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Comparison of metabolites in the follicular fluid of bovine preovulatory and cystic ovarian follicles using nuclear magnetic resonance

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ABSTRACT

Estimation of metabolites in cystic and normal preovulatory follicular fluid through proton Nuclear Magnetic Resonance (^{1}H NMR) in cattle suffering from cystic ovarian follicle is highly desirable.The trans-vaginal ultrasound guided ablation was used to collect follicular fluid from cystic (15) and normally cycling (8) dairy cattle. NMR spectra of both fluids were recorded at a resonance frequency of 500.13 MHz on a Bruker Avance-500 spectrometer equipped with solid state probe (5 mm). Spectra were phased manually, baseline corrected, and calibrated against 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid at 0.0 parts per million (ppm) using Prometab software running within MATLAB. The cystic ovarian follicle associated metabolites with variable importance in projection (VIP) scores >2 were lactate (1.98 ppm), UDP-G (5.62), pyruvate (2.34 and 2.38) and creatinine/creatine (3.14) in cystic and normal preovulatory follicular fluid.These metabolites showed identifiable peaks, and thus can be used as potential biomarkers for dairy cattle suffering from cystic ovarian follicle.

Key words: Cattle, Cystic ovarian follicle, Follicular fluid, Metabolomics, Nuclear magnetic resonance, Transvaginal ablation

Cystic ovarian follicle (COF) is one of the considerable drawbacks in high yielding dairy cattle with incidence rate of 5 to 30% (Singh *et al.* 2009). It provokes an appreciable economic loss due to reduced milk production, prolonged calving to conception interval, 22 to 64 additional days open, and an increased number of inseminations per conception (Peter 2004).The condition is often correlated with endocrine dysfunctions, infectious, and nutritional origin (Butler 2003). However, the exact pathogenesis of COF in dairy cows is still not completely understood. It is generally accepted that the etiology is multifactorial, in which genetic, phenotypic and environmental factors are involved. The most widely accepted hypothesis is based on neuro-endocrinological dysfunction of the hypothalamic-pituitary-gonadal axis (Vanholder *et al.* 2006).Therefore, the estimation of fertility in dairy cattle is important for cost effective, and to reduce economic loss to the farmers.

Following COF, the biochemical composition of various metabolites like glucose, amino acids, and proteins in the follicular fluid (FF) is disturbed. Therefore, a reliable FF assay would be useful to predict the metabolomic differences of these dairy cattle.These metabolomic changes can be accurately investigated by Nuclear Magnetic Resonance (NMR) technique that provides various magnetic properties of atomic nuclei and informative spectra about structure, and dynamics of molecules (Revelli *et al.* 2009).

Estimation of progesterone, estrogen, and growth factors in plasma, and FF are the common metrics used for studying the cause of COF throughout the world. Yet these parameters have limited value for predicting the fertility of cattle (Wathes *et al.* 2007, Grado-Ahuir *et al.* 2011). *In vivo* assessment of follicular fluid metabolites by gas chromatography (GC–MS) based metabolomics, and high pressure liquid chromatography is the most reliable-although expensive, laborious, and time-consuming method (Salvetti *et al.* 2012). However, these assays remain controversial in regards to their reliability, and accuracy in predicting fertility of dairy cattle. Though, fertility-associated metabolites have been identified in the normal preovulatory follicular fluid in mare (Gerard *et al.* 2002), bovine (Sarty *et al.* 2006) and porcine (Bertoldo *et al.* 2013), yet NMR investigations of cystic ovarian fluid in dairy cattle has been largely ignored, and is still lacking. Based on these correlations, the objective of this study was to investigate metabolomic variations in cystic and normal pre-ovulatory follicular fluid through NMR technique.

MATERIALS AND METHODS

Animals: The present study was conducted on repeat breeding Holstein Friesian cattle suffering from cystic ovarian follicle (15) and normally cyclic (8) cows selected

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from university dairy farm. The cystic cows had history of nymphomania, irregular prolonged estrus, aggressive behavior, and breeding (natural/AI) more than three times. Additionally, the data regarding parity, body condition score and milk yield was also recorded. Cystic ovarian follicle with diameter of ≥ 25 mm and wall thickness of <3mm was confirmed by trans-rectal B-mode ultrasonography (Agroscan, 5/7.5 MHz transducer).

Methodology: Selected cows based on aforesaid criteria (15; group 1) were subjected to trans-vaginal ablation by ultrasound, B-mode scanner, Aloka SSD 500 equipped with 5.0 MHz sector array transducer. Prior to ablation, the cows were subjected to caudal epidural anaesthesia (5 to 10 ml of 2% Xylocaine, Inj. Lignocaine). A trans-vaginal probe with guided aspiration needle was inserted into the vagina of the animal. A 50 cm long sterile needle (18 G) was advanced through the vaginal wall into the cystic follicle. Cystic ablation was defined by collapse of the cystic follicle following evacuation of cystic contents. The aspirated follicular fluid was centrifuged at 3,000 rpm for 15 min and stored at –20°C till NMR analysis.

Cattle (8; group 2) showing normal estrus with duration of 1 to 2 days with dominant preovulatory follicle were similarly subjected to ablation. The collected follicular fluid was centrifuged and stored similarly as in group 1. The samples were shipped to Department of NMR, Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University (JNU), New Delhi, India in a commercial insulated shipping container. Follicular fluid (normal and cystic) progesterone was estimated by liquid phase radioimmunoassay (RIA). The sensitivity of assay was 0.1 ng/ml, with mean intra- and inter-assay coefficients of variance to be 6.0 and 9.3%, respectively. Estradiol concentration was estimated using pre-coated enzyme linked immune sorbent assay (ELISA) plates. The sensitivity of assay was 2.53 pg/ml with mean intra-assay coefficient of variation as 2.43%.

Materials for NMR: NMR Sample Tubes (NorellInc, US); Deuterium oxide, D_2O (Sigma Aldrich); 3-(Trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt, TMSP (Sigma Aldrich).

NMR analysis: Follicular fluid samples were thawed on ice, vortexed and mixed in an 1.5 ml eppendorf tube as follows: 600 µl follicular fluid, 20 µl phosphate buffer saline (PBS) containing 3-(Trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TMSP; 1 mM/ml) and 80 µl D_2O. The mixture was taken into NMR tube and spectra were generated.

NMR spectra were recorded at a resonance frequency of 500.13 MHz on a Bruker Avance-500 spectrometer equipped with solid state probe (5 mm) at AIRF, JNU, New Delhi. For each sample, 16 free induction decays (FID) were accumulated over a spectral width of 6067.96 Hz with an acquisition time of 2.62 sec and relaxation delay of 1 sec at 298 K. The resulting spectra were phased manually, baseline was corrected, and calibrated against TMSP at 0.0 ppm using Prometab software running within MATLAB, version R2009a (Math Works, Natick, MA). Bins between 4.5 and 5.2 ppm containing the suppressed water resonance signal were excluded (Aich *et al.* 2007), and the spectral bin corresponding to acetone (2.22 ppm), a common laboratory impurity in NMR experiments, was removed from the model (Fulmer *et al.* 2010).

Data analysis: Hormonal data was expressed as Mean±SEM, statistical analysis was conducted using IBM SPSS statistics 21.0 windows using one way ANOVA followed by least significant difference (LSD) post-hoc analysis ($P<0.05$). A supervised partial least square-discriminant analysis (PLS-DA) was performed to identify the discriminating significant features between FF. Permutation was done to assess the strength of model by randomly assigning samples to different groups (Rubingh *et al.* 2006).

Ethics approval: This study was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) and conducted according to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi. The reference number was GADVASU/2014/IAEC/22/01805.

RESULTS AND DISCUSSION

Animals had BCS of >4. Per day milk yield (kg/day) was recorded to be 20 to 25. The parity of suffered cattle mostly varied between 2nd and 3rd. The chemical shift (ppm)

Table 1. Chemical shifts of various metabolites in cystic vs normal follicular fluids (FF)

Metabolite	Abbreviation	Normal FF	Cystic FF
Valine	Va	1–1.1	1–1.1
3-hydroxy-butyrate	3-OH-B	-	1.2
Lactate	Lac	1.98 and 4.1	1.98 and 4.1
AA (alanine, lysine and glycine)	Ala, L and Gy	1.5, 1.5 and 3.3	1.5, 1.5 and 3.3
Acetate	Ac	2	2
N-acetyl-gylycoproteins	N-ac-gly-p	2.1	2.1
Pyruvate	P	2.2	2.2
Glutamate and succinate	Glut and S	2.34 and 2.38	2.34 and 2.38
Creatine and creatinine	Cr/Crt	3	3
Sugar chains	S-chain	3.5–4	3.5–4
UDP-glucoronate	UDP-G	5.62	5.62
Glucose	Glu	5.3	-

in ^{1}H NMR spectra was compared and interrelated by reference to earlier data on other body fluids including serum/plasma with known chemical shifts of the simple metabolites in aqueous solution as shown in Table 1. The typical ^{1}H NMR spectra of normal and cystic follicular fluid are shown in Figs 1 and 2 respectively. The two dimensional score plot of partial least square discriminate analysis (PLS-DA) of cystic and normal follicular fluid spectral bins indicated a clear clustering between the groups for both follicular fluids (Fig. 3; Pr=0.03). The spectral bin data

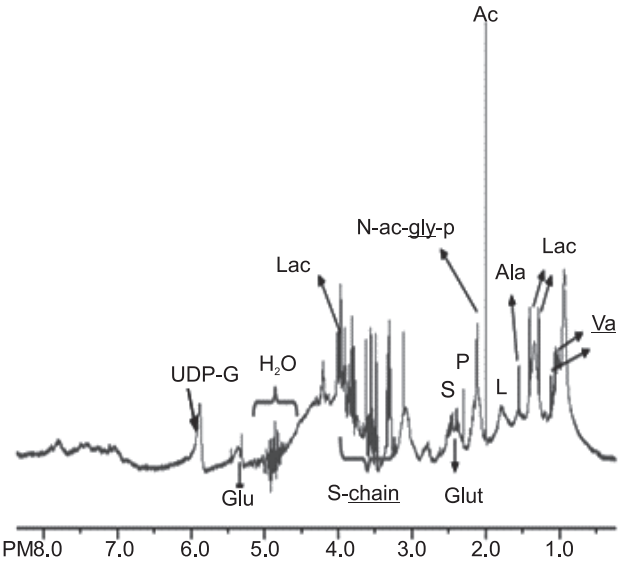

Fig. 1. One-dimensional ^{1}H NMR spectra of normal follicular fluid from dairy cattle.

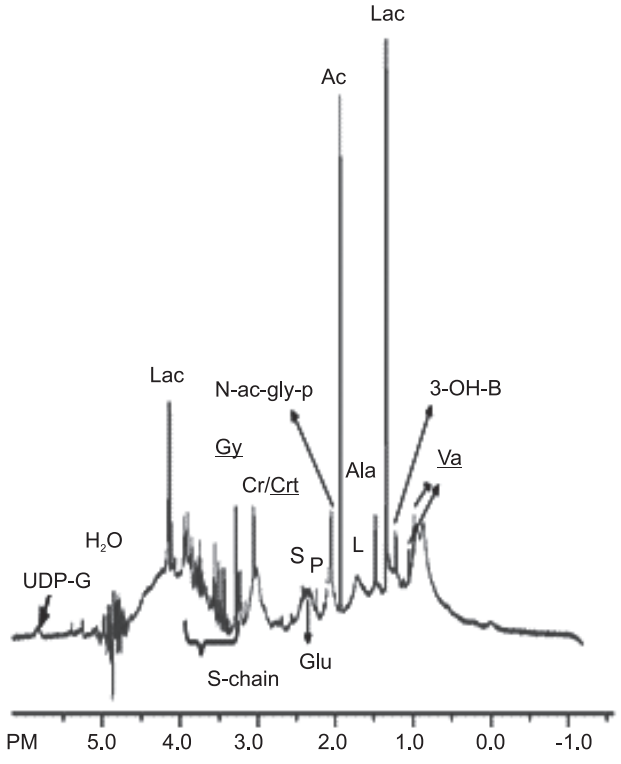

Fig. 2. One-dimensional ^{1}H NMR spectra of cystic ovarian follicular fluid from dairy cattle.

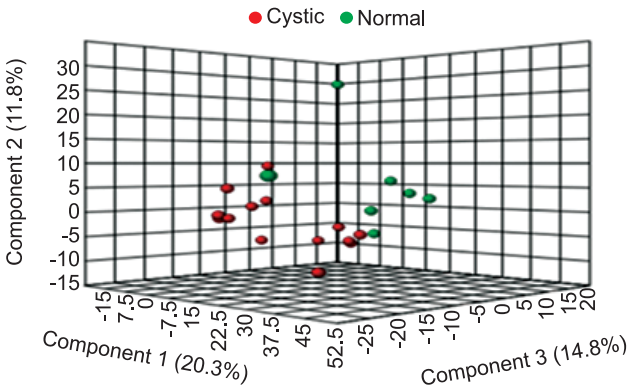

Fig. 3. PLS-DA score scatter plot of spectral bins of cystic and normal animals.

obtained from the spectra of follicular fluid were then normalized, log transformed and pareto scaled (Fig. 4). The variable importance in projection (VIP) score in cystic and normal follicular fluidare shown as a measure of their potential as a biomarker on the scale of 2 to 5 (Fig. 5).

The cystic and normal FF metabolites with a VIP score >2 included lactate (1.98 and 4.1 ppm), UDP-G (5.62), pyruvate (2.34 and 2.38) and creatinine/creatine (3.14). The VIP score indicated UDP-G was low whereas lactate, pyruvate and creatinine/creatine were higher in cystic as compared to normal follicular fluid. These metabolites have clearly identifiable peaks in one-dimensional (1D) spectra, and thus has diagnostic importance. There were significantly ($P<0.05$) higher concentration of progesterone and estradiol in cystic follicular fluid as compared to those in normal preovulatory follicular fluid (18.88±2.84 ng/ml and 626.66±136.2 pg/ml vs 7.13±2.17 ng/ml and 215.75±16.04 pg/ml, respectively).

This is the first report, according to our knowledge, on the identification of COF associated metabolites in ovarian follicular fluid of dairy cattle using ^{1}H NMR analysis, a method that can provide information about several metabolites in a biofluid at the same time (Kumar *et al.* 2015). In the present study, UDP-G (5.62 ppm) was higher in normal follicular fluid as compared to cystic FF. UDP is intermediate for Vitamin C synthesis acting as antioxidant and is required for steroidogenesis (Thomas *et al.* 2001). However, its concentration decreased in cystic follicular fluid as steroidogenesis is affected (Khan *et al.*2011), and hence UDP is utilized for vitamin C. Furthermore, there is a positive correlation of UDP and Vitamin C synthesis with COF (Haliloglu *et al.* 2008). Vitamin C in COF results in progesterone synthesis during COF (Ortega *et al.* 2009). It can be correlated with hormonal estimation (Maniwa *et al.* 2005, Khan *et al.* 2011) that revealed higher concentrations of P4 and E2 in cystic follicular fluids causing defective endocrine milieu.

In a study by Gerard *et al.* (2015), lactate concentration in normal follicular fluid of cow was found higher. However, much higher lactate was found in the present study due to prolonged cases of COF. This increase in lactate production during cystic development occurs due to the increased

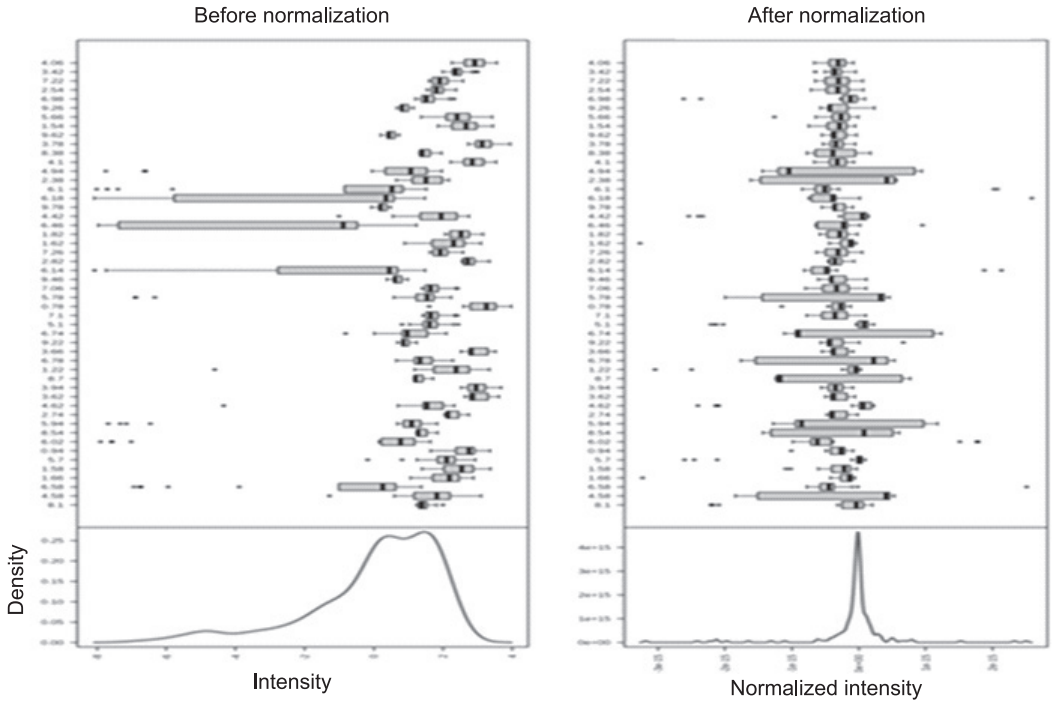

Fig. 4. Normalized, log transformed and pareto scaled spectral bin data of follicular fluids generated using Metabo-Analyst

glycolytic activity of granulosa cells (Harlow *et al.* 1987). Furthermore, an increase in follicular lactate dehydrogenase activity due to serum influx to the follicular fluid occurs as follicle increase in size. Similarly, creatine/creatinine increase occurs (AbdEllah *et al.* 2010).

Glucose metabolite is necessary for oocyte development and is metabolised by glycolysis and pentose phosphate pathways. In the present study, some prominent signals of glucose (5.3 ppm) can be seen in normal FF but could not be estimated precisely in cystic spectra. It could be due to the metabolization of glucose to produce pyruvate during COF (Fernandez *et al.* 2012). Glucose is metabolised to acetyl-CoA producing an intermediate, pyruvate that can generate lactate. Consequently, increased follicle glucose consumption leads to higher lactate and pyruvate production (Harris *et al.* 2007). As a result, ovulation is hindered and follicle continues to grow like COF. Moreover, decrease in intra-follicular glucose during preovulatory maturation was also recorded in mare (Zhao *et al.* 2012).

So it can be concluded that concentration of UDP-G, lactate and pyruvate in follicular fluid plays an important role in providing an energy source for developing oocyte. Concentration of lactate in follicular fluid accumulates during follicular growth and in case of negative energy balance, there is increased 3-hydroxybutyrate. As a result, hormonal disturbances occurs and an ovulation leads to cyst. Hence, our results support hypothesis that Nuclear Magnetic Resonance profiles of cystic fluid are dissimilar to normal preovulatory follicular fluid. However, this pilot study was conducted on fifteen chronic cases of follicular cyst and eight normal highly selected dairy cattle. The low number of animals could account for the lower resolution of some

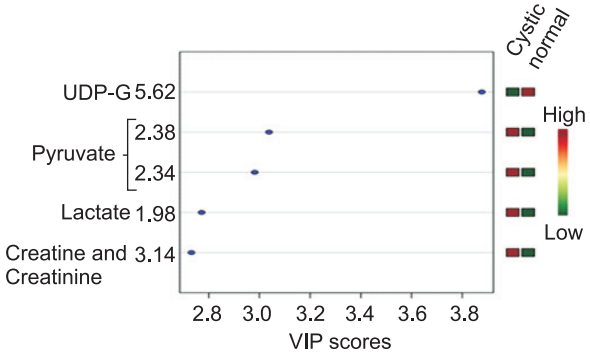

Fig. 5. Variable importance in projection (VIP) scores of follicular fluids in normal and cystic dairy cattle.

metabolites. Expansion of this study to 30–40 dairy cattle might provide clearer peaks for those metabolites. Nevertheless, among the small sample size utilized in this study, UDP-G, pyruvate, lactate, creatine, and creatinine in follicular fluid are the potential biomarkers associated with COF. These COF related metabolites should be further investigated for additional validation among a broader range of dairy cattle suffering from cystic ovarian follicle.

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