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**Electrolytes, metabolic and Acid-base Parameters in Blood and Fluids of the Reproductive
Tract during in vivo Maturation of Bovine Oocytes**

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Keywords: iSTAT, bovine follicular, oviductal and uterine fluids, metabolites, acid-base

ABSTRACT

Follicular fluid is the microenvironment that supports oocyte maturation and competence. Using Abbott iSTAT1™ and NanoDrop, we determined the dynamics of acid-base, electrolyte, metabolites, and total protein in venous blood, fluids of the dominant follicle (FF), oviduct (OF), and uterus (UF) during the window of oocyte maturation. Holstein heifers (n=36) were synchronized with PGF_{2α} on Days -11 and 0, CIDR during Days -6 to 1, and GnRH given on Day 2 after 2nd PG. Samples were collected at 24h, 48h, 60h, 72h, and 78h after 2nd PG. Most electrolytes analyzed, Cl⁻, K⁺, and Ca²⁺ were significantly affected in blood and FF ($P<0.05$) by CIDR removal. Similarly, Cl⁻ and Na⁺ also significantly changed in OF and UF across time. Glucose, lactate, and creatinine significantly changed across time points in FF compared to blood. Moreover, pO₂, pCO₂, TCO₂, and pH significantly changed across time in FF. Most parameters were not significantly correlated between blood and FF across time points except for glucose, Cl⁻ and creatinine. Furthermore, pO₂ in FF was nearly 3X higher than blood, suggesting low O₂ during *in vitro* maturation is inappropriate. In conclusion, components of the follicular fluid undergo major changes during the window of oocyte maturation.

INTRODUCTION

Bovine embryos from genetically elite animals have been produced by both in vivo and in vitro approaches for rapid genetic improvements. In vivo embryos are of better quality but are more expensive. For instance, the cost of in vivo embryos generated using X-sorted semen is 2.5 times higher than that of their in vitro counterparts¹. While less costly, in vitro embryos are of lower quality, contain fewer cells, survive cryopreservation poorly, and are less fertile^{2,3}. The average pregnancy rate from in vivo embryos is more than 60%, while it is only around 40% from in vitro embryos⁴. The poor in vitro environment in which the oocytes are matured, and embryos are cultured is the culprit of these differences. While ample research has focused on improving the embryo culture media, in vitro blastocyst development continues to linger around 20-30%⁵. The maturation condition of oocytes, however, appears to be more consequential to embryo development. Smith et al.⁶ reported a 75% blastocyst rate from oocytes matured in vivo yet cultured in vitro. Similarly, Yuan et al.⁷ reported a 4-fold increase in live birth rates of cloned pigs by improving oocyte maturation medium alone. While the currently used oocyte maturation condition regularly produces 75-85% nuclear maturation rates, the cytoplasmic maturation remains poor⁵.

Therefore, the determination of the in vivo microenvironments supporting oocyte maturation and embryo development is important in improving embryo production in vitro. Oocyte maturation and early embryo development occur in the ovarian follicle and oviduct/uterus, respectively⁸⁻¹⁰. The oviduct and uterine environments, also play vital roles in sperm transportation, reservoir, and oocyte fertilization¹¹. The fluids of the follicle (FF), oviduct (OF), and uterus (UF) are constituted from a combination of selective serum transudate and locally produced molecules, secreted from the uterine, oviduct epithelial, and granulosa cells^{8,12}.

Numerous prior studies have reported electrolytes, metabolic and acid-base parameters in bovine blood and follicular and a few on oviductal, and uterine fluids^{8,9,12}. However, the vast majority of them used slaughterhouse materials¹³. Among the few that did conduct a controlled study with clearly defined reproductive status, samples were taken from different days of the estrous cycle. No reports to date followed the progression of changes in these fluids during the specific window of time in which the oocytes mature in vivo.

The Abbott point-of-care handheld blood analyzer, iSTAT, is a convenient device for measurements of tissue fluids. A few recent studies have reported its adaption in the bovine blood and tissue fluids^{14,15,16}. In this study, we collected serum, follicular, oviductal, and uterine fluids in Holstein heifers 24h before and after the LH surge, during which the follicles undergo final development, and the oocyte matures. The dynamics of components of these fluids were determined by iSTAT. We found significant changes in most parameters studied. The data obtained here are good references to improve the in vitro oocyte maturation conditions.

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MATERIALS AND METHODS

69 1. Animals and estrus synchronization

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The present study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut. The study was reported in accordance with ARRIVE guidelines and under IACUC supervision. All methods were performed in accordance with the relevant guidelines and regulations. A total of 36 reproductively normal and healthy nulliparous Holstein heifers approximately 15.29 ± 0.54 months of age and 417.03 ± 8.27 kg in weight were purchased from local farms. Once arrived, the animals were given a 14-day acclimation and quarantine time before treatments. The animals were then allocated to one of six groups (n=6/group) for reproductive tract collection.

Fig. 1. Schematic diagram of animal handling, treatment, and tissue collection (not drawn to scale). The synchronization protocol was modified from Bo et al.¹⁷.

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To synchronize estrus, a protocol reported by Bo et al.¹⁷ was modified and the animals were treated as shown in **Fig. 1**. After the 14-day quarantine period animals were given two sets of two prostaglandin F₂α (PGF₂α; 2 ml of Lutalyse HighCon Injection, Dinoprost Tromethamine injection, 12.5 mg/ml) intramuscularly injections eleven days apart on Days -11 and 0. A vaginal CIDR device (controlled internal drug release; EAZI-Breed CIDR Cattle Insert) containing 1.38 grams of progesterone was inserted on Day -6 and removed on Day 1 (24 h after the first PGF₂α injection of the second set). On Day 2 or 48 h after PGF₂α, a single dose of 100 µg gonadotrophin-

releasing hormone (GnRH; 2 ml of Factrel injection, 50 µg/ml) was given intramuscularly to induce the LH surge. This protocol ensures tight estrus synchronization through rapid progesterone decline (after CIDR removal) and precise LH surge (induced by GnRH).

After quarantine, jugular venous blood samples were collected daily into silicone-coated vacutainer tubes without anti-coagulants. The samples were allowed to clot and then centrifuged at 2,500g for 15 min in a refrigerated centrifuge (4°C). The serum was stored at -80°C until analysis. Daily ultrasound of the ovaries using an Aloka 500 ultrasound machine equipped with a linear probe of 5 MHz was conducted starting from the second set of PGF₂α injections (Day 0). To avoid manual ovulation, ultrasound was not conducted on the day of slaughter.

The animals were sacrificed at 24, 48, 60, 72, and 78 h after the first PGF₂α injection of the second set (Day 0), and reproductive tracts were transported at ambient temperature to the lab for further processing.

2. Follicular, oviduct and uterine fluid collection

Follicular fluids from the largest growing follicles were aspirated using a syringe fitted with an 18-Gauge needle. The fluids were centrifuged at 1,000g for 5 min to remove granulosa cells then analysis was done before storing at -80°C. Theca cell layer and granulosa cells of the follicle were examined as described previously¹⁸. Briefly, the collapsed follicle was placed in PBS and rinsed in a new petri dish. Whereupon the follicle was cut into quarters with scissors, and theca and granulosa cells layer were peeled off from the rest of the follicle wall. The granulosa cells were gently scraped using a fine glass needle under the stereomicroscope. The dominant status of the follicles was verified by vascularization of the theca layer and the abundance and morphology of granulosa cells.

To collect fluids from the oviduct and uterus, the uterotubal junction and the uterine-cervical junction were first tied to avoid fluid movements. The oviducts were catheterized from the fimbriae end and oviductal fluid was collected into an Eppendorf tube with gentle massage. The cornu uteri were catheterized from the uterotubal junction and fluid was collected into an Eppendorf tube by gently massaging the cornu uterus.

Due to the small amounts, the fluids from the left and right oviducts and uterine horns were combined. Previous studies of the two sides of the reproductive tracts reported no difference between fluids ipsilateral or contralateral to the side of ovulation^{8,19,20}. The OF and UF samples were centrifuged at 2,000g for 2 minutes in a refrigerated centrifuge (4°C) and then analysis was done before storing at -80°C.

3. Measurements of total protein, gases, acid-base parameters, electrolytes, and metabolites

The NanoDrop 1000™ was used to measure the total protein in both the serum and FF. The handheld iSTAT1 analyzer from Abbott together with the CG4+ and CHEM8+ cartridges were used on all fluids when sufficient amounts were available. CG4+ was designed to determine pH, pO₂, pCO₂, TCO₂, HCO₃⁻, base excess (BE), saturated oxygen saturation (sO₂), and lactate. The CHEM8+ determines sodium (Na⁺), potassium (K⁺), chloride (Cl⁻), ionized calcium (iCa²⁺), TCO₂, glucose, urea nitrogen (BUN)/urea, creatinine, hematocrit, and hemoglobin. The iSTAT device was designed for use with human blood but has been used off-label in other species and tissue fluids^{15,16}. The caveat of this off-label use is that the cartridges were calibrated to the ranges of parameters in humans. Animal fluids may contain levels higher or lower than the maximum or minimum detection limits.

4. Statistical analysis

130 We used R[®] software²¹ to run one-way ANOVA across different time points after PGF_{2α}
131 and significance was set at $P < 0.05$. We also used Fisher's Least Significant Difference (LSD) for
132 pairwise comparisons of different time points. Linear regression and Pearson correlation were used
133 to evaluate the relationship between blood (predictor) and FF (outcome) values of different
134 parameters across time. The goodness of fit was evaluated by Adjusted R² values and the
135 regression coefficients (slopes) were used to determine the significance of correlation.

137 **1. Estrus Synchronization**

138 All animals had one growing large follicle and a CL was seen through ultrasound
139 examination (data not shown) before and after PGF_{2α} injection and at the time of tissue collection.
140 Follicles collected at 78 h post-PGF_{2α} ovulated in 4 out of 6 animals and their FF were not available,
141 thus we are only reporting the analysis of the remaining 2 animals in this group. From the
142 appearance of the theca layers and ovulation crowns, the animals likely ovulated just one or two
143 hours before collection. Since none of the animals ovulated at the 72-h point, this synchronization
144 protocol induced tight ovulation which likely occurred within an hour or two of 75 h after PGF_{2α}
145 injection (**Fig. 2**). Because we expected the LH surge to occur 2 h after the GnRH injection (given
146 at 48 h post PGF_{2α}), ovulation induced through this protocol, therefore, occurred between 22 and
147 28 h after the LH surge. This is a much tighter synchronization and earlier ovulation by 3-5 hours
148 than the original report of an average of ~ 79.1 h¹⁷ and range of 64-96 h after PGF_{2α}. It is worthy
149 to mention that our study utilized Holstein dairy heifers, yet Bo et al¹⁷. used a mixture of
150 Angus/Brangus cows and heifers.

Fig 2. Schematic diagram of treatments, the estimated timing of LH surge, and windows of in vivo oocyte development, maturation, and follicle ovulation (not drawn to scale; besides blood, no other samples were collected at the time of PGF_{2α} injection).

2. Microenvironment of the developing and maturing oocytes (FF) and blood

Changes in the microenvironment of oocytes during the periods of final oocyte development and resumption of meiosis were monitored in follicular fluids between ~26 h before and 30 h after the LH surge, respectively. Jugular venous blood is much easier to collected and used to reveal whether blood parameters could predict the follicular microenvironment.

While the FF is mainly derived from the blood serum, most parameters were very different between these two fluids. For example, total protein concentrations in serum averaged 87.80 ± 9.50 mg/ml and was unaffected by luteolysis (**Fig. 3**). This was expected as serum protein levels should not dramatically change according to ovarian development. Follicular fluid, however, contained a much less total protein with an overall mean of 51.00 ± 12.11 mg/ml. Furthermore, total protein changed with the progression of follicular development ($P=0.041$), reaching the highest level of 57.79 ± 5.20 mg/ml at the 72h time point. This higher level of protein may represent the secretion from the larger number of granulosa cells as follicles approach ovulation¹⁸.

Fig 3. Total protein concentrations in serum (black bars) and follicular fluids (gray bars) across different time points after PGF_{2 α} . Significant differences across time were only seen in follicular fluid ($P=0.041$).

Three out of four electrolytes (Cl^- , K^+ and Ca^{2+}) have significantly changed in blood with P -value of 0.037, 0.014, and 0.0008, respectively, but Na^+ with P -value of 0.079 was not significant. Surprisingly, FF parameters (K^+ , Na^+ , and Ca^{2+}) did not change significantly across time with P -value of 0.099, 0.079, and 0.096, respectively, and Cl^- was not significant with P -value of 0.143 (**Fig. 4**). Interestingly all electrolytes in FF underwent major changes between 24 and 48 h (pairwise comparison $P < 0.05$) after $\text{PGF}_{2\alpha}$ when luteolysis/CIDR removal occurred which should cause a drastic and rapid decline in progesterone levels, but not significant changes across all time points. In FF, Cl^- and K^+ significantly decreased, while Ca^{2+} and Na^+ increased. Most electrolytes in FF, however, attempted to return to pre-luteolysis levels by 60h post- $\text{PGF}_{2\alpha}$.

Fig 4. Electrolytes in blood (solid lines) and FF (dotted lines) across different time points after $\text{PGF}_{2\alpha}$ injection. In Blood, P -value (Na^+ = 0.079, Cl^- = 0.037, K^+ = 0.014, Ca^{2+} = 0.0008), and FF (K^+ =0.099, Na^+ =0.079, Ca^{2+} =0.096 and Cl^- = 0.143).

Fig 5. Partial pressures of oxygen (pO₂) (P -value= 0.0069) and carbon dioxide (pCO₂) (P -value= 0.336) in FF of different time points after PGF_{2α} injection. The * is donated to data from 2 animals, and the rest of the animals ovulated.

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Tissue fluid gases are important for pH maintenance and metabolism. The O₂ partial pressure in FF (**Fig. 5**) significantly decreased (P -value= 0.0069) upon luteolysis and stabilized thereafter. These changes in pO₂ may be a result of the accumulation of more granulosa cells and therefore higher metabolism and consumption and subsequently the gaining of vascularization by the theca layer. Nevertheless, the follicular antrum is an avascular compartment, yet pO₂ level was similar to that in arterial blood^{22,23}, suggesting active mechanisms for maintaining this high O₂ level. This observation suggests that in vitro oocyte development and maturation should employ arterial blood level of O₂ as opposed to the lower O₂ tension of 5%. The increase in pCO₂ upon luteolysis/CIDR removal was not significant (P -value= 0.336) is compatible with the decrease in pO₂, and a result of increased metabolism. As expected, blood pO₂ and pCO₂ were not significant with p -value of 0.257 and 0.198, respectively.

Fig 6. Blood (a) and FF (b) metabolites across time points after PGF_{2α} injection. *Is donated to data from 2 animals, and the rest of the animals ovulated. The significant results are FF glucose ($P=0.01$), creatinine ($P=0.026$), and Lactate ($P=0.001$). The pH did not significantly change in blood $P=0.078$ and FF $P=0.067$. Blood glucose or lactate did not significantly change. Creatinine changed significantly in both Blood ($P=0.006$) and FF ($P=0.026$).

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As expected, the blood pH was well-maintained across treatment times (**Fig. 6a**). However, the pH in FF (**Fig. 6b**) significantly decreased as follicles approached ovulation ($P=0.028$). Blood glucose, lactate, and creatinine also did not significantly change across time (**Fig. 6a**). Significant

189 changes, however, were found in glucose, lactate, and creatinine in FF (**Fig. 6b**) and are compatible
 190 with the increased metabolic activities as follicles continued the trajectory toward ovulation.
 191 During 24 to 78 h post-PGF injection, glucose significantly decreased from 38.40 to 25.83 mg/dL
 192 ($P=0.01$) due to higher metabolism. The reduction in glucose resulted in a near doubling of lactate,
 193 from 7.81 to 12.19 mM, and highly significant ($P=0.003$) across treatment groups.

Fig 7. Correlations between blood (X-axis) and FF (Y-axis) in nine parameters across time points. The black line and the gray area in each plot represent the regression line and confidence intervals of the correlations, respectively. The intercepts are; Cl⁻ 0.98 ($P=0.02$), Creatinine 0.51 ($P=0.002$), Glucose 1.21 ($P=0.046$), iCa²⁺ 0.10 ($P>0.05$), K⁺ 1.89 ($P>0.05$), Na⁺ 0.51 ($P>0.05$), TCO₂ -0.2 ($P>0.05$), pH -6.08 ($P>0.05$), and Lactate -3.03 ($P>0.05$). The adjusted R² represents goodness of fit of the regression.

194 This dramatic conversion of glucose to lactate is interesting because the FF contained
195 nearly arterial blood pO₂ levels, yet the cells seemed to be utilizing glycolysis instead of oxidative
196 phosphorylation. Nonetheless, the combined increases in lactate and pCO₂ likely resulted in the
197 reduced pH in FF. Similarly, the higher metabolism increased creatinine significantly across time
198 ($P=0.026$). As a closed and avascular microenvironment, the metabolic activities are fully reflected
199 in the metabolite levels across time.

200 One of the objectives of this study was to determine if blood parameters could be used to
201 estimate changes in FF. We calculated the correlations for nine parameters (**Fig. 7**) and found that
202 glucose ($P=0.046$), creatinine ($P=0.02$), and Cl⁻ ($P=0.02$) were significantly and positively
203 correlated. While not significant, a few parameters such as iCa²⁺, TCO₂, pH, and lactic acid were
204 negatively correlated.

205 3. Oviductal and uterine fluids

206 Oviductal and uterine fluids visually varied in thickness and clarity, most likely due to
207 different cellular debris contents. The visual characteristics were not correlated to times of sample
208 collection. When samples contained large amounts of debris, the iSTAT failed to make a
209 determination (producing <> to indicate failure of measurement), or the values were higher than
210 the upper limit of detection. We, therefore, were not able to collect data from all fluids on all
211 parameters. The number of samples that gave successful readings is included in Table 1 for each
212 time point and parameter. Across time points (**Table 1**), significant changes were observed in
213 oviductal Cl⁻ ($P=0.002$), TCO₂ ($P=0.005$) and glucose ($P=0.049$); (**Fig. 8**), as well as uterine Na⁺
214 ($P=0.0001$), Cl⁻ ($P=0.0006$), and creatinine ($P=0.002$).

Table 1. Levels ($\pm$ SEM) of electrolytes and metabolites in OF and UF (numbers of animals at each time point are in brackets)

Groups	Na ⁺ (mM)	Cl ⁻ (mM)	TCO ₂ (mM)	Urea Nitrogen (mg/dL)	Creatinine (mg/dL)
Oviductal Fluid					
24 h	103.5 $\pm$ 3.5 (6)	96.5 $\pm$ 9.5 (4)	15 $\pm$ 10 (6)	19.5 $\pm$ 4.5 (3)	4.6 $\pm$ 4.4 (6)
48 h	106.5 $\pm$ 4.5 (4)	104 $\pm$ 2 (4)	6.5 $\pm$ 1.5 (4)	23 $\pm$ 3 (3)	3.9 $\pm$ 0.9 (4)
60 h	110.5 $\pm$ 2.5 (4)	111 $\pm$ 3 (4)	6 $\pm$ 1 (4)	21 $\pm$ 3 (4)	1.55 $\pm$ 0.4 (4)
72 h	115 $\pm$ 15 (6)	108 $\pm$ 9 (3)	15 $\pm$ 1.5 (2)	16.5 $\pm$ 6.5 (3)	2.25 $\pm$ 1.7 (6)
Uterine Fluid					
24 h	107.5 $\pm$ 7.5 (6)	101 $\pm$ 2 (3)	12 $\pm$ 0.23 (4)	12 $\pm$ 2 (3)	4.125 $\pm$ 3.6 (5)
48 h	113 $\pm$ 1 (4)	98.5 $\pm$ 1.5 (4)	25 $\pm$ 1 (3)	14.5 $\pm$ 0.5 (4)	9.65 $\pm$ 0.3 (4)
60 h	128 $\pm$ 10 (5)	121 $\pm$ 11 (5)	11 $\pm$ 1 (4)	10.5 $\pm$ 3.5 (5)	1.45 $\pm$ 1.3 (5)
72 h	123 $\pm$ 7 (5)	113.5 $\pm$ 6.5 (5)	14.5 $\pm$ 2.5 (5)	11.5 $\pm$ 3.5 (5)	2 $\pm$ 1.7 (5)

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Fig 8. Glucose levels in OF significantly decreased ($P=0.049$) across time points.

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4. Differences across tissue fluids

Although a parameter may vary significantly across time points within a tissue fluid, the differences of a particular parameter across fluids were even greater (**Fig. 9**). To demonstrate these differences, we pooled data within tissue fluids across time points. While Na^+ and Cl^- were relatively similar across the four different fluids, K^+ , urea nitrogen, and creatinine were drastically different. Specifically, K^+ (**Fig. 9b**) was the highest in OF and UF. It is worthy to mention that the iSTAT has a detection limit for K^+ at 9 mM and multiple measurements for OF and UF were out of range, showing readings as >9 mM, and puzzlingly, still unattainable after dilutions. Therefore, the K^+ levels in these two fluids could be much higher than 9.0 mM. Conversely, the iCa^{2+} was the lowest in UF (**Fig. 9b**). Urea nitrogen and creatinine (**Fig. 9c**) were higher in OF than both blood and FF and highest in UF, likely a result of differences in cellular activities.

Fig 9. Levels ($\pm$ SEM) of electrolytes and metabolites in the four tissue fluids. **a)** Na⁺ and Cl⁻ were relatively similar among fluids. **b)** K⁺ is higher in FF than blood and highest in OF and UF. The* is donated to the maximal reading of iSTAT (K⁺ >9.0 mM) was reached in both OF and UF. The iCa²⁺ was lowest in UF. **c)** Urea Nitrogen was similar in blood and FF but higher in OF and highest in UF. Creatinine was higher in OF and UF than FF and blood.

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Fig 10. Pooled gas parameters in FF and blood (a) and pooled parameters in jugular blood samples (b).

236 When examining the pooled data on gases (**Fig. 10**), we found that in FF the pCO₂ and
 237 pO₂ was approximately 5X and 2X higher than the levels in venous blood, respectively. These
 238 suggest the follicle's strong mechanism in providing a near arterial blood O₂ level to the developing
 239 oocytes and the somatic cells in the follicles were highly metabolically active. The pooled

240 parameters specific for the blood, such as hematocrit, hemoglobin and sO_2 are also presented in

241 **Fig. 10b.**

DISCUSSION

1. Total protein and electrolytes

While we found that serum maintained many parameters relatively constant for homeostasis, for the first time we report that many parameters changed in fluids of the reproductive tract as a result of luteolysis/CIDR removal and the subsequent LH surge/GnRH administration. For example, all parameters except for Cl^- in FF underwent significant changes ($P < 0.05$). Two out of six parameters in OF and five out of five in UF also changed significantly across time points. Several prior studies^{24,25,26} compared protein and electrolytes among blood and fluids of the reproductive tracts. Few differences, however, were found likely because most of these studies either used slaughterhouse materials^{25,26,27} or collected samples on different days of the estrous cycle^{28,29,15}. The closest study to ours was conducted by Berg et al.¹⁵ who used the iSTAT and reported higher K^+ and low Na^+ in pre-ovulatory follicles than in dominant follicles. In oviductal fluids, Ca^{2+} ^{29,30} underwent the most changes at estrus or a few days after estrus²⁸. In the uterus, Hugentobler et al.³¹ reported striking higher K^+ and lower Cl^- and Na^+ on Day 14 than other days. None of these prior studies, however, were designed to capture tissue fluid changes during the window of in vivo oocyte maturation.

As reported previously by Hugentobler et al.³¹, we also found large differences in electrolyte levels among different fluids, suggesting local regulation on secretion and excretion. In all tissue fluids studied, Na^+ and Cl^- are the major electrolytes and appear to be maintained at comparable levels to preserve a charge-neutral environment. These two electrolytes also varied the least from one fluid to another. For example, Cl^- only varied 0.005, 3, and 11%, respectively, from blood to OF, FF, and UF. The Na^+ levels in the three tissue fluids also stayed close to those in the blood and FF, varying less than 15% in UF and OF. The most varied electrolyte was K^+ , which

was high in both UF or OF and was more than 52% and 24% lower in blood and FF, respectively. It is known that K^+ levels in the blood are tightly regulated through kidney excretion, and the concentration gradient for K^+ between serum and FF suggests an active inward transport²⁷. The high K^+ levels in the OF and UF could be a result of cellular breakdown and release of K^+ into the surrounding fluids³². It is important to point out that electrolytes measured by the iSTAT were within the ranges of those reported previously using other methods^{19,20,28}. The concentration of K^+ in OF and UF ranged between 3.5-14 mM among different studies with UF generally in the higher range. Most OF and UF samples in our study reached the detection limit of the iSTAT (9 mM). The concentration gradient across fluids could be greater if the actual readings were obtained.

2. Tissue fluid gases and acid-base parameters

Similar to electrolytes and total proteins, acid-base parameters and gases in bovine FF and blood have also been reported by many studies in samples of different days of the estrous cycle or in follicles of different developmental stages^{12,22,23,33–35}. However, no report to date has specifically characterized blood and FF parameters while oocytes undergo maturation. Here we found that during this unique window, blood parameters were well-maintained, yet FF underwent significant changes include pH, pCO_2 , and TCO_2 . Although Indrova et al.¹² collected FF at different times post PG or superovulation, these were conducted in three different experiments, and no comparisons could be made. Additionally, many studies used OPU for FF collection, which increased pO_2 due to the mixing of FF with air in the OPU tubing^{34,36,37}.

To date, only one study documented pH, pO_2 , and pCO_2 in FF after timed initiation of maturation. Fischer et al.⁹ aspirated superovulated women undergoing IVF to prevent

hyperstimulation syndrome. This is normally conducted 10-12 h post-hCG injection³⁸. As follicles increase in size, pO₂ dropped from 80 to 50-60 mmHg and pCO₂ increased from 35 to 50 mmHg, pH fell from 7.4 to 7.3. The trends of changes noted by Fischer et al. are similar to what we found here and likely reflect the increase in the number of granulosa cells by the growing follicles¹⁸, which in turn consumes more O₂, produces more CO₂ which lowers the pH.

The data from the iSTAT collected here were consistent with those studied using different methods of determination, and under different reproductive statuses in that pO₂ in FF was significantly higher than that in venous blood. It is hard to explain, however, why Hussein et al.³⁹ found pO₂ at 27 and 39 kPa in spontaneous and induced preovulatory follicles. These pO₂ levels were higher than atmospheric O₂ partial pressure of 20.95 kPa (assuming the air contains 20.95% oxygen) and other reported atrial blood pO₂ levels in the bovine.

3. Carbohydrate substrates and metabolites

We found eight prior studies which documented bovine FF glucose and most also reported lactate levels. However, all except for one used ovaries from slaughterhouses and large variations both within and among studies were found in the levels of glucose (25-96 mg/dL) and lactate (5.5-14.4 mM)^{25,26,40-43}. Nishimoto et al.⁴⁰ and Orsi et al.¹⁰ tried to categorize follicles as presumptive dominant and pre-ovulatory but did not find many changes between them in either glucose or lactate. Although Leroy et al.²⁴ used cows of known stage post-partum, it is unclear what stages the follicles belonged to. Our data that glucose decreased while lactate increased after luteolysis/LH surge are the first such finding. Because no prior studies contained information on the precise stage of follicular development, we are not able to compare our data to prior studies

with the exception that our data on glucose and lactate from the iSTAT are within the prior reported ranges.

Similarly, no prior reports can be used as references for our data on glucose and lactate in OF because the two prior studies also used slaughterhouse materials. Lamy et al.⁴⁴ reported glucose at 2.3-3.5 mM (or 41-63 mg/dL) in OF. Also using slaughterhouse materials, Jordaens et al.⁴⁵ reported glucose in OF at 40 mg/dL and in blood at 110 mg/dL. These ranges are similar to the data we found in both blood and OF.

Urea nitrogen levels were similar in blood and follicular fluid at 3.50 ± 0.03 and 4 ± 0.11 mg/dL, but highest in oviductal fluid at 17 ± 1.28 mg/dL, which was higher than the levels in uterine fluid at 9.50 ± 0.73 mg/dL. Equally drastically different among the four fluids was creatinine, which was similar in follicular fluid and blood 1.20 ± 0.04 and 1.05 ± 0.04 mg/dL, respectively, but 5 ± 0.81 and 4.60 ± 0.42 mg/dL in uterine and oviductal fluid, respectively. We found a number of prior studies in which BUN/urea or creatinine was determined in bovine follicular fluid. Using slaughterhouse ovaries slaughterhouse materials, Iwata et al.²⁵, Tabatabaei et al.⁴¹, Kor et al.⁴², and Leroy et al.²⁶ reported urea ranged between 2-20 mg/dL in follicles of different sizes as well as in blood. However, due to the lack of pre-slaughter information on the animals, it is unclear at what physiological stage these samples were obtained. Using post-partum cows and comparing urea in FF and blood, Leroy et al.²⁶ reported similar values between these two fluids. The range of urea in FF and blood found in our study are within the ranges of those reported previously especially those by Leroy et al.²⁶ using post-partum cows. Creatinine levels in FF were relatively more consistent in prior studies. Using slaughterhouse materials^{25,42,43,43} all reported creatinine at ~ 1.5 mg/dL in FF of different sizes. Our data using precisely timed samples

were within the ranges of prior data in both blood and FF although no prior study could be referenced for the significant changes we observed after luteolysis and GnRH injection.

We found only one prior study which reported urea level in OF and no data on urea or creatinine levels in UF. Kenny et al.²⁰ reported that OF urea was ~6 mM at standing heat. Our data in OF ranged from 16.5-23 mg/dL or 2.8-4 mM and changed significantly across time. This is the first time multiple, time-lapsed data are reported and yet can't be compared to prior literature.

4. Off-label use of iSTAT in tissue fluids other than the human blood

The iSTAT has been validated for the determination of pH, pCO₂, pO₂, total CO₂, oxygen saturation, base excess, HCO₃⁻, Na⁺, K⁺, ionized Ca²⁺, hematocrit, and hemoglobin concentrations¹⁴⁻¹⁶ in cattle blood against conventional instruments. The convenient handheld device has also been used for bovine follicular fluids¹⁵. While it is optimized for levels in human blood, in our hands we found it effective in most measurements of the CG4+ and CHEM8+ cartridges in bovine blood and follicular fluids. The parameters successfully detected by the iSTAT are within the ranges reported in prior studies using other instrumentations, although prior data were from slaughterhouse materials or different days of the estrous cycle.

It is worthy to mention that we also tried to use these cartridges of the iSTAT in uterine and oviductal fluids but many results, such as pO₂, pCO₂, HCO₃⁻, and other parameters from CG4+ cartridge, could not be obtained, likely due to interference by cellular debris in these samples. Most samples were so thick that even we inverted the cartridges to allow gravity to help the sample pass through the narrow capillary channel, the instrument still failed to produce readings. Other

measurement failures were caused by out-of-range levels. The iSTAT is designed for human blood and the detection ranges were optimized for this sole purpose. For example, the maximum detection limit for K^+ is 9 mM. However, multiple oviductal and uterine fluids gave the reading of >9 mM. Strangely enough, diluting the samples with deionized water did not solve the problem. The previously reported oviductal and uterine K^+ levels were 6 and 9-13 mM, respectively³¹. While we obtained good and consistent readings in blood and follicular fluids for ions, gases, and metabolites, the utility of the iSTAT especially in combination with the CG4+ cartridge in oviductal and uterine fluids is limited.

CONCLUSION

This is the first study that characterized changes in components of the blood and fluids of the reproductive tracts during the window of final follicular development and oocyte maturation. The data revealed the in vivo microenvironment presented to the oocyte for competence in fertilization and embryonic development. Moreover, this is also the first report to compare parameters across four different fluids using the same groups of animals. The data presented here provide invaluable information for the improvement of in vitro oocyte maturation conditions and references to the reproductive processes.

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CONFLICT OF INTEREST

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The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

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Omar Gungor and Saleh Salman have contributed equally to this research study. They

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planned the research protocol, hypothesized the study idea, designed the experiments, managed

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farm animal procedures and data collection, ran statistical analysis, and revised the whole

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manuscript. Saurav Ranjitkar and Delong Zhang have assisted in samples collections procedures

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and statistical analysis, and manuscript writing. Dr. Xiuchun (Cindy) Tian is the supervisor and

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corresponding author of this research. She planned the research protocol, provided and allocated

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the USDA funding, hypothesized the study idea, managed samples collections, finalized the

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experimental design, revised the statistical analysis and the whole manuscript. All authors

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contributed to make an integrated work, revised, and agreed on the final manuscript style as

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submitted.

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