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ORIGINAL RESEARCH PAPER



Plasma concentration and uterine and ovarian expressions of insulin-like growth factor-2 in dogs with cystic endometrial hyperplasia–pyometra

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ABSTRACT

The objective of this study was to investigate the plasma concentrations of insulin-like growth factor-2 (IGF-2) as well as its expression in the uterus and ovary of healthy dogs and those with cystic endometrial hyperplasia (CEH)–pyometra complex. Group 1 ($n = 10$) included bitches with open cervix pyometra, while Group 2 ($n = 7$) consisted of clinically healthy bitches in dioestrus. The number of IGF-2 immunopositive interstitial cells was significantly higher in Group 1, whereas in Group 2 there were only two cases in which a few cells were IGF-2 immunopositive. IGF-2 immunopositivity was observed in the endometrial glandular epithelium in both groups. Additionally, interstitial fibroblasts and macrophages in the endometrium were also positive in Group 1. The concentration of plasma IGF-2 was higher in Group 1 than in Group 2 ($P < 0.05$). The concentration was positively correlated with IGF-2 expression in the endometrial glands ($r = 0.926$; $P < 0.001$) in Group 1. However, a negative correlation was present between plasma IGF-2 concentration and IGF-2 expression in the interstitial endocrine cells of the ovary in Group 1 ($r = -0.652$; $P < 0.05$). The results suggest that IGF-2 plays an important role during the inflammatory process occurring in bitches with CEH–pyometra complex as well as in the endometrium of healthy bitches in dioestrus.

KEYWORDS

cystic endometrial hyperplasia–pyometra, dog, insulin-like growth factor-2, ovary, uterus

INTRODUCTION

The cystic endometrial hyperplasia (CEH)–pyometra complex is a hormonally mediated post-oestral disorder of adult intact bitches (Feldman and Nelson, 1996; Johnston et al., 2001) and may present either with a vaginal discharge (open-cervix pyometra) or without a vaginal discharge (closed-cervix pyometra) (Smith, 2006; Pretzer, 2008). The factors contributing to pyometra include bacteria, CEH, progesterone- (P4-) dominated phase of the ovarian cycle, and exogenous progesterone and oestrogen administrations (Feldman and Nelson, 1996). Progesterone leads to endometrial proliferation and the accumulation of

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uterine glandular secretions and suppresses the contractions of the myometrium, thus creating an intrauterine environment predisposed to bacterial growth (Smith, 2006). In other words, bacterial growth increases due to the inhibition of leukocyte response to infections in the progesterone-dominated uterus (Feldman and Nelson, 1996). Furthermore, endogenous P4 or synthetic progestins may cause hypersecretion of growth hormone (GH) of mammary origin, hyperplastic ductular changes in the mammary gland and CEH in dogs (Bhatti et al., 2007).

Non-hormonal factors such as growth factors may effectively contribute to the pathogenesis of CEH (De Cock et al., 2002). There are two forms of insulin-like growth factor (IGF): insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-2 (IGF-2) (Rinderknecht and Humbel, 1978; Engström et al., 1998). The former is expressed after birth and is produced in the liver, while the latter is expressed in somatic tissues during the early embryonic and fetal developmental process in mammals (Engström et al., 1998; Bergman et al., 2013). Both IGF-1 and IGF-2 are present in the circulation and can be measured in plasma. Additionally, the IGF family is an important factor for the regulation of cell proliferation, growth, migration and differentiation (Bergman et al., 2013; Agaoglu et al., 2016). Dabrowski et al. (2015) indicated that there was no statistically significant difference in serum IGF-1 concentrations between dioestrous dogs and dogs with pyometra. In contrast, Jitpean et al. (2014) stated that IGF-1 concentrations were decreased in dogs with pyometra due to inflammatory response or decreased appetite. Although the relationship between IGF-1 expression and the CEH-pyometra complex was demonstrated, the possible role of IGF-2 in the pathogenesis of the CEH-pyometra complex in the bitch was not investigated. To the best of our knowledge, this is the first report concerning the plasma concentrations of IGF-2 as well as its expression in the uterine and ovarian tissues of healthy bitches and those with CEH-pyometra complex.

MATERIALS AND METHODS

Animals

The study included seventeen privately owned female dogs which had not been treated with any exogenous hormones. Ethical approval was obtained from the Animal Ethics Committee, Ondokuz Mayıs University, Samsun, Turkey (approval number 2013/49). All dogs underwent ovariectomy under general anaesthesia.

Group 1 ($n = 10$) consisted of bitches with open cervix pyometra, aged 7.45 ± 2.8 years. The breeds were two mongrels, two Norfolk Terriers, two Pekineses, one Doberman Pinscher, one Golden Retriever, one English Setter, and one Miniature Pinscher. The diagnosis of pyometra was based on sanguinopurulent vaginal discharge, peripheral leukocytosis as well as cytologic, vaginoscopic and ultrasonographic (Falco Vet, Pie Medical Imaging, Maastricht, The

Netherlands) examinations. The bitches showed no clinical signs other than vaginal discharge. The vaginal discharge had been observed by the owners for 3–7 days before the examination.

Group 2 (controls, $n = 7$) consisted of clinically healthy dioestrous bitches aged 2.07 ± 0.88 years and representing the following breeds: four mongrels, one Pointer, one English Setter, and one Dogo Argentino. The bitches in this group were diagnosed to be in dioestrus by cytological, vaginoscopic and ultrasonographic examinations. They were brought to the clinic by their owners for spaying and were healthy at presentation. Routine physical, haematological and ultrasonographic examinations did not reveal any abnormalities.

Blood samples for P4 and IGF-2 measurements were taken from the cephalic vein into heparinised tubes before the surgery in both groups.

P4 measurement

The plasma was separated after centrifugation at $1,550 \times g$ for 10 min, then transferred into labelled microcentrifuge tubes and stored at -20°C until assayed. P4 concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) method using canine-specific commercial kits (MyBioSource, Inc., San Diego, CA, USA). All plasma samples were analysed twice according to the manufacturer's recommendations. Both intra-assay and inter-assay variability for the assay was lower than 15%. The ELISA plate was read at 450 nm on a microplate reader (Digital and Analog Systems, RS 232, Rome, Italy). The concentration of P4 was calculated with reference to a standard curve that was generated by plotting the average optical density (450 nm) obtained from each standard on the horizontal axis versus the corresponding each standard concentration on the vertical axis. Results were expressed as ng/mL of plasma.

IGF-2 measurement

The blood samples were processed within 2 h of collection by centrifugation at $1,550 \times g$ at 4°C for 10 min. Plasma was transferred into a previously labelled microcentrifuge tube and stored at -80°C until analysis. The quantitative measurement of IGF-2 in plasma was performed using a canine-specific enzyme-linked immunosorbent assay (ELISA) kit (MBS740813, MyBioSource, Inc. San Diego, CA, USA), according to the manufacturer's protocol. The minimum detection limit of IGF-2 with the kit was 1.0 ng/mL, the range was 10–250 ng/mL. The average intra-assay coefficient of variation was <6%, and the inter-assay CV was <10%. Assays were performed concurrently in duplicate. Briefly, 100 μL of plasma samples or standards were dispensed into each well of the microtitre plate and incubated with 50 μL of enzyme conjugate at room temperature for 1 h. The wells were rinsed with diluted wash solution. Fifty microlitres of substrate A solution and 50 μL of the substrate B solution were then dispensed into each well and the plate was incubated at 22°C for 15 min. The enzymatic reaction was terminated by adding 50 μL of stop solution

into the centre of each well. The absorbance was measured at 450 nm using an ELISA microtitre plate reader (Infinite F50, Tecan Austria GmbH, Grödig, Austria).

Sample collection and histopathological examination

Both ovaries and uterine horns of each dog were fixed in 10% neutral formalin immediately after the surgery. In order to standardise the histopathological and immunohistochemical evaluations, the left and right uterine horns were cut transversally and divided into two equal parts. Then, 0.5 cm tissues were taken from the middle of each half of the horns. Thus, uterine cells of four different parts were counted, considering the fact that the sections were sufficient since there are numerous tubular glands throughout the endometrium. Then the tissues were embedded in paraffin using standard methods and 5- μ m sections were prepared and stained with haematoxylin and eosin (HE) (Luna, 1968). The uterine sections were examined under light microscope (Nikon Eclipse E600, Nikon Instruments Inc., Tokyo, Japan). Histological examinations were performed to confirm healthy tissue or the presence of CEH-pyometra.

Immunohistochemistry for IGF-2

A rabbit anti-IGF-2 polyclonal antibody was used for immunostaining. A streptavidin-biotin detection system (Zymed Histostain Plus Bulk Kit, Cat. no. 85-9043, San Francisco, CA, USA) was used for the demonstration of IGF-2. Sections were dewaxed in xylene and hydrated through graded alcohols. Endogenous peroxidase activity was blocked with H₂O₂ 3% in methanol for 15 min. All steps were performed in a humidified chamber at room temperature. The sections were rinsed with phosphate-buffered saline (PBS, pH 7.2) and subsequently heated (800 W in a microwave oven) in citrate buffer (pH 6.0) for 10 min for antigen retrieval. Following protein blocking for 10 min, sections were incubated with rabbit anti-IGF-2 polyclonal antibody (1:64) (Bioss, Cat. no. Bs-0015R, Massachusetts, USA) overnight at 4 °C. The sections were then incubated with an anti-rabbit biotinylated polyvalent secondary antibody for 10 min followed by the addition of the horseradish peroxidase enzyme for 10 min and then 3-amino-9-ethyl carbazole (AEC) in H₂O₂ chromogen for 10–15 min (controlled by visual observation with a microscope). Primary antibodies were omitted from negative control sections, which were incubated with either PBS or diluted normal serum from the species in which the primary antibody was raised. Sections were counterstained with Mayer's haematoxylin, rinsed with distilled water, and mounted with aqueous mounting medium. The distribution of immunoreactive cells was examined with a Nikon Eclipse E600 microscope.

Semiquantitative analysis was modified and performed according to Klein et al. (2001). For each case, the quantification of immunostaining was made by a semiquantitative method. Five microscopic fields were consecutively examined at $\times 400$ magnification in a Nikon Eclipse E600 light microscope. At least 200 ovarian (granulosa cells, theca cells,

luteal cells of corpus luteum) and uterine cells (epithelium of endometrial glands, fibroblasts, macrophages, smooth muscle cells of the myometrium), for each case, were counted by two blinded pathologist (MY and MS). The observers classified the cells as immunopositive or negative. Thus, the percentage of IGF-2 immunopositive cells was determined according to the following equation: 'the percentage of IGF-2 immunopositive cells = (number of IGF-2 immunopositive cells/total counted cells) \times 100 = [%]'.

The values of IGF-2 immunopositive cells were categorised into 4 scores as follows: 0 = absence of immunopositive cells, 1 = 1–10%, 2 = 11–40%, and 3 = above 40% of immunopositive cells.

Statistical analyses

Statistical analyses were performed using SPSS software (Sigma Stat, Systat Software, Inc., San Jose, CA, USA). Normal distribution and homogeneity of variances were confirmed by Shapiro–Wilks and Levene's tests, respectively. Significance of differences between groups was analysed by the Mann–Whitney *U* test. Data were presented as mean \pm standard deviation. Correlations were determined by Pearson's correlation. *P* values less than 0.05 were considered statistically significant.

RESULTS

Clinical findings

Group 1 had mucopurulent vulvar discharge accompanied by leukocytosis but no fever and vomiting. Uterine discharge and cervical patency were observed during vaginoscopic examination. Ultrasonographic examination revealed uterine enlargement as well as anechoic to hypoechoic fluid. In Group 2, the bitches had no signs of illness and the appearance of the uterus was normal on ultrasonographic examination. The vaginoscopic appearance of mucosal folds was flattened. Additionally, vaginal smears of both groups displayed only intermediate and parabasal cells and neutrophils. Based on the history, cytological findings and plasma progesterone concentrations, the bitches were considered to be in late dioestrus. Therefore, uterine and ovarian expressions of IGF-2 were compared for the normal uterine horns and for uterine horns with CEH-pyometra in the bitches that were in the same stages of the oestrus cycle.

Plasma IGF-2 and P4 concentrations

The concentration of plasma IGF-2 was higher in Group 1 than in Group 2 (24.76 ± 5.62 and 16.90 ± 6.36 ng/mL, respectively; *P* < 0.05). The concentration was positively correlated with IGF-2 expression in endometrial glands (*r* = 0.926; *P* < 0.001) of Group 1. However, a negative correlation was present between plasma IGF-2 concentration and IGF-2 expression in the interstitial cells of the ovary in Group 1 (*r* = –0.652; *P* < 0.05). Additionally, there was no significant correlation between plasma IGF-2 concentration

and age either in Group 1 ($r = -0.245$; $P > 0.05$) or in Group 2 ($r = -0.229$; $P > 0.05$).

The plasma P4 concentration was higher in Group 1 than in Group 2 (10.24 ± 5.31 and 5.20 ± 1.00 ng/mL, respectively; $P < 0.01$). The plasma progesterone concentration was positively correlated with plasma IGF-2 concentration in Group 1 ($r = 0.583$; $P < 0.05$).

Histopathological and immunohistochemical findings

Thickening and cystic irregular elevations on the endometrial surface and chronic endometritis were characterised by lymphocyte infiltration in Group 1. The expression of IGF-2 in the corpus luteum (Fig. 1a), theca cells (Fig. 1b) and interstitial endocrine cells were observed in the ovaries of dogs in both groups. The expression in the corpus luteum and theca cells had the same values (3.0 ± 0.0) in both groups ($P > 0.05$). IGF-2 immunopositive interstitial cells were significantly higher ($P < 0.001$) in Group 1 (2.30 ± 0.67); however, in Group 2 (0.29 ± 0.49) there were only two cases in which a few cells were immunopositive (Fig. 1c and d). Immunopositivity was detected in the endometrial glandular epithelium of both the healthy dioestrous bitches and in those with pyometra, but the epithelial cells of the cystic endometrial glands were not stained (Fig. 2a and b). IGF-2 expression in the endometrial glandular epithelium was lower in Group 1 (1.50 ± 1.08) than in Group 2 (2.57 ± 0.53), although the difference between the groups was not statistically significant ($P > 0.05$). Additionally, interstitial fibroblasts (Fig. 2b) and macrophages (Fig. 2c) in the

endometrium were also strongly stained in Group 1. IGF-2 expression in fibroblasts was markedly higher ($P < 0.001$) in Group 1 (2.00 ± 0.82) than in Group 2 (0.29 ± 0.49). In addition, the myometrial and vascular smooth muscles were positive for IGF-2 in both groups (Fig. 2d).

DISCUSSION

It is well known that canine pyometra occurs in bitches older than four years, although its incidence is higher particularly in bitches older than 6 years. The age and the size of the bitches differed between and within the groups of this study. However, no differences in plasma IGF-2 concentrations depending on the body size of dogs have been found so far (Maxwell et al., 1998; Reynaud et al., 2010), although many studies described a positive correlation between plasma IGF-1 concentrations and dog size and body weight (Maxwell et al., 1998; Reynaud et al., 2010; Greer et al., 2011). Greer et al. (2011) showed a decrease in IGF-1 levels with increasing age. On the other hand, IGF-2 is known to be less age dependent than IGF-1 because it is less GH-dependent than IGF-1 (Cooke et al., 2016). Accordingly, there was no significant correlation between plasma IGF-2 concentration and age both in Group 1 ($R = -0.245$; $P > 0.05$) and Group 2 ($R = -0.229$; $P > 0.05$) in our study.

Sex steroid hormones and their receptors lead to morphological changes in the endometrium (De Cock et al., 2002). The prolonged influence of natural or synthetic P4 or

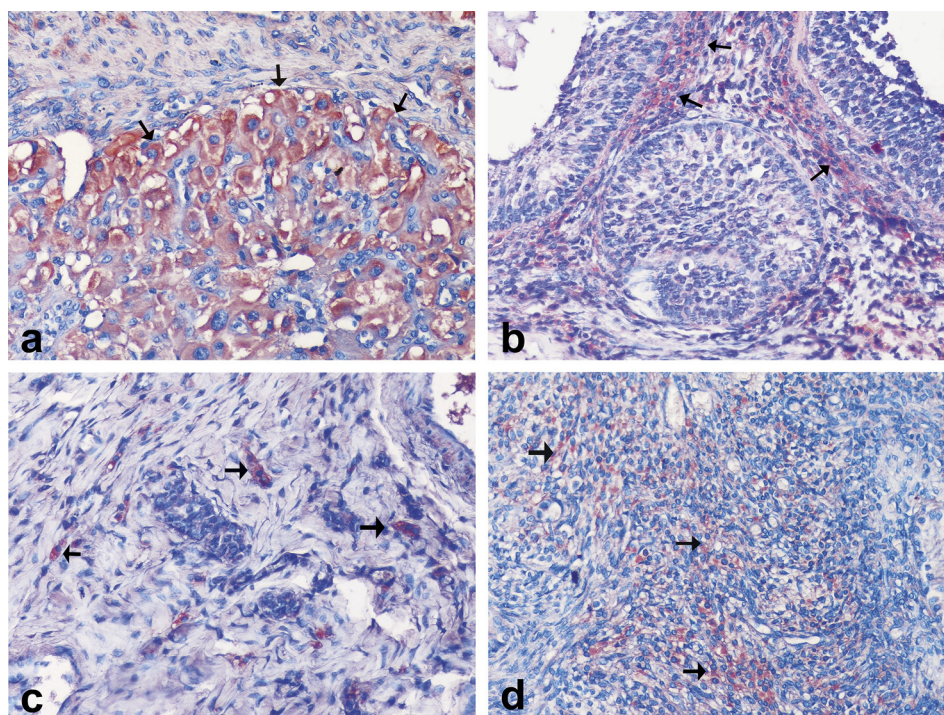


Fig. 1. Expression of IGF-2 in the ovary of dogs in the two groups. Intense IGF-2 positive luteal cells in the corpus luteum (arrows) (a), in theca cells on the walls of follicles (arrows) (b), a few interstitial endocrine cells in the medullar area (arrows) in the ovary of controls (c). Many interstitial endocrine cells in the medullar area (arrows) in the ovary of dogs with pyometra (d). ABC peroxidase counterstained with Mayer's haematoxylin, $\times 20$

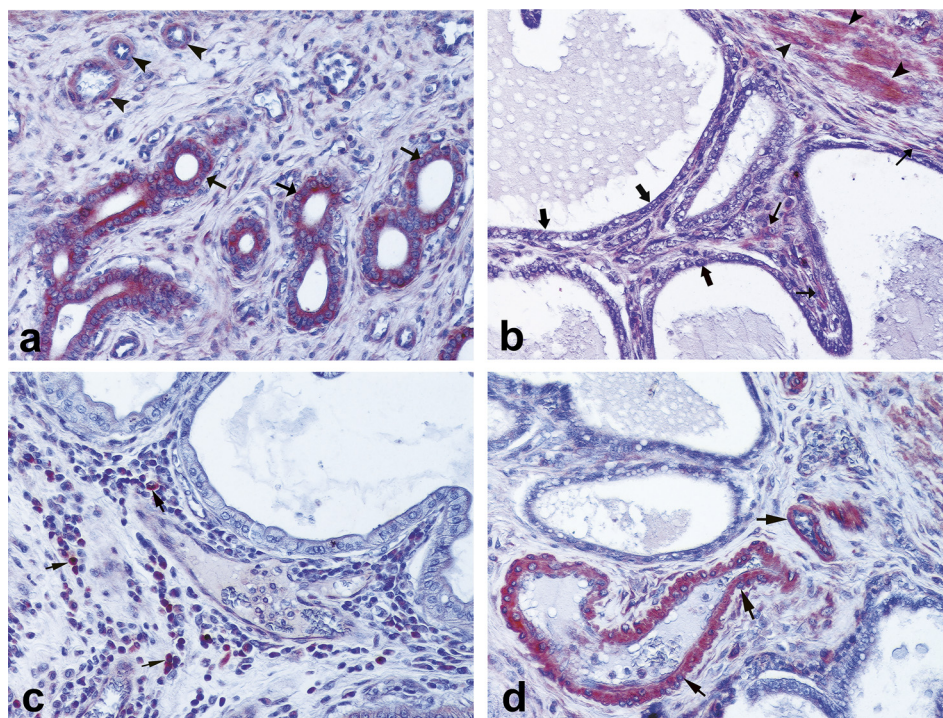


Fig. 2. Expression of IGF-2 in the uterine tissue of dogs in the two groups. Intense IGF-2 positive epithelial cells of the uterine glands (arrows) and smooth muscles of vessels in the controls (arrow heads) (a). Intense IGF-2 expression in fibroblasts (thin arrows) and smooth muscles of the myometrium (arrow heads). Cystic endometrial gland epithelium is negative (thick arrows) in the endometrium with cystic endometrial hyperplasia–pyometra (b); intense IGF-2 expression in macrophages (arrows) in the interstitial area of the endometrium with cystic endometrial hyperplasia–pyometra (c); intense IGF-2 expression in the smooth muscles of vessels (arrows) in the endometrium with cystic endometrial hyperplasia–pyometra (d). ABC peroxidase counterstained with Mayer's haematoxylin, $\times 20$

oestrogen causes proliferative cystic endometrial alterations in bitches (De Cock et al., 1997; Bigliardi et al., 2004). Besides, endogenous P4 or synthetic progestins may induce overproduction of GH related to increasing IGF-1 concentration (Selman et al., 1994). Both IGF-1 and IGF-2 are mitogenic factors and their uterine expressions are arranged by steroidogenic hormones (De Cock et al., 2002). In humans, the effects of oestrogen on endometrial proliferation during the proliferative phase of the endometrial cycle are mediated by IGF-1, whereas the effects of P4 during the secretory phase of the cycle are mediated by IGF-2 (Di Pietro et al., 2013). Selman et al. (1994) showed that synthetic progestin treatment leads to similar increases in GH and IGF-1 levels and results in a similar degree of insulin resistance in bitches. Moreover, the administration of progestins in dogs also increases the plasma concentration of IGF-2 even before GH concentration begins to increase (Mol et al., 1997). The dogs included in the present study did not receive progestin injections; however, plasma P4 concentration was positively correlated with plasma IGF-2 concentration in the dogs with pyometra. Additionally, IGF-2 was shown to stimulate the proliferation of granulosa cells, hence to produce oestradiol and progesterone (Cara, 1994). Reynaud et al. (2010) reported that plasma P4 level and intrafollicular IGF-2 were positively correlated in dogs at the pre-luteinising hormone (pre-LH) stage. Similarly, in our study, the mean plasma P4 concentration was higher in

Group 1 than in Group 2, and the concentration was positively correlated with plasma IGF-2 concentration in Group 1. This suggests that high progesterone concentrations result in high IGF-2 concentrations and IGF-2 seems to be involved in the control of endometrial growth.

It was shown that increased IGF-1 as a mitogenic effect in the endometrium might play an important role in the development of CEH in dogs. IGF-1 was located around the epithelial cells of the endometrium in dogs with CEH (De Cock et al., 2002). In contrast, IGF-2 immunopositivity was not found in the epithelial cells of cystic endometrial glands in our study. However, the endometrial glandular epithelium was immunopositive in both groups, suggesting that IGF-2 regulates tissue growth during both dioestrus and pyometra in the dog. In the immune system, IGF-2 promotes granulocyte–macrophage colony formation (Livingstone and Borai, 2014), and this might explain why macrophages in the endometrium were also strongly stained in Group 1.

Many studies have shown that IGF-2 is implicated in the development of different types of cancer such as breast cancer, gastrointestinal stromal tumour, colorectal and lung cancer as well as metabolic and endocrine diseases in humans (Nosho et al., 2004; Bergman et al., 2013; Tas et al., 2016; Livingstone and Borai, 2014; Yamasaki et al., 2020). Similarly, plasma IGF-2 concentration was found to be increased in a dog with pancreatic islet cell tumour (Finitello et al., 2009), in canine mammary tumour (Amini et al.,

2020; Noguchi et al., 2020) and in canine demodicosis (Yarim et al., 2015). It has been suggested that the stimulation of human bone cell proliferation by P4 may be related to increased IGF-2 concentration (Tremollieres et al., 1992). Growth factors, particularly IGF-2, can stimulate tumour growth (Bergman et al., 2013). These data show an obvious relationship between increased IGF-2 levels and uncontrolled growth. In our study, IGF-2 immunopositivity was determined in the endometrial glandular epithelium of both the healthy dioestrous bitches and in those with pyometra, suggesting that IGF-2 plays an important role during the inflammatory process in bitches with CEH–pyometra complex as well as in the endometrium of healthy dioestrous bitches.

The morphological and functional differentiation of the uterus during oestrous cycles and pregnancy is associated with highly co-ordinated changes in the endometrial and myometrial mRNA expression of IGFs, IGF receptors and IGF-binding proteins (IGFBPs), i.e. the IGF system. It was demonstrated that IGFBP-2 and IGF-2 are potent local mitogens for the porcine uterine epithelium (Badinga et al., 1999). In cows, Robinson et al. (2000) found that IGF-2 mRNA expression in the endometrial stroma, myometrium and uterine glands on day 16 of the oestrous cycle was higher than during pregnancy. It was also reported that IGFs lead to endometrial cell proliferation and neoplasia (Calle et al., 2003; Tas et al., 2016). Similarly, we found that glandular epithelial cells were well stained for IGF-2 in both control dogs and dogs with CEH–pyometra. However, epithelial cells in the cystic endometrial glandular epithelium were negative. Additionally, IGF-2 immunostaining was intense in the endometrial mucosal layer in dogs with CEH–pyometra. Besides, myometrial smooth muscles and vascular smooth muscles were positive for IGF-2 in dogs with CEH–pyometra and in healthy control dogs. Furthermore, we found intense IGF-2 staining in interstitial fibroblasts and macrophages in the endometrial mucosal layer that might be the source of the high plasma concentrations of IGF-2 in dogs with CEH–pyometra. In the development of CEH–pyometra, deregulation of the IGF axis might have a significant role and this could be a part of future treatment strategies. Further studies are needed for getting a deeper insight into the factors governing IGF-2 expression in CEH–pyometra.

Schams et al. (1999) reported that GH, locally produced IGFs and IGFBPs may have an important role in the control of ovarian function in large farm animals. It was also found that exogenous progestins or endogenous progesterone may cause increased plasma concentrations of GH in the luteal phase of the oestrus cycle in bitches (Eigenmann et al., 1983). Our study determined that the expressions of IGF-2 in the CL and theca cells were at the same levels in both groups, and this finding was thought to be associated with the fact that the bitches of both groups were in dioestrous. However, IGF-2-immunopositive interstitial cells were markedly increased in dogs with CEH–pyometra compared to healthy control dog ovaries.

In conclusion, a clear expression of IGF-2 in the uterus of both healthy dioestrous bitches and bitches with

CEH–pyometra shows that IGF-2 plays a role in tissue regulation. On the other hand, increased IGF-2 expression might be a part of pathological changes during CEH–pyometra. Increased plasma P4 and IGF-2 concentrations have growth-promoting function during embryonic development; however, this function might be a disadvantage during the development of CEH–pyometra. The focus of future investigations therefore should be on growth factor inhibitors for the treatment of canine pyometra.

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