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Investigation of The Effects of Some Intrafollicular Growth Factors (GDF-9, GATA-4, GATA-6, IGF-I and IGF-II on Etiopathogenesis of Cystic Follicular Ovarian Degenerations in Cows

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Abstract
The aim of the presented study was to investigate the efficacy of intrafollicular GDF-9, GATA-4, GATA-6, IGF-I and IGF-II levels on etiopathogenesis of follicular cystic degenerations in dairy cows in accordance with some puerperal physiology parameters. After calving, all the cows were screened for the preovulatory and cystic follicles via ultrasonography with 5 MHz transrectal linear probe for the evaluation of ovarian activity at the days of postpartum 14, 24, 34, 44 and 55. Preovulatory and cystic follicular fluids were collected by aspiration via transvaginal ovum pick up method. Furthermore, uterine involution and vaginal discharges were evaluated by clinical examinations. Intrafollicular expression of GDF-9, GATA-4, GATA-6 and IGF-II were estimated by Western-blot assay and IGF-I levels were determined by ELISA. In cyst group, densitometric evaluation revealed that the expression of intrafollicular GDF-9, GATA-4 and GATA-6 and IGF-I levels were significantly lower than the control group ($p<0.01$). According to ovarian examinations, in cyst group, it was observed that at the day of postpartum 14 and 24, the difference in follicular diameters were significant between groups ($p<0.01$) and the follicles moved directly to cystic structure at the days of postpartum 24 or 34, is originated from same follicles at the day of postpartum 14. Involution process was observed to be slightly lower in the cyst group, but, it was determined that there was no difference in uterine involutions among the study groups ($p>0.05$). In follicular cyst group, especially at the days of postpartum 24 and 34, the observation of severe mucotic vaginal discharges was found noteworthy. As a result, dairy cows presenting cystic ovaries, have lower intrafollicular GDF-9, GATA-4, GATA-6 and IGF-I levels than those of the preovulatory follicle group and also it has been suggested that these proteins may play an active role in the etiopathogenesis of ovarian cysts and these findings may be associated with physiological parameters as well.

Keywords: Cow, postpartum, follicular cyst, follicular fluid, transforming growth factor family, IGF-1, ELISA, western blot.

INTRODUCTION

Follicular cysts of the ovary are anovulatory structures, which are identified for many mammalian species as well as cows. The incidence of ovarian follicular cysts in dairy cattle showed a range from 5.6% to 18.8% (Lopez-Diaz et al. 2002). The time which deflection lapse between diagnosis, treatment and insemination of the cows with follicular cyst can take up to 50-70 days. This situation causes significant economic losses in terms of dairy cattle industry (Vanholder et al. 2005). The effect of disruptions in some possible mechanisms in the process of formation of anovulatory follicles and permanent ovarian cysts in cows are aimed to be explained. These are categorized under three main themes such that preovulatory luteinizing hormone (LH) response from the pituitary to the estradiol in the new preovulatory follicles developing in the postpartum period is not observable, LH release problems occur due to the inadequacy of gonadotropin release from the hypothalamus despite the presence of estradiol, and sometimes anovulation problems arise from the lack of ovarian response despite the presence of gonadal response (Rizzo et al. 2010; Paredes et al. 2011).

During folliculogenesis, gonadotropins and metabolic hormones interact with local gonadal somatic cells at the receptor level, and thus externally they control the stimulation and inhibition of autocrine and paracrine signals. However, such a control mechanism is ensured by some intraovarian factors. For example, the ovarian somatic cells and some factors secreted from the oocyte in this interaction directly affect the function of the hypothalamic-pituitary-gonadal axis although to which size the follicle will grow, and at which stage it will be ovulated is closely related to the amount of intra-follicular estradiol. This situation highlights the effects of intraovarian regulation mechanisms on folliculogenesis and ovulation process (Scaramuzzi et al. 2011). Follicular development as a fertility parameter along with pubertas is a phenomenon during which oocyte gains the ability to ovulate. One of the evidences and most important indicators for this is the rapid increase in the production and activities of transforming growth factor-beta family proteins (TGF-β) secreted from the oocyte and other functional somatic cells of the ovary. These proteins play an important role in the intraovarian regulation mechanisms. Some of these duties are; granulosa and theca cell proliferation, follicle selection, follicle development and structural strength, steroidogenesis, oocyte maturation, ovulation and luteinization (Hunter et al. 2004; Webb et al. 2007; Webb and Campbell, 2007). Growth and Differentiaon Factor-9 (GDF-9), which plays a role in regulating the phenotype of the oocyte, is synthesized from the oocyte and surrounding cumulus cells. The process of regulation of this phenotype involves supporting of a healthy formation of the oocyte and its gradual maturation. Thanks to the Cumulus cells and the follicular cells which face the lumen, such a

gradual maturation is regulated (Juengel & McNatty, 2005). GATA 4 and 6 play a key role in directing endocrine, paracrine and autocrine signals in the ovary (Tremblay & Viger, 2003). It is highly probable that the follicle which is under the extreme effect of FSH (follicle-stimulation hormone) or highly susceptible to FSH stimuli due to differentiation properties of FSH characterized for anti-apoptotic, proliferative, aromatase enzyme production and the formation of LH receptors in the granulosa cells of the follicle would be the dominant follicle (Son et al. 2011). Follicle stimulating hormone and Insulin Like Growth Factor-I (IGF-I) support each other's effects on proliferation, and reproduction of steroids, activin and inhibin in bovine granulosa cells (Fortune et al. 2004; Beg and Ginther, 2006). IGF actions increase as the follicles grow. As the concentration of IGF-I in circulation increases, follicular growth also increases (Nicholas et al. 2002). In cows and sheep, IGFs are secreted from somatic cells of the follicle under the influence of insulin as well as oocyte-induced GDF-9 and Bone Morphogenetic Protein-6 (BMP-6) and BMP-15, together with insulin (Webb et al. 2007, Scherzer et al. 2009). While plasma IGF-I level is of critical importance for follicular development, it is also directly related to energy status and liver health (Butler, 2000; Zulu et al. 2002).

In the presented study, it is aimed to compare the expression levels of intra-follicular GDF-9, GATA-4, GATA-6, IGF-I and IGF-II, which have important roles in the intraovarian regulation mechanisms in the physiological process in dairy cows with high metabolic activities and are thought to have an effect on the etiopathogenesis of cystic ovarian degenerations, in cystic and healthy dominant follicles, within the framework of postpartum involution, ovarian activity and puerperal physiology.

MATERIALS AND METHODS

Animals and Grouping

The study was conducted in a commercial farm engaged in intensive dairy cattle breeding. Lactating *Holstein* cows in the postpartum period, which have given at least 1 and at most 4 births, were used as animal material. Cows in the dairy were housed in separate sections according to their yield characteristics and age. In the study, ovarian activities were monitored by means of regular gynecological examinations at ten-day intervals from the 14th day of postpartum, performed for every cow which has given birth to a calf. The diagnosis of cystic follicles was made according to Day's (1991). Accordingly, in the postpartum period, the cows with a persistent follicular structure of 2.5 cm and more in diameter in their ovaries as identified in the examinations performed at ten-day intervals, have formed the cyst group (n=10), whereas the cows with a physiological preovulatory follicular structure of 1.5-2.0 cm in diameter in their ovaries and indicating the clinical findings of oestrus have formed the control group (n=10).

Gynecological Examinations

The postpartum period, was monitored through the rectal palpation and ultrasonographical (ALOKA® PS2) examinations of the ovaries, uterine horns, uterine body and cervix uteri starting from the 14th day. Measurements of uterine horns diameters were made according to Kamimura et al. (1993). During the examinations, 8 mm and larger follicles in both ovaries of the cows were recorded.

Follicular Fluid Aspiration

Follicular fluid aspiration was performed transvaginally using the Ovum Pick Up (OPU) method (Seneda et al. 2003). For the purposes of follicle aspiration, 6 cm (length) 18 G (1.250 mm outer diameter) cannulas. Follicular fluids were transferred into pure serum tubes (BD Vacuteiner®-365815) and centrifuged at 1300 g for 13 minutes and storedat-20ºCuntilanalysis.

Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Methods

Proteins were separated in 10% SDS-PAGE. The same volume of follicular fluid was applied to each well. After electrophoresis, proteins were transferred to nitrocellulose membranes (Nitrocellulose Pure Transfer Membrane, Santa Cruz Biotech) and blocked with 3% bovine albumin in TBST. Goat polyclonal IgG anti-mouse GATA-4, goat polyclonal IgG anti-human GATA-6, GDF-9 and IGF-II were used as a primary antibody and bovine anti-goat IgG-HRP (Santa Cruz Biotech) was used as a secondary antibody. Proteins were detected by Luminol Reagent (Santa Cruz Biotech). Protein bands were scanned using a Bio-Rad GS-800 densitometer and signal intensity was determined with Quantity One Software (Bio-Rad) to compare expression levels among groups.

IGF-I (Insulin-like Growth Factor I) analysis of Follicle Fluid

IGF-I levels in follicular fluids were measured using the ELISA (Enzyme-linked immunosorbent assay) technique using a commercial kit (Cusabio®, CSB-E08893b). The absorbance values of the samples were measured at 450 nm with the ELISA MAT-2000® microplate reader. Absorbance values were calculated in nanogram/milliliter using Ridawin® package program.

Statistical Analysis

The SPSS was used for analysis of data (v14.1; Chicago, IL). Firstly, data were checked for equality of variance trough Levene's test. The Shapiro-Wilk test was performed for normality of original and logarithmic values. Student's t-test was used when data were normally distributed, and the Mann-Whitney U test was used when data were not normally distributed. Descriptive statistics for each variable were calculated and presented as the "mean±SEM." Probability values of $P<0.05$ were considered as statistically significant.

RESULTS

Ultrasonographical Findings

Although there was no statistically significant difference in terms of both uterine horns and cervix involution between cyst and control groups, it is determined that uterine measurement values were higher in the cyst group on 54th day of postpartum period compared to the control group ($p>0.05$). It was observed that the course of the uterine involution process was similar in both groups. However, it was determined that the rate of involution occurred in a more horizontal curve in the cyst group (Table 1). In the ovarian examinations of the cyst and the control group, which were performed by means of ultrasonography from the 14th day until 54th day of postpartum period; luteal structures were identified in the examinations of generally aspirated follicular structures, which conducted ten days later. It was observed in the examinations of these cases, which were conducted 20 days after aspiration, that corpus

luteum disappeared and a new follicular structure bigger than 8 mm was formed. While this observation revealed a statistical difference in terms of follicular numbers compared to the control group on 44th day of postpartum period during which the first cycles were concentrated after aspiration in the cystic group ($p<0.01$), it was found that the said difference disappeared in the examinations conducted on the 54th day of postpartum period when normal cycles started to occur (Table 2). In the cyst group, the follicle diameters which would transform into a cystic structure were found to be significantly larger compared to the control group as of 14th day of postpartum period ($p<0.01$) (Fig1).

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Table 1. Ultrasonographical findings of cervix and uterine horns during post calving periods in cystic and control cows. ($\overline{x}\pm S\,\overline{x}$): mean±standard error.

Examination days (post calving days)	Cervix (cm) ($\overline{x}\pm S\,\overline{x}$)		Uterine horns (cm) ($\overline{x}\pm S\,\overline{x}$)	
	Cystic Group (n=10)	Control Group (n=10)	Cystic Group (n=10)	Control Group (n=10)
14th	4,08±0,36[a]	4,04±0,19[a]	6,90±0,67[a]	6,33±0,50[a]
24th	3,84±0,39[a]	3,62±0,33[a]	5,80±0,62[b]	6,46±0,52[b]
34th	4,14±0,33[a]	3,90±0,21[a]	5,60±0,48[c]	4,65±0,17[c]
44th	4,42±0,41[b]	3,37±0,21[a]	5,30±0,43[d]	4,65±0,33[d]
54th	4,36±0,41[b]	3,70±0,14[a]	5,10±0,44[e]	4,80±0,28[e]

[a,b,c,d,e]: Means with different superscripts in a column are significantly different ($P<0.05$).

Table 2. Mean numbers of follicles in cystic and control cows during post calving period. ($\overline{x}\pm S\overline{x}$): mean±standard error.

Examination days (post calving days)	Cystic Group(cm) (n=10) ($\overline{x}\pm S\,\overline{x}$)		Control Group(cm) (n=10) ($\overline{x}\pm S\,\overline{x}$)	
	Right Ovary	Left Ovary	Right Ovary	Left Ovary
14 th	2,14±0,26[a]	2,57±0,37[a]	1,85±0,34[a]	2,75±0,31[a]
24 th	2,30±0,30[a]	2,30±0,21[a]	1,70±0,21[a]	2,40±0,27[a]
34 th	2,40±0,34[a]	2,30±0,21[a]	1,70±0,30[a]	2,60±0,27[a]
44 th	1,70±0,26[a]	1,90±0,23[a]	2,00±0,26[b]	2,80±0,25[b]
54th	1,80±0,25[a]	2,20±0,20[a]	2,20±0,29[a]	2,30±0,30[a]

[a,b:] Means with different superscripts in a line are significantly different ($P<0.05$).

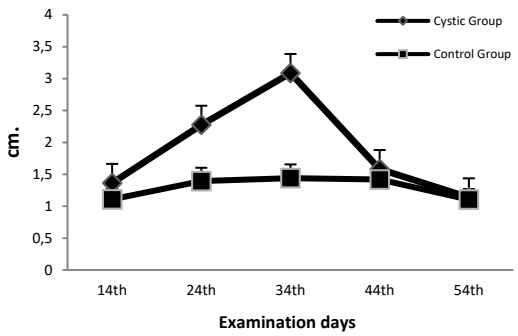

Figure 1. Follicular diameters in cystic and control groups.

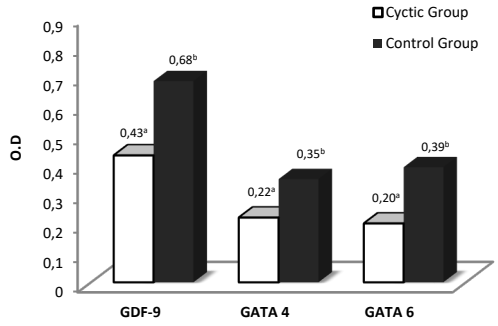

Fig 2. Expression levels of proteins in follicular fluids of bovine cystic follicles .
[a,b]: Means with different superscripts are significantly different *(P<0.01).* O.D: optical density.

Intra-follicular IGF-I and IGF-II Findings in Cyst and Control Groups

In the cyst and control groups, intra-follicular IGF-I levels were calculated to be 20.9±1.01 ng/ml in the cyst group and 27.7±1.55 ng/ml in the control group. Average intra-follicular IGF-I levels were determined to be statistically significant between the groups ($p<0.01$). As for IGF-II, it could not be determined by Western Blot Method.

Western Blot Findings in GDF-9, GATA-4 and GATA-6 Proteins in Follicle Fluid and Densitometric Evaluation of Bands

It was determined that the densitometric values of GDF-9, GATA - 4 and GATA - 6 protein bands obtained from follicular fluids of cyst group were lower compared to the control group ($p < 0.01$). Densitometric measurement values of intra-follicular growth factors such as GDF-9, GATA-4 and GATA-6 are shown in Figure 2.

DISCUSSION

In the examinations made for cyst group on the 24th, 34th, 44th and 54th days of postpartum period, follicular cysts with a follicular diameter of 2.5 cm and above showed a steady growth curve in the following examinations, while the follicles below 2.5 cm showed a faster growth curve. In this group, the recurrence was determined for two out of ten subjects which underwent aspiration. Corpus luteums occurred in eight cases which did not exhibited recurrence after aspiration. It was determined that the life span of the corpus luteum was within a normal cycle for two cases which could be monitored during the study period and that it was directed to a healthy cycle in the 20-day period following aspiration. Completion of the postpartum period within physiological limits is the only way to achieve economic goals in dairy cattle breeding. Controlling the factors which affects this process ensures that fertility parameters remain within acceptable limits (LeBlanc et al. 2002; Ingvartsen and Moyes, 2012). Bacterial lipopolysaccharides produced particularly by gram negative bacteria in the presence of contamination and infection of the uterus in the process of involution negatively affect ovarian and pituitary functions (Sheldon et al. 2002).

Although postpartum uterine dimensions vary according to the breed and genetic characteristics of the cow, it is generally identified in ultrasonographic measurements of the uterus to be approximately 15 cm, 9-1 cm, 7-8 cm, and 5-6 cm on the 2nd, 10th, 30th and 60th days of postpartum period respectively (Arthur et al. 1982). Olson et al. (1986) reported these measurements to be 3-4 cm and 2-3 cm on the 30th and 40th day of postpartum period respectively. In the presented study, uterine horn diameter was reduced from 6.9±0.67 cm to 5.1±0.43 cm measured for cyst group respectively on14th and 54th day of postpartum period; whereas it was reduced from 6.33±0.5 cm to 4.8±0.28 cm measured for control group respectively on14th and 54th day of postpartum period; As for cervical uteri diameters; it was determined to be 4.08±0.36 cm and 4.36±0.41 cm measured for cyst group respectively on14th and 54th day of postpartum period; whereas it was determined to be 4.04±0.19 cm and 3.70±0.14 cm measured for control group respectively on14th and 54th day of postpartum period. These measurements for both study groups are similar to the measurement findings by Zhang et al. (2010), Olson et al. (1986) and Senosy et al. (2009). The cervix measurements, which started to decrease in parallel with the control group on the 14th day of postpartum period, exhibited a different increase than the control group on the 24th day of postpartum period. The slow involution process in the cyst group and the increase in cervix diameters as of the 24th day of postpartum period, cysts began to form structurally; the edema which exhibits increase in the uterus tissue especially under the reflection of intra-follicular steroids after the 24th day of postpartum period when cysts start to form structurally is considered that it could change the measurement values and involution curve in cases with tendency towards the cyst.

The follicular activity in the ovaries in the postpartum period and selection of dominant follicle depend on the frequency of gonadotropic hormone release (Crowe 2008), and the selection of the dominant follicle occurs as of the 10th day after birth. The first ovulation after delivery is formed in the second week of postpartum period (McDougall et al. 1995). However, the first cycles in the postpartum period are short whereas the life span of the corpus luteum after possible ovulation may be even shorter (Wathes et al. 2003). Thiengtham et al. (2008) reported that the first dominant follicle selection and early ovulation occurred between 12th to 15th days of the postpartum period, while Sakaguchi (2011) reported that they observed initial ovulation on the 18th day of the postpartum period, but the first cycles were short. Lee and Kim (2006) stated that how many times a cow gives birth to a calf and its milk yield play an important role in the formation of cystic follicular structures; accordingly, they proposed that the incidence of cyst also increases as the number of births given increases.In this study, the fact that the ovaries were active for the majority of the subjects on the 14 th day of postpartum period and the follicle diameters were generally larger than 1.5 cm in the cyst group was in parallel with the findings of Sakaguchi (2011) and Thiengtham et al. (2008). However, it was determined to differ from the control group due to the fact that initial ovulations are not observed in the early period. For the cyst group in the presented study, the mean diameter of the largest follicles was measured to 1.85±0.28 in the examinations made on the 14th day of the postpartum period and it was significantly different from the healthy group ($p < 0.01$).

Insulin and IGF-I receptors are specifically available in the cow ovary. Insulin plays an indirect role in follicle development by acting on IGF-I receptors (Spicer and Echternkamp, 1995). Insulin-like growth factors influence the release of FSH and LH and thus support the steroidogenesis process in follicular cells, the increase in the number and function of LH receptors. However, IGF (insulin-like growth factor) system plays an important role in follicle selection and ovulation by stimulating the aromatase system together with carrier proteins (IGFBP = Insulin-like growth factor-binding protein) (Zulu et al. 2002b). The IGF-I system plays a central role in the regulation of TGF-β bioactivity during the growth and development of the follicles, thanks to specific extracellular proteases. While IGF-I plays an important role in the selection of dominant follicles, intra-follicular IGF-I levels show a positive correlation with the follicle diameter in physiologically healthy follicles. Intra-follicular IGF-I levels in cystic structures and physiological dominant follicles differ in cows (Perks et al. 1999). Eden et al. (1988) reported that intra-follicular IGF-I levels in women with polycystic ovary syndrome are the same as healthy individuals and that there is no intra-follicular difference, while Ortega et al. (2008) stated that the opposite is valid for the cows. Bovine granulosa cells can synthesize IGF-I carrier proteins (Santiago et al. 2005). Possible insufficiency of the expression of carrier proteins in granulosa cells in follicles with tendency towards cystic structure and irregularity in the IGF system suggests that the cystic structures may be an indicator of the decrease in intra-follicular IGF levels in the postpartum period. In this study, the lack of ovulation in cystic subjects on the 14th day of postpartum period can be associated with the increase in the number LH receptors due to a probable decrease in IGF-I level in blood and lack of response to LH, and low intra-follicular IGF-I levels in cystic cases,

LH surges and preservation of the presence of oocyte with a view to inhibiting premature differentiation of granulosa cells as a result of increase in intra-follicular estradiol upon second release of FSH.

The functions of GDF-9, a member of the TGF- β parent family, ensure that proteins and binding proteins bind to receptors during follicle development. These receptors are frequently available in oocyte, granulosa cell and theca cells. However, these receptors interact with different proteins at different stages of development of the follicles, and their distribution differs depending on the species (Dijke et al. 2000). GDF-9, which plays a role in regulation of the quality and function of the oocyte, is produced by oocyte cumulus cells. GDF-9 takes a part in promoting oocyte health and its gradual maturation (Elvin et al. 2000). Juengel and McNatty (2005) reported that the gradual maturation was regulated by means of the lumen-facing cells of the cumulus cells and follicles, and the factors released from oocyte were restricted and the concentration level of oocyte-derived factors was controlled during this process. Regarding cows and sheep,GDF-9, BMP-6 and 15 are expressed by the oocyte in the early stages of the primordial follicle (McGrath et al. 1995; Jaatinan et al. 1999; Bodensteiner et al. 1999; McNatty et al. 2001). It is reported that GDF-9 deficiencies cause follicular development disorders in women and GDF-9 expression decreases in women with polycystic ovary syndrome (Zhao et al. 2010). In this study, it is anticipated that the results obtained for follicular cysts and after treatment of follicular fluids extracted from preovulatory healthy follicles with GDF-9 antibody are similar to the findings of Juengel and McNatty (2005), and the oocyte could be degenerated and aged in cystic structures, and accordingly, the required signals for establishment of relation between oocyte and somatic cells and protein expressions could not be made satisfactorily and remain persistent in the ovaries with no tendency for ovulation. In the study, it is known that inhibin alpha expression is high in follicular fluid analyzes and immunohistochemical studies on cystic and granulosa cell tumors, although the level of inhibin alpha expression is not checked (Matzuk et al. 1992). As is known, inhibin alpha uses common receptors with members of the TGF-beta family regarding its intra-follicular actions (Stenvers and Findlay, 2009), and it is suggested in this study for the cyst group that the preferable binding of the probably high inhibin alpha to these receptors might have indirectly blocked the actions of GDF-9.

GATA-4 and 6 are released from granulosa cells and theca cells in the ovary (Heikinheimo et al. 1997; Laitinen et al. 2000). In the presented study, the fact that the expression levels of GATA-4 and GATA-6 proteins in follicular cysts were found to be significantly lower densitometrically compared to the control group ($p<0.01$) can be associated with the inadequacy of gonadotropin receptors in the cystic follicles. Additionally, the fact that there may be no cellular differentiation at the ovarian level (receptor up regulation) despite the tonic and pulsatile gonadotropin releases in the cyst group can be considered as problems in response to gonadotropins and consequently anovulation of the follicles.

It can be concluded that GATA-4 and GATA-6 expressions and activations are suppressed as a result of gonadotropin receptor deficiency in the presence of possibly low IGF-I levels in terms of intra-follicular molecular regulation mechanisms as well as systemic metabolic changes such as bacterial endotoxins, insulin resistance in the formation of cystic follicular structures in the early postpartum period. Furthermore, it is suggested that the follicles with tendency towards cysts in the early postpartum period are shaped from the first follicular wave which develops as of 14th day of the postpartum period, and they are intensified in clinical presentation between 24th and 34th days of the postpartum period.

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