Egevet, Turkey) was applied intramuscularly. General anaesthesia was induced with isoflurane (ISO Forane®, Abbott, UK) in oxygen at a concentration of 2% and they were closely monitored during the surgery with electrocardiography. Analgesia was achieved with carprofen (3 mg/kg, i.m.) (Rimadyl®, Pfizer, İstanbul, Turkey). Laparotomy was performed by a suprapubic incision. The ovarian artery and vein were isolated by breaking down the mesovarium and ligated. Ligatures were placed on the ovarian pedicles and the body of the uterus adjacent to the cervix. Ovaries and uterus were removed carefully. The peritoneum, muscles and skin incision were closed with 1 absorbable and 1 non-absorbable suture materials, respectively.

Tissue Collection and Preservation

Immediately after operations, tissue samples from the middle part of cornu uteri showed cystic structures measuring 2x1x1 cm were excised and rinsed with wash buffer, and phosphate-buffered saline (1xPBS, pH 7.4). Specimens were fixed overnight in 10% neutral phosphate-buffered formalin for 24 h and subsequently dehydrated in a graded ethanol series. They were finally embedded in paraffin and used for routine histopathology and immunohistochemical staining. For total RNA isolation, small pieces of the uterus (1x1x1 cm) were snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Histopathological Examination

The histopathological examinations were completed with Haematoxylin and eosin (H&E) stained sections and they were classified according to Munson et al.[25] and De Bosschere et al.[3]

Total RNA Isolation

Deep-frozen (-80°C) uterine tissue samples were pulverized with a sterile mortar and pestle under liquid nitrogen. Total RNA was isolated from 15 mg of tissue powder using innuPREP RNA Mini Kit (Analytik Jena AG, Jena, Germany), including the elimination of genomic DNA, following the manufacturer's instructions. On ethidium bromide-stained 1% agarose gel, RNA integrity was tested by the presence of intact bands of 18S and 28S. mRNA purity and quantity were determined by optical density (OD) measurement (NanoDrop 2000c, Thermo Scientific, USA). The OD 260/280 ratio of all samples was between 1.8-2.0.

Real-Time PCR Measurements

Total RNA (adjusted to be 1.0 µg/11 µL with nuclease-free water) and 1 µL (100µM) Random Hexamer (RevertAid RT Reverse Transcription Kit, Thermo Fisher Scientific, USA) were incubated at 65°C for 5 min and snap cooled on ice. Twelve microliters of total RNA (1.0 µg) in 20 µL

final volume were then reverse transcribed with 4 µL 5xReaction Buffer, 1 µL RiboLock RNase Inhibitor (20 U/µL), 2 µL 10 mM dNTP Mix and 1 µL RevertAid RT (200U/µL) (all from RevertAid RT Reverse Transcription Kit, Thermo Fisher Scientific, USA). Real-time PCR was performed in a LightCycler® 480 II thermocycler (Roche Applied Science, Germany) by measuring each sample in triplicate. Reactions were performed in a final volume of 10 µL using 5 µL LightCycler® 480 SYBR Green I Master (Roche Applied Science, Germany), 10 µM of each primer, 1.9 µL PCR-graded water and 2.5 µL cDNA. Amplification conditions were the same for the target and the reference genes: initial denaturation for 5min at 95°C followed by 45 cycles at 95°C for 10s, 57°C for 10 s and 72°C for 10 s.

For each sample, amplification and melting curves were obtained using Absolute Quantitation/Second Derivative and Tm Calling analysis modes in the LightCycler® 480 II software. Melting curve analysis was used for each primer pair to confirm the gene-specific peak and the lack of primer dimer. Results were evaluated by E-method. To determine the relative expression of genes Ct value was normalized by β-Actin and GAPDH. The values in the negative control group (CG) were compared with CEH-P and CEH groups and fold change values were calculated (fold change>2= Target Upregulation; fold change<0.5= Target Downregulation). Primer pairs for the target genes $PTGS_2/COX_2$, PTGES, PTGER2, PTGER4, PGFS, PTGFR, PGR and the reference genes β-Actin and GAPDH were designed using NCBI *(https://www.ncbi.nlm.nih.gov/)* – Ensemble *(https://www.ensembl.org/index.html)* databases and purchased from Macrogen Inc. (Korea). *(Table 2)*.

Immunohistochemical Staining

Sections prepared from formalin-fixed paraffin-embedded uterine tissue samples were immunostained for the detection of the estrogen receptor protein (ER) and progesterone receptor (PR). Two µm tissue sections were incubated in a pre-treatment system (PT Link, DAKO, Denmark) at 97°C for 20 min for antigen retrieval before immunohistochemistry. To inactivate endogenous peroxidase, tissues were treated with 0.3% hydrogen peroxidase in methanol for 5 min at room temperature. Primary antibodies used were the following for 30 minutes: Monoclonal mouse anti-human progesterone receptor (1:250 dilution; Clone PgR, DAKO, Denmark) and monoclonal rabbit anti-human estrogen receptor α (ready-to-use; Clone EP1, DAKO; Denmark). Horseradish peroxidase streptavidin conjugate was used for 30 min and visualization was performed by using the substrate DAB chromogen mix for 10 min at room temperature.

Quantitative Evaluation

Sections were evaluated concerning ERα and PR localization semi-quantitatively using a light micro-

Table 2. *Primers used in Real-Time PCR analysis*

Target Gene	Gene ID	DNA Sequences of the Primers (5'-3')		Product Size (bp)
PTGS2/COX2	442942	Forward	CTGTACCCGAACAGGATTCTAC	120
		Reverse	CCCTTGAAGTGGGTAAGTATGT	
PTGES	480698	Forward	CAAGTGAGGCTTCGGAAGAA	102
		Reverse	GAGGCAGCGATCCACATC	
PTGER2	403797	Forward	CACCTCATTCTCCTGGCTATT	118
		Reverse	GAGCTTGGAGGTCCCATTT	
PTGER4	403589	Forward	CAGATGGTCATCCTGCTCAT	107
		Reverse	TTCACCAAACGTGGCTGATA	
PGFS	497070	Forward	GGACATCATCCTGACTGCATAC	101
		Reverse	CATTGAGAACTGGGTCCTTCAG	
PTGFR	479981	Forward	GCATTTGCTGGAGTCCATTTC	117
		Reverse	TTTGATTCCACGTTGCCATTC	
PGR	403621	Forward	TACCAGCCGTACCTCAACTA	132
		Reverse	GACACCATAATGACAGCCTGAT	
GAPDH	403755	Forward	GAACATCATCCCTGCTTCCA	129
		Reverse	CAGGTCAGATCCACAACTGATAC	
β-Actin	403580	Forward	GCCAACCGTGAGAAGATGA	97
		Reverse	CAGAGGCGTACAGGGACA	

scope. Three different fields, endometrial glands, endometrial stoma and myometrium, of each section per specimen at 40× magnification were evaluated for immunohistochemical staining. Values were recorded as percentages of positively stained target cells in each of the four intensity categories as follows: no staining; +, weak; ++, moderate; +++, strong staining. HSCORE value was calculated by summing the percentages of cells that stained at each intensity category and multiplying that value by the weighted intensity of the staining using the formula HSCORE = ∑(I X PC), where I is the intensity score and PC is the corresponding percentage of the cells.

Statistical Analysis

The statistical analysis of Real-Time PCR data was analyzed by means of Kruskal-Wallis variance analysis (P<0.05). Differences by Kruskal-Wallis Test were compared by the Mann-Whitney U test. Results were expressed as mean ± SE for each category. The Real-Time PCR data were subjected to statistical analysis using $2^{-\Delta\Delta Ct}$ values to determine statistically significant differences in all gene expressions. Expression values in CEH-P and CEH groups were checked against the values in the control group (CG).

The statistical analysis of immunohistochemical staining of the endometrial gland and myometrium samples were carried out using Kruskal-Wallis variance analysis, whereas endometrial stroma immunostaining scores were analyzed by ANOVA. Differences between Kruskal-Wallis Test and ANOVA were compared by Mann-Whitney U and Tukey test, respectively. All statistical analysis was performed with SPSS software (SPSS for Windows. Standard version release 11,5. Copyright SPSS Inc., 2002, the USA).

Results

Gene Expression of Prostaglandins

Gene transcription of $PTGS_2/COX_2$, PTGES, PTGER2, PTGFR and PGR were statistically different between groups [(P<0.05); (P=0.006), (P=0.038), (P=0.007), (P=0.002), (P=0.007), respectively]. The highest expression of $PTGS_2/COX_2$ mRNA was detected in CEH-P compared with CG [(P<0.05); (P=0.008)], whereas approximately 8-fold increased expression of $PTGS_2/COX_2$ mRNA in the CEH group was also statistically different compared with CG [(P<0.05); (P=0.008)] *(Fig. 1)*. The only difference of PTGES mRNA expression was found between CEH and CG groups [(P<0.05); (P=0.008)], although the expression of PTGES was 15- and 6.5 fold higher than

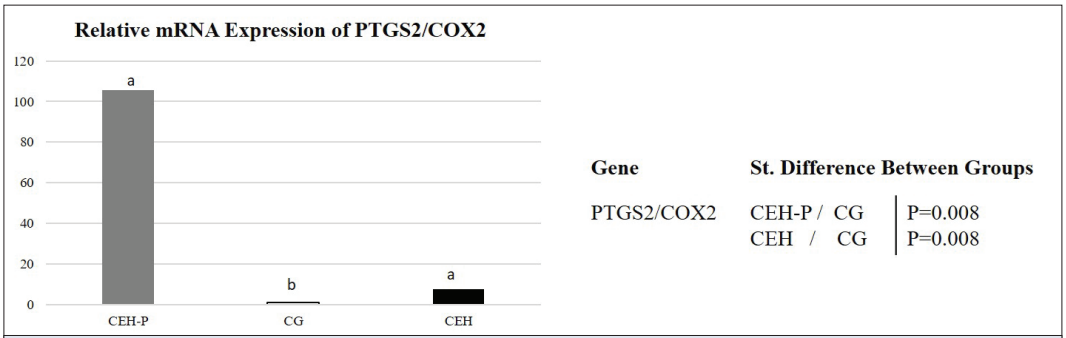

Fig 1. qPCR of PTGS2/COX2 in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown as fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group. a,b indicates the difference between groups, P<0.05

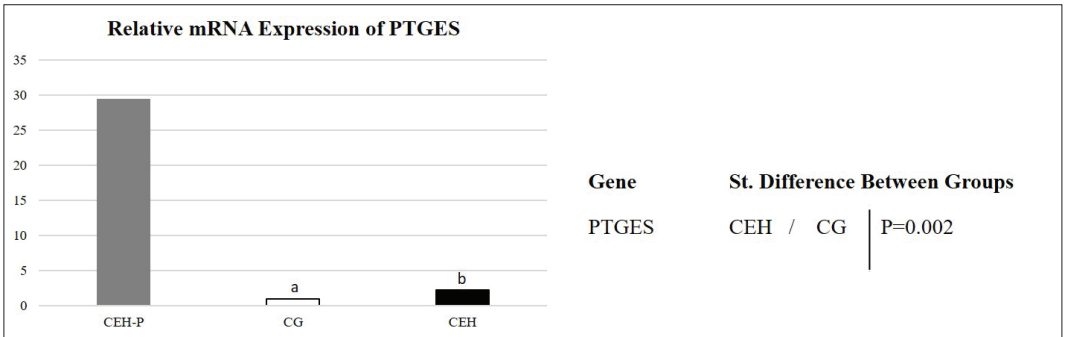

Fig 2. qPCR of PTGES in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown as fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group. a,b indicates the difference between groups, P<0.05

CG and CEH groups, respectively *(Fig. 2)*. A higher transcriptional level of PTGER2 was observed in CEH-P than CEH and CG groups [(P<0.05); (P=0.008)], whereas no statistical difference was detected of PTGER4 expression between groups (P>0.05) *(Fig. 3, Fig. 4)*. The PGFS and PTGFR (FP) mRNA expressions were significantly increased in CEH compared with other groups and the statistical difference in PTGFR mRNA was observed between groups (P<0.05) *(Fig. 5, Fig. 6)*. Expression of progesterone receptor mRNA (PGR) was highest in CEH and statistically different from the CEH-P group [(P<0.05); (P=0.016)] *(Fig. 7)*.

Immunohistochemical Localization of Estrogen and Progesterone Receptors

Immunohistochemical staining of ER receptor was

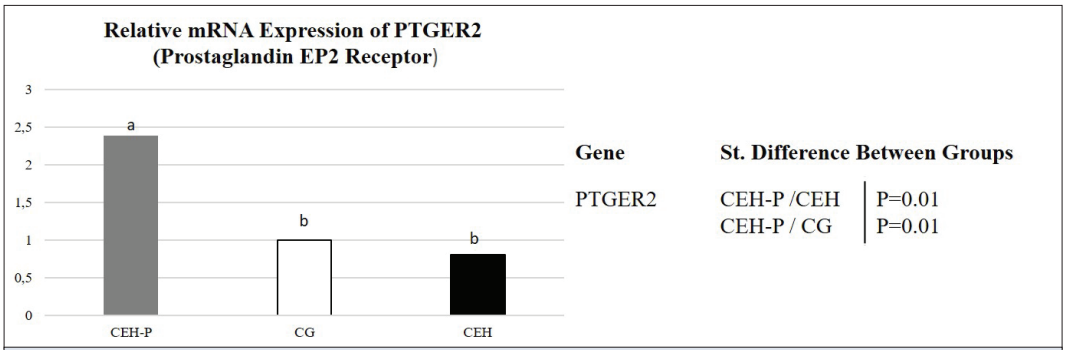

Fig 3. qPCR of PTGER2 in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group. a,b indicates the difference between groups, P<0.05

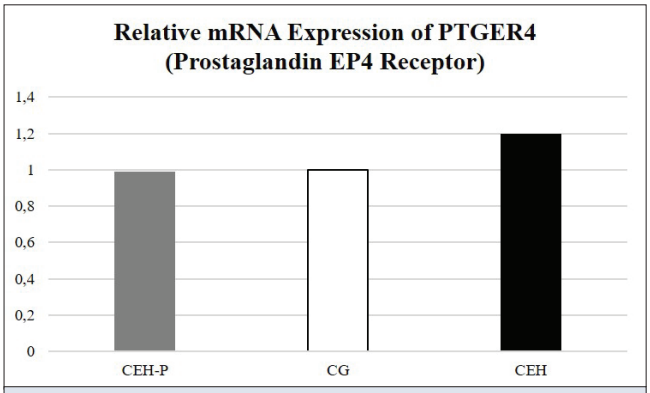

Fig 4. qPCR of PTGER4 in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown as fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group, P>0.05

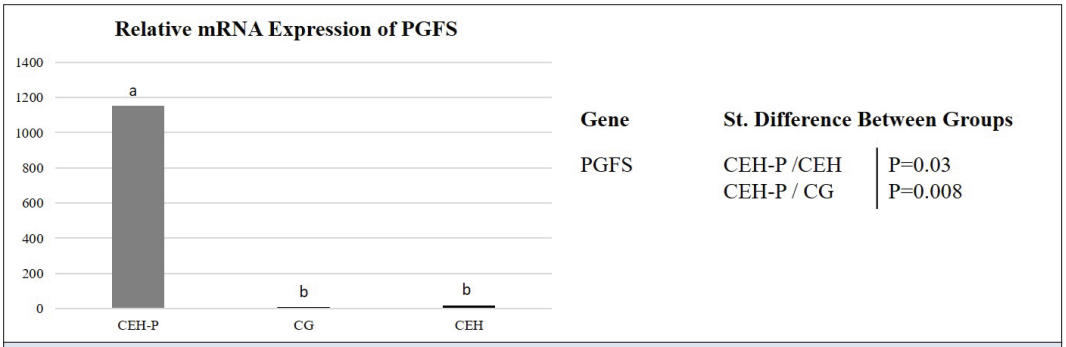

Fig 5. qPCR of PGFS in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown as fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group. a,b indicates the difference between groups, P<0.05

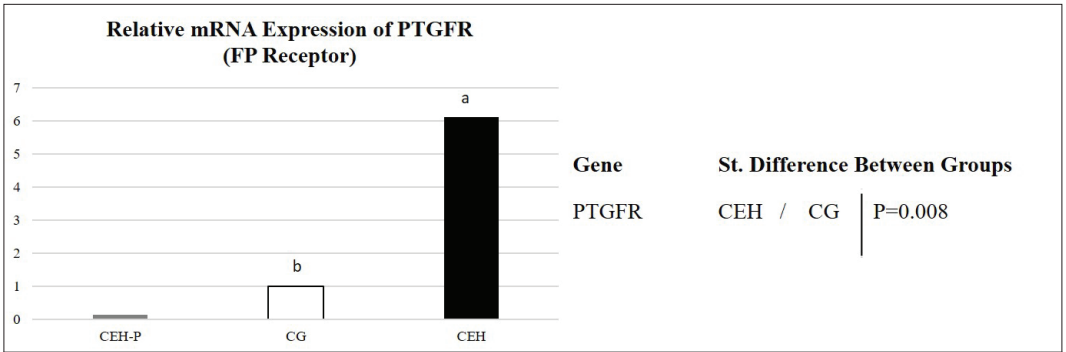

Fig 6. qPCR of PTGFR in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown as fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group. a,b indicates the difference between groups, P<0.05

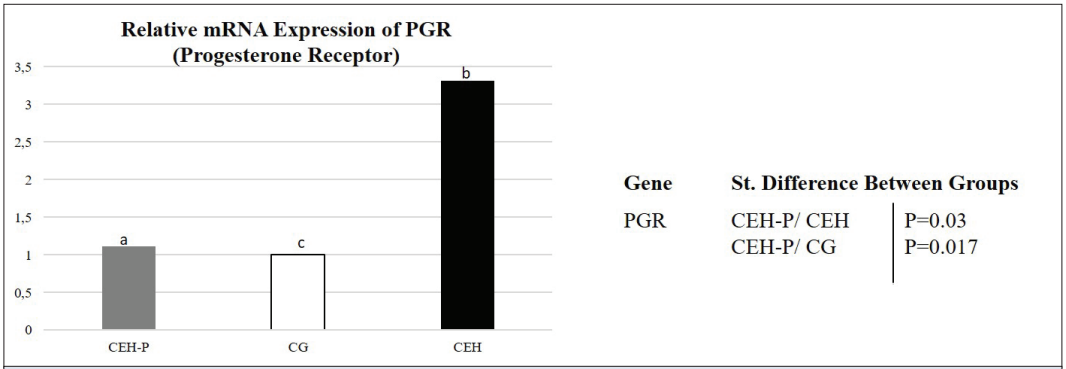

Fig 7. qPCR of PGR in CEH-P (Cystic endometrial hyperplasia-Pyometra group) and CEH (Cystic endometrial hyperplasia group). Results are shown as fold change relative to the CG (Control Group). Gene expression of CG was set at 1, and related to negative group. a,b,c indicates the difference between groups, P<0.05

statistically different among groups in endometrial glands, endometrial stoma and myometrium [(P<0.05); (P=0.030); (P=0.011); (P=0.011), respectively] *(Table 3)*. However, the only statistical difference in the immunostaining score of ER was detected between CEH-P and CG in all regions of the uterus (P=0.008) *(Fig. 8, Fig. 9, Fig. 10)*. Epithelial cells in the mucosal area and glands in propria mucosa had high estrogen receptor scores, whereas hyperplasic glands in propria mucosa had lower or no estrogen receptor scores *(Fig. 11)*. No PR immunostaining was observed in all groups.

Histopathological Examination Findings

In the sections examined in the CEH group, it was observed that the cuboidal cells in the lamina epithelialis protrude into the lumen (hyperplasia) by producing two-three-layer epithelial cells. In addition, similar formations were also found in the glandular epithelium of the lamina propria. It was also noted that the gland lumens filled with eosinophilic (proteinous) fluid and became cystic, and the cuboidal cells were flattened. In addition to lesions similar to the above, neutrophil leukocyte infiltrations were found in the gland lumens in CEH-P group *(Fig. 12)*.

Table 3. *HSCOREs of ER and PR staining in endometrial gland, endometrial stroma and myometrium in CEH-P, CG and CEH groups. a, b: Different letters in superscript in the same line indicate statistically significant difference*

Examined Microscopic Fields		CEH-P	CG	CEH	*P*
ER	Endometrial Gland	207.10±89.48[a]	300[b]	248±71.55[a,b]	0.030
	Endometrial Stroma	133.20±93.11[a]	300[b]	230.60±95.37[a,b]	0.011
	Myometrium	258.76±33.01[a]	300[b]	282±44.24[a,b]	0.011
PR	Endometrial Gland	No staining	No staining	No staining	-
	Endometrial Stroma				
	Myometrium				

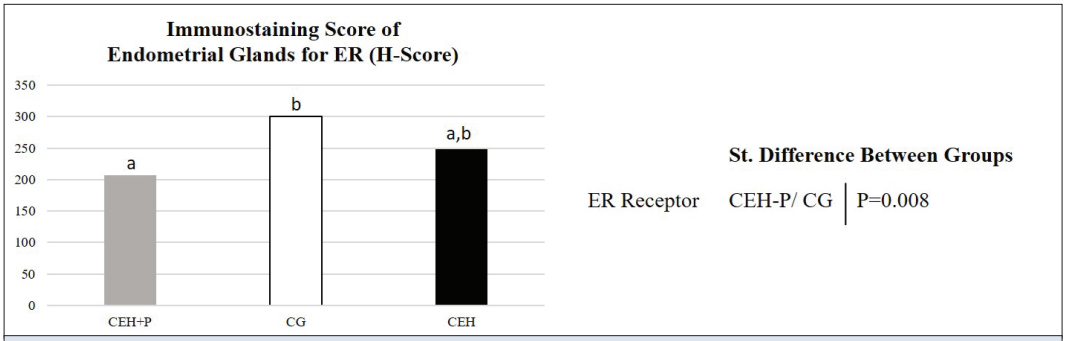

Fig 8. HSCOREs of ER reactions of endometrial glands in control group comparing with CEH-P and CEH groups. a,b indicates the difference between groups, P<0.05

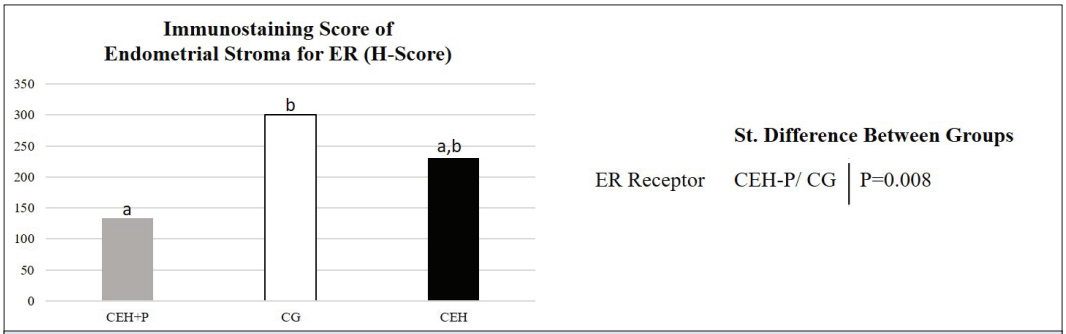

Fig 9. HSCOREs of ER reactions of endometrial stroma in control group comparing with CEH-P and CEH groups. a,b indicates the difference between groups, P<0.05

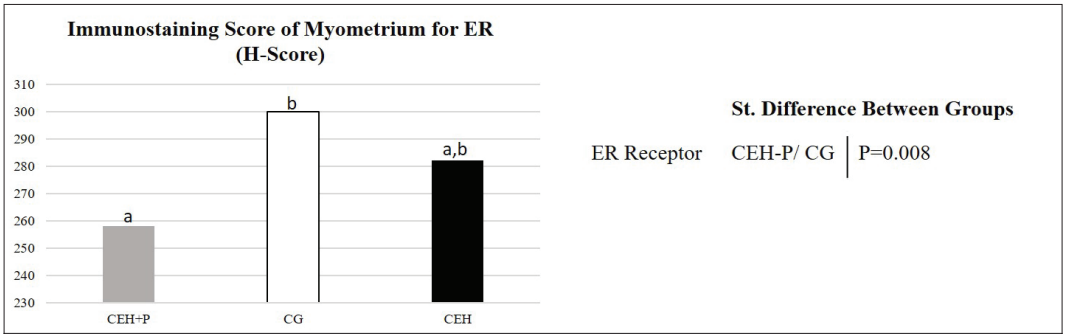

Fig 10. HSCOREs of ER reactions of myometrium in control group comparing with CEH-P and CEH groups. a,b indicates the difference between groups, P<0.05

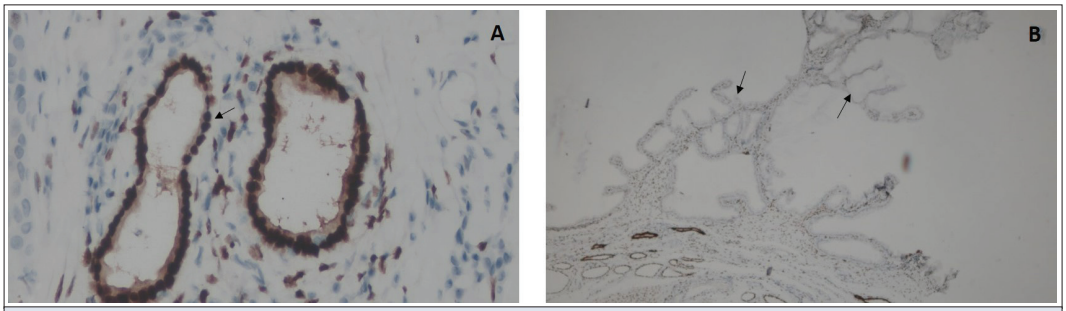

Fig 11. Immunpositive staining of endometrial gland epithelial cell nuclei (ER+) x40 magnification (A). Unstained cells with ER in hyperplastic areas (B) x20 magnification

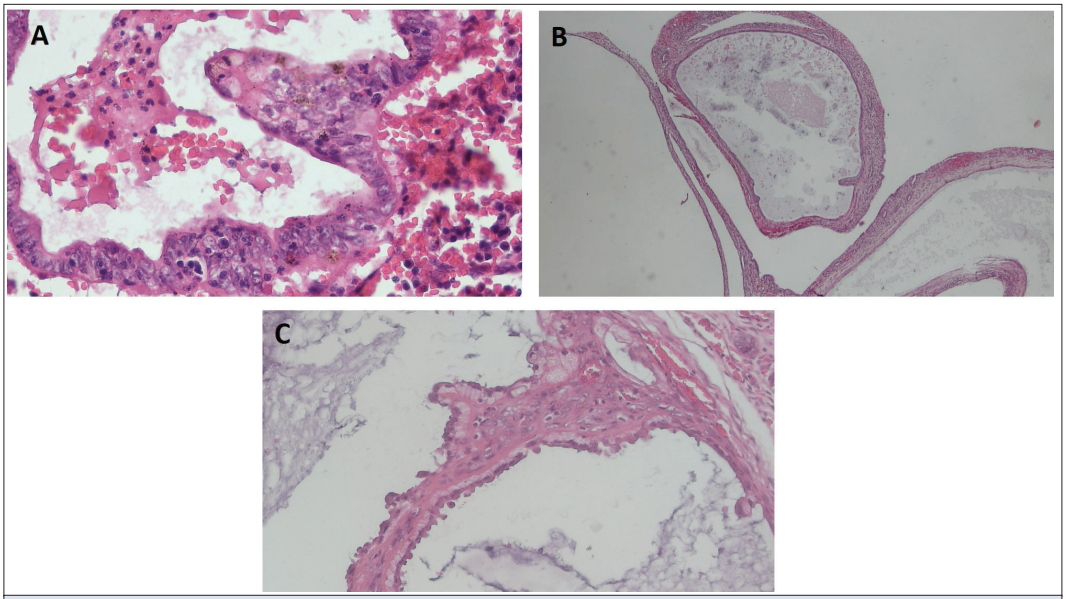

Fig 12. Haematoxylin and eosin (H&E) stained sections demonstrating (A) cystic endometrial hyperplasia-pyometra (CEH-P) (x20 magnification) and (B&C) cystic endometrial hyperplasia (CEH) (x4 magnification; x20 magnification)

Discussion

This paper reports, the regulation of the genes related to prostaglandin synthesis and its receptors in spontaneously occurring CEH cases by comparing CEH-P and CG groups. The expressional profiles of the genes are measured in CEH without any uterine accumulation and/or infection. All genes and receptors in prostaglandin synthesis were evaluated in terms of whether the reason could be leading to severe differential changing of endometrium, for the first time.

The important roles of cyclooxygenases and prostaglandins have been revealed in animal cancers. The overexpression of COX_2 has been thoroughly examined in many cancer types in animals, however, $PTGS_2/COX_2$ is known as an important enzyme, playing role in inflammatory diseases [11,14]. In the present study, extremely high $PTGS_2/COX_2$ gene transcription has been measured in CEH-P cases in dogs, which is in accordance with previous studies [14]. Further to that, 8 times higher $PTGS_2/COX_2$ transcription is being detected in spontaneously occurring CEH cases without infectious. On the other hand, a similar expression pattern was observed in PGES gene transcription. The higher PGES content was measured in bitches with CEH-P, possibly due to infection, whereas the endometrium in the CEH group had significant upregulation of the PGES gene.

Via cell surface G protein-coupled receptors (EP1-EP4), PGE_2 influences many intracellular signaling pathways, which contribute to various stages and different types of cancer [27]. The importance of EP subtypes in animal tissue differentiation is a rather new field, however, elevated levels of EP2 and EP4 biosynthesis have already been detected in response to PGE_2 in human endometrial carcinoma [27]. PTGER4 (EP4) expression was higher in the CEH group, whereas PTGER2 (EP2) expression was downregulated compared with CG. The low expression of PTGER2 in CEH cases is unknown. Other EP receptors should be taken into consideration; as various EPs could contribute to different pathologies by inducing different pathways. The upregulation of PTGER4 and downregulation of PTGER2 might follow a different pathway in CEH development, as this pathology is not been accepted as a canine cancer type.

In relation to infectious, high expression levels of PGFS and PTGFR could be the consequence of increased local inflammatory response in CEH-P cases [11,28,29]. $PGF_{2\alpha}$ acts through PTGFR and PGFS, leading to neutrophil infiltration [30]. The evidence of the high capacity of PGFS and PTGFR in CEH cases without infection compared to CG production could be a new debate in controlling endometrial changes in bitches. Though CEH is not a malignancy in canine uterine disorders, the regulation pattern of prostaglandin enzymes is different from CEH-P and healthy bitches.

CEH-P develops through the influence of sequential progestational stimulations in diestrus [31]. The strong immunoreaction for estrogen receptors (ER) and moderate reaction for progesterone receptors (PR) have been revealed in CEH with infection cases [32]. No immunostaining was observed in the immunostaining of PR in this study, whereas strong PGR gene expression was detected in CEH cases compared with CEH-P and CG. Downregulated PGR gene expression was an interesting

finding in bitches with CEH-P, which is not in accordance with previous results.

The detection of active gene expression of progesterone receptors by quantitative PCR but no immunostaining on protein level was also reported in human endometrial and breast cancers and mammary tumors in dogs [33,34]. The mismatch could be related with the isoforms of progesterone receptors or/and non-coding of truncated receptor variants by alternatively spliced transcripts [33,35,36]. The genes coding for progesterone receptors could be alternatively spliced by exon-skipping or exon duplication [33]. The defined two isoforms of progesterone receptor; isoform A (PRA) and isoform B (PRB) were transcribed from a single gene under the control of different promoters. The unexpected results are possibly related to alternative splicing as it's known as not every splice variant is translated into protein in malignant cases [33]. The different immunostaining patterns or no staining in canine mammary tumors had been revealed, suggesting that the regulation from gene to protein could be lost during malignant transformation. Further studies are needed by DNA sequence analysis.

On the other hand, distinct staining for ER was localized to endometrial glands, endometrial stroma and myometrium. Besides, decreased or no ER staining was another important finding in bitches with CEH. Long-term follow-up studies have shown that a lower percentage of ER staining has a poor prognosis with higher metastatic potent in human breast cancer [37]. On the other hand, malign tumors had decreased ER expression in canine mammary tumors [38]. The expressional alterations of ER in the hyperplastic field of the uterus should be evaluated whether they are preliminary differentiation of endometrium in further studies.

Canine adenocarcinomas are rare uterine neoplasms [21]. The dog was reported with cystic endometrial hyperplasia-pyometra complex with adenocarcinoma by Janowski et al.[23]. A similar case of multiple pathologies of the feline uterus was also available [22]. The relationship between CEH and adenocarcinoma is still unknown in bitches and queens as they are rare cases in these species. However, mild or simple hyperplasia is known as a risk of becoming endometrial cancer in women. Until now CEH cases are not accepted as neoplasia in dogs. The two pathologies in a bitch might bring new questions although no studies on the molecular level had been carried out. The high levels of prostaglandin gene expression without infectious could be related to the first step of preliminary differentiation of endometrium. CEH and pyometra cases are mostly treated by elective ovariohysterectomy operations when they are diagnosed. If it could be possible to follow these patients for a longer time, the results could easily answer the questions of the relationship between two endometrial changes in dogs and the high expressional levels of prostaglandins in CEH cases.

In summary, the high levels of prostaglandin enzymes and low ER scores in CEH could be a preliminary step for the next stages of severe differentiation of endometrium.

Availability of Data and Materials

The data sets during and/or analyzed during the current study are available from the corresponding author (G. R. Özalp) upon reasonable request.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contribution

YK: Experimental design, collection of the data, writing manuscript. ÖY: contribution to experimental design, histopathological examination, editing manuscript. AA: statistical analyses, editing manuscript. BB: Real-Time PCR analysis. MÖÖ: contribution to experimental design, histopathological examination, editing manuscript. RGÖ: Experimental design, writing manuscript & editing, supervision. All authors read, revised, and approved the final manuscript.

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