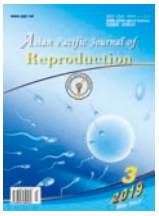

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Follicular fluid composition of ovulatory follicles in repeat breeder Holstein dairy cows

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ABSTRACT

Objective: To examine differences in the metabolite, steroid and lipopolysaccharide of follicular fluid collected from the ovulatory follicle of Holstein repeat breeding cows, lactating cows, and virgin fertile heifers.

Methods: Estrus was induced in animals possessing functional corpus luteum by intramuscular administration of prostaglandin F2α. Six to twelve hours after detection of the standing estrus, cervical samples were collected to detect subclinical endometritis *via* counting neutrophils on stained smears of cervical swabs. Then, follicular fluid of ovulatory follicles and serum samples were collected from repeat breeding cows (n=11), lactating cows (n=8) and virgin fertile heifers (n=10). Sodium and potassium were measured with a flame photometer method. Urea, total protein, glucose, cholesterol and β-hydroxybutyric acid were assayed with commercial spectrophotometry kits. Chloride concentration was also measured with titration of samples against silver nitrate. Progesterone, estradiol-17β and lipopolysaccharide concentrations were measured using enzyme-linked immuno sorbent assay kits.

Results: All analysis of follicular fluid samples showed that repeat breeding and lactating cows had a mean higher lipopolysaccharide concentration than that of the virgin fertile heifers (P<0.05). But concentration of serum estradiol-17β in repeat breeding and lactating cows was lower than that of virgin fertile heifers (P<0.05). In addition, the mean percentage of neutrophils in the cervical secretion of repeat breeding cows was higher than that of lactating cows (P<0.05).

Conclusions: High follicular fluid concentration of lipopolysaccharide in ovulatory follicles results in the occurrence of repeat breeding syndrome in dairy cows. Further, a lower serum estradiol-17β concentration and a higher percentage of neutrophil in the cervical secretion on the day of artificial insemination may have resulted in the occurrence of repeat breeding syndrome in dairy cows.

1. Introduction

Repeat breeding (RB) syndrome is considered as one of the most important causes of low reproductive performance in dairy herds throughout the world. The occurrence of a high incidence of RB syndrome results in increased calving to conception interval, calving interval, a high number of services per conception, and the high risk of early involuntary culling. All these outcomes detrimentally impact the profitability of the dairy industry[1,2]. RB cows are

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routinely found as clinically healthy with normal estrous cycles. No abnormalities including anatomical and uterine infections are palpably found in these animals. Based on a conventional definition, when cows do not conceive after either continuous artificial inseminations at least three times or natural mating using semen from well-known fertility bulls, they are categorized as repeat breeders[1]. Epidemiological studies report various incidences of RB syndrome in dairy cows in different regions of the world[1,3-5]. The large variation in the reported incidence of RB syndrome could be mainly ascribed to differences in the definitions, different managements and regions. Subclinical endometritis[6], nutritional deficiencies[7], abnormal heat behavior or improper heat detection[8] mismanagement in artificial insemination[9], and endocrine dysfunctions[8] are considered as potential causes of the RB syndrome in dairy cows.

Follicular fluid and its components play a vital role in the final development of the ovulatory follicles and oocytes[10-12]. The sources of follicular fluid are transudated plasma and follicular cells[10,13]. Thus, the contents of follicular fluid are similar to plasma with some differences which are vital for ovarian functions such as steroidogenesis, follicle growth, oocyte maturation, ovulation and oocyte transport in the oviduct[14,15]. As the follicle grows, the composition of follicular fluid is also altered[12]. Plasma biochemical elements including magnesium, chloride (Cl^-), zinc, copper and mineral phosphate with similar concentrations are found in the bovine follicular fluid; however, sodium (Na^+) and potassium (K^+) concentrations are higher than plasma. Lactic acid, cholesterol, vitamins C and A have also been detected in bovine follicular fluid. Vitamins may be related to hormonal prosynthesis and synthesis during follicular growth and development[16,17].

It has been shown that the metabolic profile of follicular fluid is influenced by season, stages of lactation, uterine and metabolic diseases and genetic merit for fertility and parity of dairy cows[18-20]. In addition, many studies have shown that pathological changes in the serum of postpartum dairy cows may affect the follicular fluid composition and this in turn may affect the quality of both oocytes and granulosa cells[21-23]. Increased β -hydroxybutyric acid (β -HBA) concentrations in follicular fluid during negative energy balance affected oocyte quality and this resulted in lower fertility of dairy cows[13]. Further, heat stress aggravated negative energy balance in early postpartum and altered biochemical contents including cholesterol, glucose, insulin growth factor-1 (IGF-1), and urea in follicular fluid of the dominant follicle in high-yielding dairy cows, which may result in inferior oocyte and granulosa cell quality and hence poorer fertility[17]. Recently, proteomic analysis of preovulatory follicular fluid revealed differentially abundant proteins in less fertile dairy cows[14]. Eight novel proteins that could influence follicular function have been found. In RB dairy heifers with a history of at least five unsuccessful artificial inseminations, inferior quality of follicular fluid of ovulatory follicles was evidenced by a low reduction in *in-vitro* nuclear maturation, fertilization and blastocyst yield[24]. To our knowledge, no detailed study has examined the follicular fluid composition of the ovulatory follicle in RB Holstein dairy cows. Therefore, the study was designed to examine metabolic, biochemical and hormonal changes in follicular fluid in RB cows, lactating cows, and virgin fertile (VF) heifers.

2. Materials and methods

All procedures used in the present study were licensed by the Ethical and Research Committee of the School of Veterinary Medicine, Shiraz University (IACUC No: 4687/63).

2.1. Animals and clinical examination

All cows and heifers (n=29) used in the present study were selected from a Holstein dairy herd located in the south of Shiraz, Fars, Iran. The animals were reared using a standard feeding schedule and ration (National Research Council, 2001). The cows were milked twice daily at 07:00 and 16:00. Manual rectal palpations and trans-rectal ultrasonography did not reveal any detectable pathology in the reproductive tract of all animals. They were assigned in three groups: 11 RB cows, 8 lactating dairy cows and 10 VF heifers that had normal and regular estrous cycles (with mean 13.6 months of age). All lactating and RB cows had a history of normal previous calving with 3.75 and 4.10 mean lactation numbers, respectively. Collection of samples was performed on mean days in milk [(60.9±5.5) days] in lactating cows. All RB cows had a history of at least three unsuccessful artificial inseminations using semen from high fertile bulls. The mean days in milk were (147.0±47.0) days in RB cows. All cows had been estrous synchronized (using 500 μg cloprostenol sodium, intramuscular, Estroplan Parnell Living Science, Australia) before they aligned to this study. So, heifers or cows at the same time of estrous phase were selected and sampled. All VF heifers were inseminated after our samplings in their next estrus. The VF heifers were diagnosed as pregnant after artificial insemination. Lactating cows also became pregnant in less than three artificial inseminations in their next estruses after our samplings. Subclinical endometritis was detected using criteria described by Parkinson *et al*[25] and Sheldon *et al*[26] *via* counting neutrophils in cytology samples on stained smears of cervical swabs.

2.2. Collection of follicular fluid and serum

Estrus for all animals was defined as the period when the animal stood to be mounted by another animal. Six to twelve hours after detection of the standing estrus, ovarian ultrasonography was performed to assess the presence of an ovulatory follicle (12-17 mm diameter). Then, a sample of follicular fluid was aspirated by using a long fine-needle covered by a hard plastic tube *via* rectum under the caudal epidural anesthesia (2% lidocaine hydrochloride; 0.2 mg/kg). In addition, blood samples from coccygeal vein were also collected to confirm estrus and examine the estradiol-17 β , IGF-1, and lipopolysaccharide (LPS) concentrations. Blood samples were centrifuged at 3 000 rpm for 10 min and then serum was separated. All samples of sera and follicular fluids were frozen at -20 ℃ until assayed for hormones, LPS, and other metabolites.

2.3. Assay of follicular fluid and serum LPS

LPS was measured with enzyme-linked immuno sorbent assay (ELISA) kit and sensitivity 3.86 EU/L (Hangzhou, Eastbiopharm CO., LTD, USA) following the manufacturer's guidelines. Briefly, the kit used a double-antibody sandwich ELISA to examine the level of LPS in serum, plasma and other related tissue liquid samples. All samples including the standard, blank and follicular fluid were assayed in duplicate. Internal recovery as determined using positively spiked follicular fluid samples was >80% and the intra- and inter-assay coefficients of variation were <10% and <12%, respectively.

2.4. Determination of follicular fluid and serum biochemical contents and metabolites

Na^+ and K^+ were measured with a flame photometer method (Flame photometer Jenway PFP7, UK). Urea, total protein, glucose, and cholesterol were assayed with commercial photometric kits (Roch, GMbH, Mannheim, Germany). Cl^- concentration was measured with titration of samples against silver nitrate. β -HBA was assayed with spectrophotometry method and a commercial kit (Randox Laboratories, Crumlin, Antrim, UK)[27].

2.5. Hormone assay

Progesterone and estradiol-17 β concentrations in follicular fluid and blood serum were measured with validated commercial ELISA kits (Immunotech, France). Progesterone and estradiol-17 β concentrations in follicular fluid were not determined in VF heifers as the amount of the samples was inadequate. For progesterone ELISA kits sensitivity, intra-assay and inter-assay coefficients were 0.05 ng/mL, 5.8% and 9.0%, respectively. Sensitivity, intra-assay and inter-assay coefficients of estradiol-17 β kits were 6.0 pg/mL, 12.1% and 11.2%, respectively. IGF-1 concentration was assayed by using a commercial kit (Diasorin Kit, Italy) and immune analyzation method. Sensitivity, intra-assay and inter-assay coefficients of this kit were 0.07 μg/mL, 7.5% and 12.0%, respectively.

2.6. Statistical analyses

Data were analyzed using one-way analysis of variance and least significant difference *post hoc* test was used for comparison of different biochemical, metabolic and hormonal concentrations among groups. In all cows and heifers, correlations between different factors were analyzed with two variable Spearman's *rho* correlation test. Data were analyzed with SPSS®10.0 for windows (Chicago, IL, USA). Data were presented as mean ± standard deviation (mean ± SD) and $P<0.05$ was considered as level of statistical significant difference.

3. Results

In this study, all aspirated ovulatory-sized follicles in cows were considered dominant as their estradiol : progesterone ratio in follicular fluid was greater than 1. The results of the present study are presented in Tables 1 and 2.

3.1. Serum LPS concentrations

Mean serum LPS concentration was numerically greater in RB cows than that of lactating cows and VF heifers. Further, the difference between RB and lactating cows was not statistically significant (Table 1).

3.2. Follicular fluid LPS concentrations

Mean follicular fluid LPS concentration in ovulatory follicles of the VF heifers was lower than that of RB cows ($P<0.05$), whereas no difference was observed in follicular fluid LPS concentrations between the VF heifers and lactating cows. In addition, mean follicular fluid LPS concentration in ovulatory follicles of lactating cows tended to be lower than that of the RB cows. Furthermore, 82.0% of RB cows had higher amount of LPS in follicular fluid of their ovulatory follicles compared to the mean LPS concentration in follicular fluid for VF heifers, while only 37.5% of lactating cows had high LPS concentrations in follicular fluid compared to the mean LPS concentration in follicular fluid of VF heifers (Table 2).

3.3. Metabolite and hormonal concentrations in blood serum

Mean serum estradiol-17 β concentration was higher in VF heifers than that of the lactating and RB cows ($P<0.05$). In addition, mean serum estradiol-17 β concentration of RB cows was lower than that of the lactating cows ($P<0.05$). The progesterone concentration in VF heifers was higher than that in RB cows. The mean percentage of neutrophils in cervical mucus of RB cows was greater than that of lactating cows ($P<0.05$). No difference was observed in serum IGF-1 concentrations between RB and lactating cows (Table 1).

Table 1. Serum lipopolysaccharide, hormones concentrations and cervical neutrophils percentages in RB cows, lactating cows and VF heifers.

Groups	Lipopolysaccharide (EU/L)	Hormones			Neutrophils (%)
		Estradiol-17 β (pg/mL)	Progesterone (ng/mL)	IGF-1 (μg/mL)	
RB cows (n=11)	1065.00 ± 943.4[a]	36.00 ± 11.27[a]	0.36 ± 0.66[a]	49.70 ± 23.21[a]	13.00 ± 5.30[a]
Lactating cows (n=8)	846.20 ± 690.22[a]	56.60 ± 37.04[b]	0.64 ± 0.56[ab]	57.80 ± 18.94[a]	3.90 ± 4.24[b]
VF heifers (n=10)	636.30 ± 154.62[b]	75.70 ± 24.34[c]	1.00 ± 0.28[b]	ND	ND

Data are expressed as mean±SD. Different superscript letters (a, b, c) in each row indicate significant differences ($P<0.05$); IGF-1 = insulin growth factor-1; ND = not determined as the heifers were virgin. RB: repeat breeding; VF: virgin fertile.

Table 2. Lipopolysaccharide, biochemical, hormones and metabolite concentrations in follicular fluid of ovulatory follicles in RB cows, lactating cows and VF heifers.

Follicular fluid		RB cows (n=11)	Lactating cows (n=8)	VF heifers (n=10)
Lipopolysaccharide (EU/L)		549.00 ± 163.47^{a}	436.00 ± 111.98^{ab}	409.00 ± 99.28^{b}
Biochemicals	Glucose (mg/dL)	62.00 ± 7.42^{a}	54.87 ± 8.20^{ab}	73.50 ± 9.16^{b}
	Cholestrol (mg/dL)	49.20 ± 23.31^{a}	48.70 ± 25.45^{a}	41.30 ± 22.76^{a}
	Total protein (g/dL)	6.78 ± 0.53^{a}	6.83 ± 0.67^{a}	6.28 ± 0.18^{b}
	K^+ (mEq/L)	4.35 ± 0.46^{a}	4.00 ± 2.06^{ab}	3.96 ± 0.12^{b}
	Na^+ (mEq/L)	140.72 ± 1.79^{a}	138.60 ± 1.75^{b}	139.87 ± 1.83^{ab}
	Cl^- (mEq/L)	113.00 ± 11.27^{a}	120.80 ± 3.40^{a}	97.90 ± 3.73^{b}
Hormones	Estradiol-17 β (ng/mL)	265.00 ± 32.96^{a}	265.00 ± 36.19^{a}	ND
	Progesterone (ng/mL)	109.80 ± 14.92^{a}	113.00 ± 18.66^{a}	ND
	IGF-1(μg/mL)	65.30 ± 19.89^{a}	61.20 ± 18.09^{a}	135.30 ± 50.90^{b}
Metabolites (mg/dL)	β-HBA	461.00 ± 280.86^{a}	473.20 ± 216.05^{a}	434.00 ± 225.76^{b}
	Urea	24.45 ± 4.97^{a}	28.50 ± 10.46^{a}	14.78 ± 6.32^{b}

Data are expressed as mean±SD. Different superscript letters (a, b) in each row indicate significant differences (P<0.05); K^+ = potassium; Na^+ = sodium; Cl^- = chloride; IGF-1 = insulin growth factor-1; β -HBA = beta-hydroxybutyric acid; ND = not determined as the amount of samples was inadequate.

There was a significant and positive correlation in IGF-1 concentration (Y = 1.142 9X + 8.571 4, r=0.64, P<0.05) and LPS concentrations (Y = 0.266 7X + 200, r=0.548, P<0.05) between follicular fluid and serum samples when the data of the animals were pooled (Figure 1).

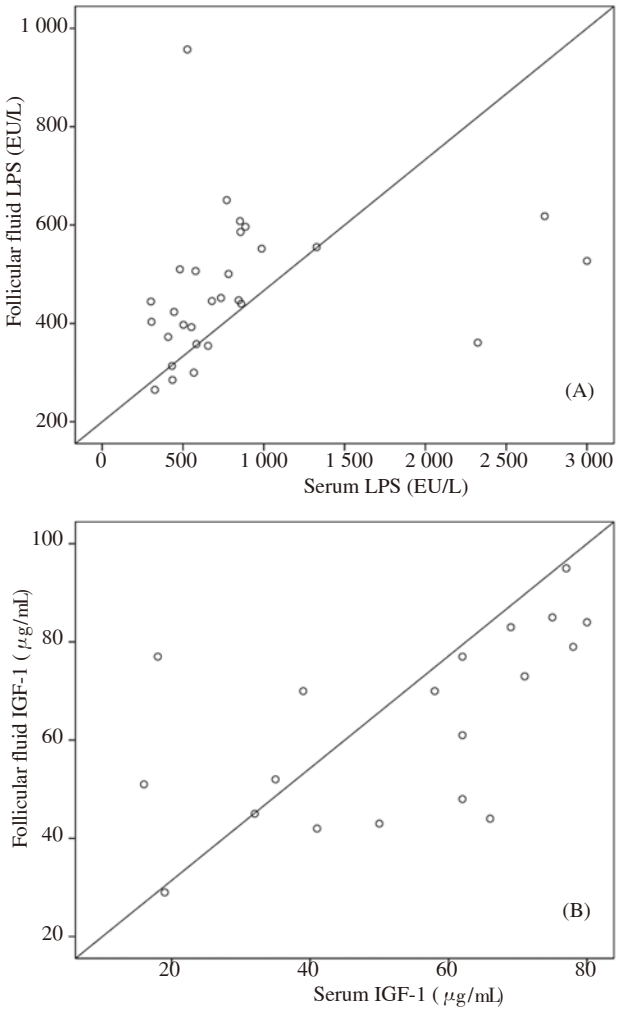

Figure 1. Correlation between (A) lipopolysaccharide (LPS) concentration in follicular fluid and blood serum and (B) IGF-1 concentration in follicular fluid and blood serum in all cows.

3.4. Biochemical, metabolic and hormonal concentrations in follicular fluid

The mean glucose concentration was greater in follicular fluid of ovulatory follicles of VF heifers than that of the RB cows (P<0.05). Mean total protein concentration in follicular fluid of ovulatory follicles in VF heifers was lower than that of the lactating and RB cows (P<0.05). Mean follicular fluid concentrations of K^+ and Na^+ in ovulatory follicles of RB cows were higher than those of the lactating cows and VF heifers (P<0.05). However, Cl^- concentration in follicular fluid of VF heifers was lower than that of lactating and RB cows (P<0.05). Mean follicular fluid urea concentration in ovulatory follicles of VF heifers was lower than that of lactating and RB cows (P<0.05). Mean follicular fluid concentration of IGF-1 in ovulatory follicles of VF heifers was higher than that of lactating and RB cows (P<0.05). β -HBA concentration in VF heifers was lower than that in RB and lactating cows. No significant difference was observed in follicular fluid progesterone concentrations between RB and lactating cows. Further, no difference was found in follicular fluid estradiol-17 β concentrations between RB and lactating cows (Table 2).

4. Discussion

Results of the present study showed a higher mean percentage of neutrophils in the cervical mucus of RB cows compared to that of the lactating cows. Collection of cervical swabs for detection of neutrophils has been regarded as an effective method for diagnosing chronic uterine infection in dairy cows[26,27]. The adverse effect of uterine bacterial infections on the growth of the dominant follicle was previously shown in postpartum dairy cows[28]. Our results confirmed that subclinical chronic endometritis was the major cause of the occurrence of RB syndrome in dairy cows. Similarly, subclinical endometritis was found to be the major cause of the occurrence of the RB syndrome in Holstein dairy cows[29–31]. The higher LPS concentration in follicular fluid of the ovulatory follicle in the RB cows compared to that of the VF heifers and lactating dairy cows (albeit approaching significant difference) could explain

lower fertility in the RB cows used in the present study. Bromfield and Sheldon[32] showed that bovine cortical ovarian follicles produced inflammatory mediators including interleukin 1, 6 and 8 in a LPS concentration-dependent manner. Interleukins are proteins and signal molecules that are secreted by leukocytes and many other body cells. Therefore, the presence of higher protein concentrations in the ovulatory follicles in RB and lactating cows in the present study could be due to the presence of high LPS concentrations in the ovulatory follicles in these animals. Our findings further showed that 82.0% of RB cows had higher amount of LPS in follicular fluid of their ovulatory follicles compared to the mean LPS concentration in follicular fluid for VF heifers, while only 37.5% of lactating cows had high LPS concentrations in follicular fluid compared to the mean LPS concentration in follicular fluid of VF heifers. Follicular fluid LPS concentrations were significantly lower in the ovulatory follicles of VF heifers than those in the RB dairy cows in the present study. Furthermore, no difference was observed in follicular fluid LPS concentrations between the VF heifers and lactating cows. Exposure of bovine theca cells to LPS under *in vitro* conditions suppressed the level of gene expression of luteinizing hormone receptor which resulted in a reduced production of progesterone concentration[16]. In addition, Magata *et al*[16] showed that the follicular fluid concentration of estradiol-17β was lower in follicles with a high level of LPS compared to those in follicles with a low level of LPS. Later, it was observed that transcripts of steroidogenic enzymes such as cytochrome P450 17-alpha-hydroxylase/17,20 lyase and aromatase P450 are reduced in bovine follicles with a high level of LPS[17].

Results of the present study also showed a lower mean follicular fluid concentration of estradiol-17β in RB cows compared to those of VF heifers. Williams *et al*[33] showed that exposure of bovine granulosa cells to LPS *in vitro* resulted in low production of estradiol-17β. Furthermore, Bromfield and Sheldon[32] detected an accumulation of LPS secreted from Gram-negative bacteria in follicular fluid of postpartum cows with uterine infections. LPS in the follicular fluid of cows with uterine infections has been proposed to reach the ovarian follicles from the uterus by the counter-current vascular mechanisms[34]. Then, LPS may perturb the endocrine function of the granulosa cells of the ovulatory follicles[4,32]. Our findings, together with that of the Bromfield and Sheldon's, indicate that the presence of LPS in follicular fluid of the ovulatory follicles may cause ovarian dysfunction, resulting in the occurrence of RB syndrome in dairy cows[32]. The positive and significant correlation between the serum and follicular fluid LPS concentrations means a direct transfer of LPS from circulation to the ovarian follicles. The higher serum LPS concentration implies that LPS may translocate from the digestive tract[35,36] as well as the uterus into blood circulation. Another possibility could be the fact that values for blood quantitates of LPS should be interpreted with caution. This is because most LPS assays cannot distinguish between lipopolysaccharide-binding protein bound to LPS or the unbound LPS[37].

As expected, the follicular fluid concentrations of IGF-1 in the ovulatory follicles were significantly higher in the VF heifers than those of lactating and RB dairy cows in the present study. Many researchers have shown a reduced blood IGF-1 concentration in lactating cows experiencing negative energy balance, potentially due to changes in liver function during early lactation[38]; however, very few researches have determined follicular fluid IGF-1 concentrations in ovulatory follicles in RB cows[39]. Our findings of higher IGF-1 concentrations in follicular fluid of the ovulatory follicle of VF heifers compared to lactating cows are in agreement with previous studies[40,41]. The important role of IGF-1 in ovarian function and follicular development has been well documented in the bovine[42]. Glucose and IGF-1 concentrations in follicular fluid of RB cows were significantly lower than those in VF heifers in the present study. Adequate and vital concentrations of IGF-1 and glucose for optimal follicular development[40] and oocyte growth[41] and their stimulatory roles for follicular growth have been described in the bovine[17,43]. Serum estradiol-17β concentrations of ovulatory follicles at the time of estrus in the RB cows were less than those of VF heifers and lactating cows in the present study. Serum estradiol-17β concentration at the time of estrus has been shown to be lower in less fertile dairy cows, potentially due to higher steroid metabolism in the liver[44,45]. The positive and significant correlation between serum and follicular fluid IGF-1 concentrations suggests a direct transfer of the IGF-1 from blood circulation to the follicles. However, the higher IGF-1 in the follicular fluid in our study shows that there is also a local mechanism to produce IGF-1 in the follicles as well[46]. In addition, the higher amount of IGF-1 in the ovulatory follicles has been previously shown in cows[47, 48].

The follicular fluid β-HBA concentration was significantly higher in RB and lactating cows than that of the VF heifers in the present study. Higher concentrations of β-HBA in follicular fluid are associated with decreased follicular steroidogenesis and lower *in vitro* oocyte competence[49,50]. Our results further confirmed that low fertility in RB cows could be attributed to the higher β-HBA in follicular fluid of their ovulatory follicles. In addition, the low serum estradiol-17β concentration in association with high follicular fluid β-HBA concentrations can explain low fertility in RB and lactation cows than the VF heifers.

Urea concentrations in follicular fluid of ovulatory follicles in lactating and RB cows were significantly greater than those of VF heifers in the present study. The high blood urea concentrations are mostly due to high-crude protein diets which are routinely fed in lactating dairy cows[51]. The concentration of urea in plasma is well correlated with that of the follicular fluid[52]. The lower follicular fluid urea concentrations in VF heifers can explain the higher fertility in virgin heifers in the present study. High urea concentrations have been shown to result in the formation of inferior quality oocytes[21], reduction in the uterine pH and these in turn cause the development of inferior quality embryos[53]. Cholesterol is a well-documented precursor of steroid synthesis in follicular fluid of follicles in the

mammalian ovary[54]. The steroid concentration in follicular fluid increases as the cholesterol concentration increases[55]. Therefore, as expected, there was no difference in the estradiol concentration in the ovulatory follicles among animal groups in the present study.

Total protein concentration was significantly greater in follicular fluid of ovulatory follicles in RB and lactating cows than that of the VF heifers in the present study. There is a high correlation in follicular fluid total protein content and serum in the bovine which suggests that a substantial part of the protein content in follicular fluid originates from serum.

The Cl^- has been shown to initiate the luteinizing hormone-stimulated steroidogenesis in the chicken granulosa cells, amphibians oocytes and steroidogenesis in adrenal glands in rats[56]. The lower Cl^- concentration in the ovulatory follicles of RB and lactating cows in our study may explain the lower steroidogenesis in lactating cows comparing with the dairy heifers at the time of estrus reported by other research groups[45,57]. Na^+ is essential to the active transport of glucose across the plasma membranes (Na^+ glucose-linked transporter), which acts against an electrochemical gradient. Later, Alves *et al*[10] reported a positive correlation between Na^+ and glucose in follicular fluid of large follicles in lactating cows. Further, it was shown that the steroidogenic activity of the granulosa cells needs high glucose utilization[58]. Our results in the present study showed a higher concentration of glucose in the ovulatory follicles of the heifers. Unexpectedly, we observed an insignificant difference in Na^+ concentration in the ovulatory follicle in VF heifers and the RB and the lactating cows, which remained to be explained. Uterine pathogens such as *Trueperella pyogenes* may secrete pyolysin, which forms pores in the plasma membrane of endometrial cells. This may result in the leakage of K^+[59]. Follicular fluid K^+ concentration in the ovulatoty follicles of RB cows was significantly higher than those of VF heifers in the present study. Adams *et al* showed that LPS is able to form micron-size pores in the cell membrane *via* destabilization of the lipid bilayer assemblies[60]. This finding implies that the same may be true for the granulosa cells in the ovulatory follicles of RB cows. This needs further investigation.

In conclusion, our results show that the inferior quality of the microenvironment of ovulatory follicles should be considered as a developmental disadvantage compared with the microenvironment of ovulatory follicles in Holstein lactating cows and VF heifers. Furthermore, subclinical endometritis and low serum estradiol-17 β on the day of artificial insemination were the main causes for the occurrence of RB syndrome in dairy cows in the present study.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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