Original article

Preliminary results of blood growth differentiation factor-9 (GDF-9) measurement in cats: future aspects of GDF-9 on stage of the cycle and spaying history

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Abstract

Growth differentiation factor-9 (GDF-9), an oocyte-derived member of the TGF-β superfamily, plays an essential role in regulation of follicular development. This study aimed to determine the cyclic changes in serum GDF-9 concentration, compare its levels before and after ovariohysterectomy (OHE), and investigate its potential as a tool in ovarian remnant syndrome (ORS) diagnosis in cats. GDF-9 measurements were performed on 50 cats referred for routine OHE. The stage of the estrous cycle was determined by vaginal cytology and measurement of serum estradiol and progesterone levels was carried out to detect the cyclic changes in circulating GDF-9. One week after OHE, serum samples were collected again from 30 cats to reveal differences in GDF-9 levels. GDF-9 levels in the follicular phase were significantly higher than those in the interestrus (p<0.05). The postoperative analysis could be performed. GDF-9 levels slightly decreased one week after OHE (p=0.053). In conclusion, blood GDF-9 levels change during the estrous cycle, and may decrease with age in cats. However, further studies are needed to reveal the efficiency of GDF-9 in ORS diagnosis.

Key words: cats, growth differentiation factor 9, ovarian cycle, ovary

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Introduction

Ovarian remnant syndrome (ORS) is a complication caused by the failure to completely remove the ovarian tissue during ovariectomy or variohysterectomy (Wallace 1991, Ball et al. 2010). Predisposing factors for ORS are a small incision line for gonadectomy, misalignment of the ovarian tissue, iatrogenic factors such as revascularization of accidentally dropped tissue into the abdominal cavity, or rarely, the presence of accessory ovarian tissue (Miller 1995, DeNardo et al. 2001). Nevertheless, faults in surgical techniques are the most common factor for ORS (Ball et al. 2010).

Continuation of the cyclic activity after gonadectomy is the primary clinical finding observed in ORS. However, the time span from gonadectomy to recurrence of sexual cycling may vary. ORS can be diagnosed by anamnesis, clinical, vaginal cytological and ultrasonographic findings, hormonal analysis, and exploratory laparotomy (Miller 1995, Ball et al. 2010).

Scientific interest in the transforming growth factor-β (TGF-β) superfamily members and their role in regulating ovarian function has increased in recent years. In addition to the endocrine signaling pathways in the hypothalamic-pituitary-ovarian axis, the oocyte itself affects the microenvironment of the developing follicle by paracrine and autocrine mechanisms (Juengel et al. 2005). Growth Differentiation Factor-9 (GDF-9), a member of the TGF-β superfamily, is the first oocyte-derived growth factor found to be indispensable for somatic cell function and fertility (McPherron et al. 1993, Dong et al. 1996).

Although studies on GDF-9 have been conducted on various animal species such as laboratory animals (McPherron et al. 1993, McGrath et al. 1995, Dong et al. 1996, Elvin et al. 1999), ruminants (Bodensteiner et al. 1999, Hanrahan et al. 2004, Hu et al. 2010, Polat et al. 2015, Tang et al. 2018), mares (Dal et al. 2013, Davis et al. 2018, Stefaniuk-Szmukier et al. 2018), and bitches (Fernandez et al. 2016, Palomino et al. 2016), studies on cats are very limited. In a study examining the TGF-β superfamily members through immunohistochemical methods in feline ovarian tissue, GDF-9 is reported to be a potent growth factor in follicle maturation (Bristol et al. 2004). Recent studies have indicated that analysis of oocyte-derived factors, in addition to gonadal steroids, has a new diagnostic value in the spaying history of cats. It is shown that Anti-Müllerian hormone (AMH), another member of the TGF-β superfamily, is a diagnostic parameter that can be used to determine the presence of ovaries in cats and dogs (Axner and Holst 2015, Yılmaz et al. 2015, Pir Yagci et al. 2016). In the literature review, no study investigating the potential relevance of GDF-9, an intraovarian factor like AMH whose essential effect on follicle development and fertility is determined, in the clinical diagnosis of reproductive cases in cats was found. It was hypothesized that GDF-9 might be a potential biomarker in diagnosing feline ovarian remnant syndrome. It was aimed at determining the cyclic changes in serum concentrations of GDF-9, to measure its levels before and after OHE, and accordingly, to investigate its potential as a diagnostic tool in ORS in cats.

Materials and Methods

The study was conducted with 50 healthy cats referred to the Small Animal Obstetrics and Gynaecology Clinic for routine OHE. A signed informed consent form was obtained from each client. The study was approved by the Istanbul University-Cerrahpasa Unit Ethics Committee (protocol no. 2020/16).

Experimental design

All cats underwent physical examination, complete blood count, and serum biochemistry profile before OHE for their health status. Vaginal exfoliative cytology which was taken by swabbing, in addition to serum estradiol and progesterone analyses performed to determine the stage of the sexual cycle. Pre-operative blood serum samples were collected from all cats for GDF-9 analyses (n=50). The cats were premedicated with atrophine sulphate, and the anaesthesia was then inducted with propofol. The cats were intubated and the anaesthesia was maintained with isoflurane (Isofluran CP®) + O2. A ventral midline laparotomy was performed for ovariohysterectomy. Pre- and postoperative pain management was achieved using butorphanol (Butomidor®). One week after OHE, post-operative blood serum samples were collected only from 30 individuals among all cats.

Vaginal smears were stained with Diff-Quik staining protocol according to the manufacturer's instructions. Slides were examined under a light microscope at X40 magnification. Blood samples were collected from vena cephalica antebrachii into silicone-coated tubes to analyse estradiol, progesterone, and GDF-9 concentrations. The tubes were centrifuged at 3000 rpm for 10 min as mentioned in the kit's manual. The sera of the blood samples were stored at -20°C until analysis.

Commercial radioimmunoassay (RIA) kits were used for estradiol and progesterone measurements (Beckman Coulter, Lot no: 210322C, United States). Enzyme-linked immunosorbent assay (ELISA) was used for analysing GDF-9 concentrations (BT Lab Cat Growth Differentiation Factor 9 ELISA Kit, Cat.
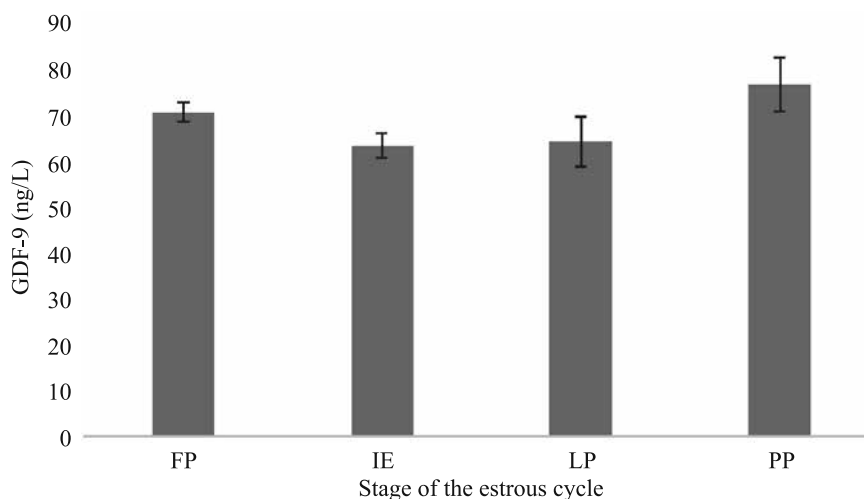
Fig 1. The mean GDF-9 concentrations according to stage of estrous cycle (FP: Follicular phase, IE: Interestrus, LP: Luteal phase, PP: Prepubertal). Bars and error bars represent means and SD respectively.

No E0078Cat, China). The sensitivity of the ELISA kit was 1.59 ng/L. The intra- and inter-assay precision of the kit were <8% and <10%, respectively. The analyses were performed according to the manufacturers' instructions by a private laboratory.

Power analysis

Statistical power analysis was performed to determine the sample size based on the data obtained from the study. For the pre-operative and post-operative GDF-9 concentrations, the minimum number of samples required was determined as 27 with an effect size of 0.50 (using the Cohen criteria), alpha=0.05, and power=80%. When the GDF-9 concentrations during the follicular phase and interestrus were compared, the effect size was 0.80 (using the Cohen criteria), alpha=0.05, and power=80%, and the required minimum number of samples was determined as 42 in this study.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) 13.0 analysis program was used for statistical analysis. Pre-operative and post-operative GDF-9 concentrations in 30 cats were compared by paired sample t test. The independent sample t-test was used to compare GDF-9 concentrations during the follicular phase and interestrus in 50 cats. Each bar represents mean ±SD. The significance level was accepted as $p < 0.05$.

Results

The mean concentrations of GDF-9 before and one week after OHE were 70.38±2.38 ng/L and 63.68±2.17 ng/L, respectively. Although GDF-9 levels

tended to decrease after OHE, no statistically significant difference was detected (p=0.053). According to the results of the clinical examination, vaginal exfoliative cytology, serum estradiol, and progesterone analyses, 20 cats were identified to be in the follicular phase, 18 cats were in interestrus, 6 cats were in the luteal phase, and 6 cats were in the prepubertal period.

The mean GDF-9 concentrations during the follicular phase were significantly higher than in interestrus ($p < 0.05$). However, no statistical analysis could be performed to compare GDF-9 levels of cats in the luteal phase or at prepubertal age due to the small sample size. The mean GDF-9 concentrations according to the sexual stage of cats are given in Fig. 1.

Discussion

Surgical sterilization of cats and dogs is routinely performed by traditional ablation of the uterus and ovaries, namely OHE (Howe 2006). ORS is one of the complications of OHE, which often results from inappropriate techniques performed during the surgical procedure (Wallace 1991, Miller 1993, Howe et al. 2006, Ball et al. 2010). Several methods have been described for diagnosing the remnant ovarian tissue. Although anamnesis and clinical findings provide guidance, the diagnosis should be confirmed by vaginal cytology, ultrasonography, hormonal analysis and/or laparotomy (Wallace 1991, Miller 1995, Howe 2006, Sontas et al. 2007, Ball et al. 2010). Among them, observation of cornified vaginal epithelial cells by vaginal cytology is the easiest and cheapest method (Howe 2006, Sontas et al. 2007). However, it is important to perform vaginal cytological examination during the follicular phase for accurate diagnosis (Miller 1995, Ball et al. 2010). In the present study, the Diff-Quik

staining protocol was used to determine the stage of the cycle before the OHE procedure. Vaginal cytology showed the most reliable results during the follicular phase with a clear background accompanying abundant pyknotic nucleated-anucleated superficial cells with/ /without corfinification.

Detection of ORS by ultrasonographic examination can be difficult depending on the size of the residual tissue (Olivera et al. 2012), stage of the estrous cycle, and the experience of the practitioner (Ball et al. 2010). Therefore, this method is mainly recommended for large breed dogs (Sontas et al. 2007). Ultrasonographic examinations of the remnant ovaries reveal acoustic enhancement, anechoic follicles, and/or cystic structures. However, it should be noted that inflammatory reactions caused by the suture material at the site of ligation may be misinterpreted as ORS (Ball et al. 2010). Nevertheless, a reproductive ultrasonographic examination was not within the scope of this study.

Measurement of circulating estradiol and progesterone hormones plays an important role in the detection of ovarian residue (Wallace 1991, Ball et al. 2010 Olivera et al. 2012). Serum estradiol concentration higher than 10 pg/mL and serum progesterone concentration higher than 2 ng/mL indicate the presence of functional ovarian tissue (Wallace 1991, Olivera et al. 2012). However, since cats mostly exhibit induced ovulation, low serum progesterone concentrations in unmated cats are insufficient in diagnosing ORS. In the analyses performed 1-3 weeks after the stimulation of ovulation with GnRH or hCG, an increase in progesterone and a decrease in estradiol were more effective in confirming the presence of ovarian tissue (Wildt et al. 1981, Wallace 1991, Sontas et al. 2007, Axner et al. 2008). As all cats participating in this study were intact animals, hormonal analyses were performed to determine the reproductive status of cats to assess GDF-9 levels at different stages of the estrous cycle. Estradiol levels higher than 20 pg/mL and lower than 15 pg/mL were interpreted as follicular phase and interestrus, respectively, while progesterone levels higher than 1 ng/mL indicated the luteal phase as described in Goodrowe (1989) and Little (2012). Prepubertal cats were 3-4 months, had not yet shown any signs of heat. These cats had progesterone levels lower than 1 ng/mL; on the other hand, they had detectable estradiol levels below 20 pg/mL with prominent parabasal cells at vaginal cytology, which were in line with Belhan and Gülyüz (2013).

Members of the TGF-β superfamily regulate fundamental biological processes such as somatic cell proliferation-differentiation, oocyte maturation, and ovulation (Juengel et al. 2005). Among these, GDF-9 is a crucial paracrine factor for female fertility in both mono-

vular and polyovular animal species (Dong et al. 1996, Hanrahan et al. 2004, Sontas et al. 2007). In one of the first studies on GDF-9 knockout mice, it was observed that follicle development was interrupted at the primary stage, and its deficiency was reported to cause infertility. The researchers concluded that GDF-9 is an indispensable factor for further development of the follicle via its paracrine effect on somatic cells (Dong et al. 1996). Its roles are listed as granulosa cell proliferation and differentiation during primordial and primary follicle development (McGrath et al. 1995, Bodensteiner et al. 1999, Knight et al. 2003), regulation of various granulosa cell enzymes necessary for the enhancement of cumulus expansion and maintaining the optimal oocyte microenvironment required for ovulation and fertilization (Elvin et al. 1999). As reviewed by Bristol--Gould and Woodruff (2006) follicular morphology changes as the oocyte grows. Folliculogenesis starts during fetal life and proceeds to primordial follicle formation after birth. Primordial follicles are classified into three types. The C class, also called primary follicle, develops to secondary and antral follicle stages as granulosa cells proliferate and the theca layer surrounds the follicle. In cats, GDF-9 protein was detected intensively in the cytoplasm of C class primary and small antral follicles. GDF-9 receptor, on the other hand, was found in primary follicles and, to a lesser extent, in small antral follicles, but most intensely in the cytoplasm of the primordial follicles. Researchers have suggested that GDF-9 is an effective growth factor in the early stages of feline follicle maturation (Bristol et al. 2004). This study is the first to examine circulating GDF-9 levels in female cats. Serum GDF-9 analysis revealed that its concentration during the follicular phase is significantly higher than during the interestrus ($p<0.05$). It has been reported that an antral follicle count by ultrasonography revealed a single follicular wave during estrus in cats. However, it is unknown whether the interestrus period includes follicular dynamics (Malandain et al. 2011). The higher GDF-9 levels during the follicular phase in this study are considered to be caused by follicular dynamics developed during this period, leading to an increase in the number of antral follicles.

Cats are seasonally polyestrous animals with induced ovulation. They ovulate in response to vaginal stimulation; however, spontaneous ovulation may also be seen. The luteal phase describes the diestrus period of pregnant cats or pseudopregnancy situation following ovulation without successful fertilization (Feldman et al. 1996, Bristol-Gould et al. 2006). Only 6 cats were identified to be in the luteal phase, having progesterone levels higher than 1 ng/mL in the present study. Additionally, 6 cats were in the prepubertal period. Although

statistical analysis could not be performed due to the small sample size, the mean GDF-9 concentations during the luteal phase were found similar to interestrus. Strikingly, the highest mean GDF-9 levels were achieved in prepubertal cats among all animals. A study on cat oocytes revealed that the oocyte recovery rate was higher in kittens than in adults. Besides, the gonads of prepubertal cats were indicated as a potentially rich source of good-quality oocytes for assisted reproductive techniques. Studies have emphasized that the number and quality of oocyte decrease with age (Kochan et al. 2021). The highest GDF-9 levels in prepubertal cats suggest that GDF-9 may reflect the number and quality of oocytes, and aging may affect the concentrations of this factor. Further studies are needed to determine the usability of circulating GDF-9 measurements for the assessment of potential fertility, oocyte quality, and ovarian reserve in animal species.

It was also aimed to compare GDF-9 levels before and after gonadectomy in female cats. It was hypothesized that the determination of GDF-9 in circulation might have potential as a tool in the detection of ovarian residue in addition to other diagnostic methods since it is an indispensable oocyte-derived factor for fertility. Despite the postoperative decrease in GDF-9, no statistical difference was found between GDF-9 levels in blood samples collected before and 1 week after OHE (p=0.053). Under the circumstances, the results of this study indicate that GDF-9 is not efficient in the diagnosis of ORS. Further studies involving repeated measurements over an extended period may reveal whether the withdrawal of GDF-9 after gonadectomy needs a longer time.

Recently, serum GDF-9 measurements have been investigated in women. Despite the impression of an age-dependent decline in GDF-9, the large variations observed between individuals – even elevated GDF-9 levels in some postmenopausal women – were unexpected and confusing. It is suggested that a possible extraovarian origin of GDF-9 should be considered (Riepsamen et al. 2019). Research performed on mice showed GDF-9 expression in testis, pituitary, and adrenal cells. It has been reported that GDF-9 regulates the expression of various genes in extraovarian cells via similar signalling pathways as in the ovary. However, the expression of relevant receptor proteins in these cells was suggested to be inadequate to respond to GDF-9 (Wang et al. 2009). In sheep, GDF-9 expression was found in various tissues (Hu et al. 2010, Tang et al. 2018). However, Tang (2018) reported that the highest expression was detected in ovaries. Nevertheless, extraovarian expression of GDF-9 has not been reported in cats with the exception of its localization in the cytoplasm of round spermatids and pachytene spermatocytes in the adult cat testis (Zhao et al. 2011). Since GDF-9 measurements did not decrease to basal levels after OHE in this study, the extraovarian expression of GDF-9 should not be ignored, as mentioned by Riepsamen (2019). However, whether the possible extraovarian expressions of GDF-9 are efficient enough to affect the circulating concentrations is also an issue that needs to be investigated.

In conclusion, the novelty of this study is the measurement of circulating GDF-9 concentrations in cats for the first time. The statistically higher GDF-9 levels achieved in the follicular phase were suggested to be correlated with follicular dynamics. The highest GDF-9 levels seen in prepubertal cats may reflect the greater pool of oocytes. Further studies will reveal whether GDF-9 is an age-dependent tool in the measurement of ovarian reserve in animal species. However, the potential of GDF-9 as a diagnostic tool in ORS in cats is not clear.

Acknowledgements

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