Characterization of ovarian follicular fluids of sheep, pigs and cows using proton nuclear magnetic resonance spectroscopy

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Summary. Proton NMR spectra were produced for Graafian follicular fluids obtained by aspiration from sheep, pig and cow ovaries. The following low molecular mass, non-protein-bound metabolites were detected at concentrations exceeding 0.1 mM: acetate, alanine, creatinine/creatine, glycine, D-3-hydroxybutyrate, lactate, valine. Glucose was difficult to quantify and N-acetyl sugars gave a broad resonance at 2.06 ppm, presumably representing side-chains of glycoproteins. Ethanol was detected at up to millimolar concentrations in some specimens, though the physiological significance of this finding was not clear. The concentrations of all metabolites were comparable to those of plasma. These results have therefore shown that NMR spectroscopy is useful for gaining a broad and semiquantitative impression of the more abundant metabolites in the fluids of preovulatory Graafian follicles.
Key words. Ovary; follicular fluid; NMR spectroscopy; sheep; pig; cow.

The chemical composition of extracellular fluid in Graafian follicles is a matter of importance because this medium bathes developing oocytes and is an indicator of the secretory activities and metabolism of granulosa cells. Most attention has been paid to the fluid accumulating in the follicular antrum since the interstitial space, which is potentially more interesting, is sparse and easily contaminated during sampling. Despite this qualification and uncertain physiological significance, antral fluid provides a useful indication of the requirements for granulosa cell and oocyte growth and maturation and may be used as a provisional guide for formulating cell culture conditions.

Antral fluid is a complex mixture consisting of water and solutes derived from plasma with metabolites of follicular cells[1-3]. Whereas its protein and electrolyte composition have been investigated extensively, little information exists for the small organic molecules (with the important exceptions of steroid hormones) other than by inference from the fact that the follicle wall is highly permeable[4].

Nuclear magnetic resonance (NMR) spectroscopy has a number of potential applications in reproductive biology but, hitherto, has mainly been used to estimate ATP and intracellular pH using ^{31}P[5,6]. Proton NMR has been shown to provide opportunities for obtaining simultaneously both qualitative and semi-quantitative information about a wide variety of organic compounds in body fluids. In this study informative spectra have been obtained from ovarian follicular fluids of three species of farm animals for a number of the most abundant metabolites.

Materials and methods

Follicular fluid and ovarian venous blood were withdrawn by hypodermic syringe and 26-gauge needle during mid-ventral laparotomy of 3 Scottish Blackface sheep and 5 Large White × Landrace pigs[7]. Anaesthesia was induced with sodium pentobarbitone and maintained with a mixture of oxygen-halothane-nitrous oxide. The animals were sampled within 24–36 h of ovulation as judged by the large sizes of the Graafian follicles sampled and by oestrous behaviour in the presence of a male. Fluid was also obtained from the largest follicles in 4 cows at unknown stages of the cycle and within 90 min of slaughter at a local abattoir. Samples with blood contamination were discarded. Fluids were centrifuged immediately at 2000 × g for 10 min and the clear supernatant fluids were stored at −20 °C.

Samples of approximately 0.5 ml derived from one large or preovulatory follicle per animal were thawed and analysed after the addition of 10% deuterium oxide at 25 °C with a Brüker WH-360 NMR spectrometer. Spectra were obtained using the spin-echo sequence $D1-90°-\tau-180°-\tau-FID$ to suppress the broad resonances from the higher molecular mass components (particularly proteins). The intensity of the water resonance was reduced by saturation during the pre-excitation delay D1 (3–10 s) and an interpulse delay of 68 ms was used to maintain clear phasing of most resonances. Thus, signals were obtained mainly from low molecular mass non-protein-bound metabolites at concentrations in excess of 0.1 mmol l^{-1}. Chemical shift values obtained using a water-soluble reference compound (sodium 2,2-dimethyl-2-silapentane-5-sulphonate) were assigned to proton resonances.

Quantitative estimates of the molar concentrations of a number of compounds with well-resolved signals were obtained by reference to the concentration of lactate, which was used as a standard because it was detectable in all samples and could be measured independently using a lactate dehydrogenase assay (Boehringer Mannheim). The concentrations of other compounds were estimated from the ratio of their NMR signals to that of lactate.

Results

Interpretation of spectra obtained from whole follicular fluids was assisted by reference to earlier data on other body fluids, including serum/plasma and urine[8-10] and to known chemical shifts of the simple metabolites in aqueous solution. The following were detected as well-resolved signals in some or all samples: acetate (CH_3; 1.94 ppm, singlet), alanine (CH_3; 1.48 ppm, doublet), creatine and creatinine (CH_3; 3.06 ppm, singlet), dihydroxyacetone (CH_2; 4.43 ppm, singlet), ethanol (CH_3; 1.20 ppm, triplet), α-glucose ($H-1$; 5.26 ppm, doublet. $H-2$ to $H-6$, 3.2 to 3.9 ppm, several multiplets) β-glucose ($H-1$, 4.65 ppm, doublet. $H-2$ to $H-6$, 3.2 to 3.9 ppm, several multiplets), glycine (CH_2; 3.58 ppm, singlet), D-3-hydroxybutyrate (CH_3; 1.24 ppm, doublet), lactate (CH_3; 1.34 ppm, doublet. CH; 4.13 ppm,

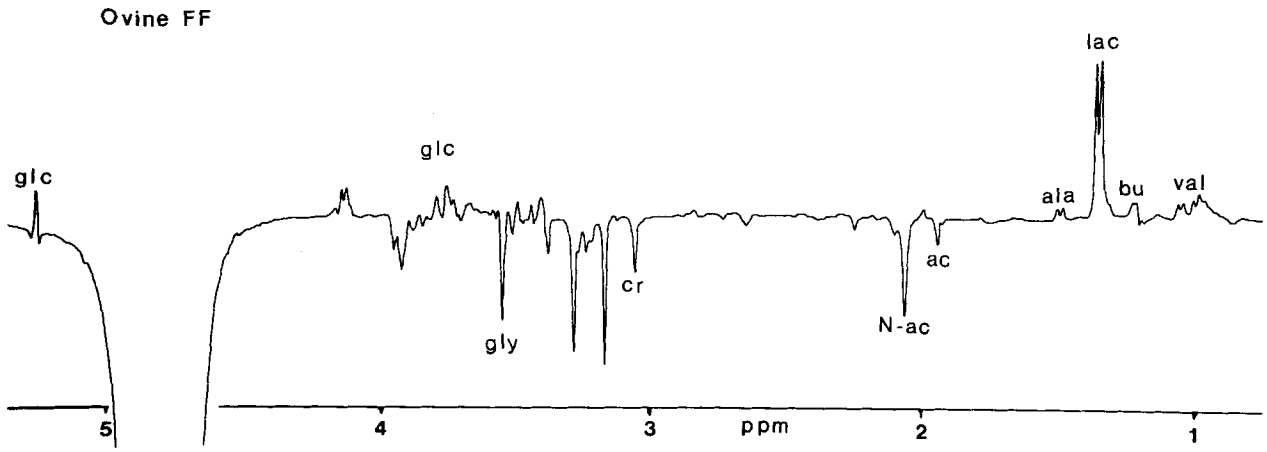

Figure 1. 360 MHz spin-echo ^1H NMR spectrum of ovine follicular fluid. Signals could be assigned to valine (val), D-3-hydroxybutyrate (bu), lactate (lac), alanine (ala), acetate (ac), N-acetyl sugar (N-ac), creatinine/creatine (cr), glycine (gly) and α- and β-glucose (glc) which present complex signals further to the left of the spectrum. Prominent signals at approximately 3.2–3.4 ppm indicate N-methyl groups of proteins.

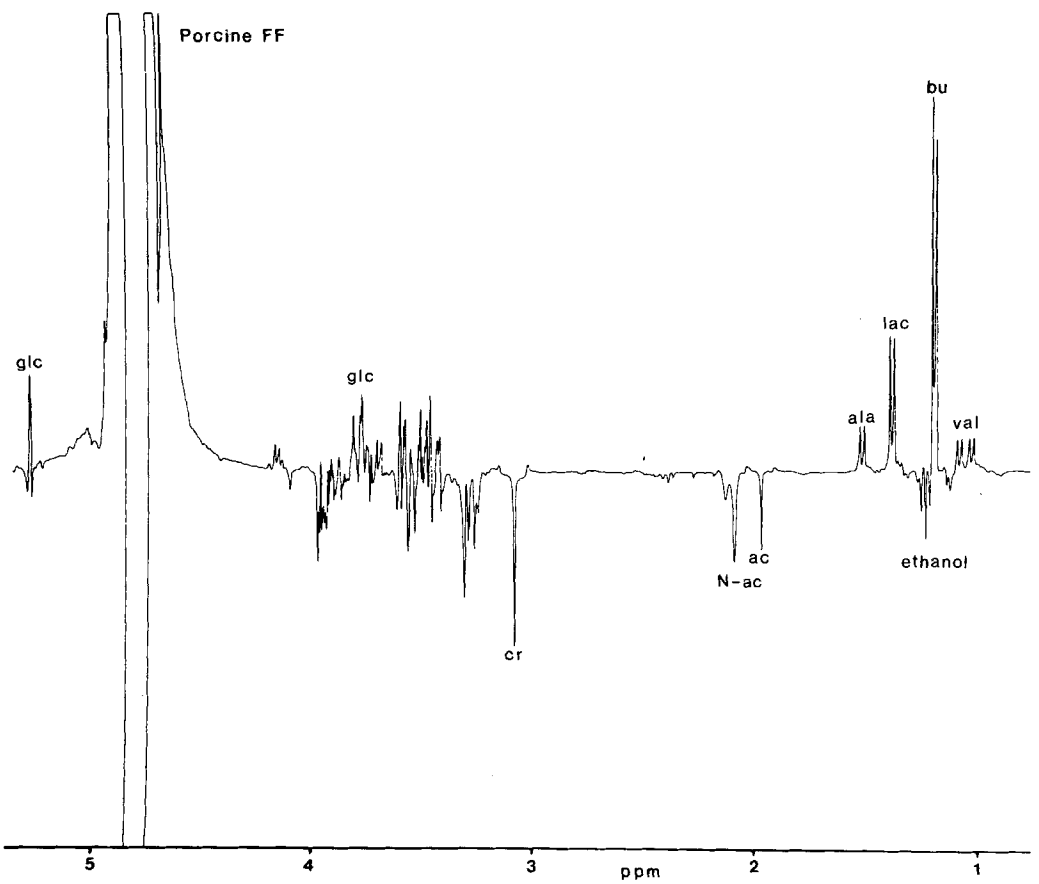

Figure 2. 360 MHz spin-echo ^1H spectrum of porcine follicular fluid. This specimen produced a prominent doublet signal for D-3-hydroxybutyrate and a notable triplet resonance for the CH_3 of ethanol at approximately 1.2 ppm.

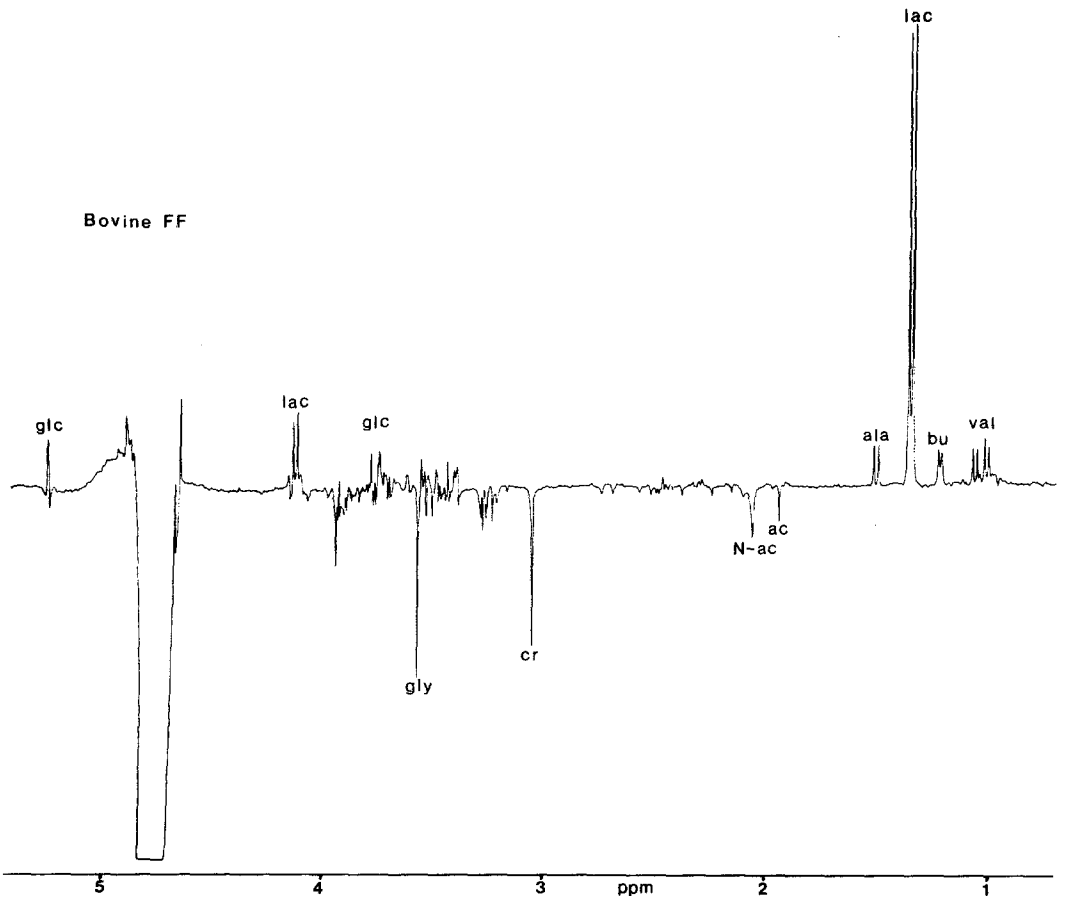

Figure 3. 360 MHz spin-echo ^{1}H NMR spectrum of bovine follicular fluid. Proton resonances were similar to those in the other species, the most conspicuous difference being a strong lactate signal with a chemical shift of 1.3 ppm and a smaller signal at 4.1 ppm.

Range of concentrations of small metabolites (mmol l^{-1}) detected by ^{1}H NMR spectroscopy in ovarian follicular fluids of three species of farm animals. Lactate, which was measured independently by enzyme assay, was used as a reference compound.

Species	Sheep	Pig	Cow
No. of animals	3	5	4
Alanine	0.28–0.33	0.24–0.88	0.42–0.82
Glycine	1.5 –2.1	0.26–1.4	1.1 –2.5
Valine	1.0 –1.9	1.1 –2.7	1.4 –3.0
Acetate	0.17–0.31	0.34–1.5	0.19–0.38
D-3-hydroxybutyrate	0 –0.31	0 –4.2	0 –0.69
Creatinine	0.86–1.5	0.71–1.5	1.0 –3.1
Ethanol	0 –1.5	0 –3.5	0 –1.63
Lactate	3.2 –4.3	2.3 –15.5	4.6 –8.7

quartet), valine (CH_3; 1.02 and 1.04 ppm, 2 doublets). A broader resonance at 2.06 ppm is attributed to N-acetyl groups of mobile carbohydrate side-chains of glycoproteins[10], but the complexity of the spectra did not allow any further information concerning the sugars.

Representative results for each of the three species are shown in figures 1–3. The concentrations of metabolites have been represented by the ranges because of considerable individual variation. Variations of ethanol and the ketone body, D-3-hydroxybutyrate, which were the most marked, ranged from undetectable to millimolar concentrations (table). Concentrations of glucose could not be estimated precisely because the resonances occurred in relatively crowded regions of the spectrum. Hypoxanthine and other purines were not detected.

The concentrations of the metabolites in ovine and porcine follicles were similar to those of ovarian venous plasma, though few blood specimens were available to study. A comparison of the results with extensive published data for plasma/serum[11,12] confirms that the concentrations of small organic molecules are of similar magnitude in body fluids on either side of the follicular wall.

Discussion

This study demonstrates a new application of ^{1}H NMR spectroscopy to analysis of the chemical composition of ovarian follicular fluids. One major advantage of this technique is the number of physiologically important metabolites that can be analysed simultaneously, and the second is that samples require minimal preparation before analysis. The relatively low sensitivity of existing technology is, however, a disadvantage which precludes analyses of smaller follicles unless samples are pooled.

The results substantiate a general conclusion arising from assays of other non-hormonal constituents of follic-

ular fluid, namely, that concentration gradients of small molecules between antral fluid and blood/lymph are small, if significant. Although more sensitive methods will be required to determine whether subtle differences exist (particularly in the sparse interstitial fluids of the follicle epithelium and cumulus oophorus), it appears reasonable to predict that the concentrations of sugars, amino acids and fatty acids in follicular fluid are indicated by those of the blood perfusing the ovary. Exceptions to the generalisation exist beside steroids, which have remarkably high concentrations in follicular fluid [1, 2, 13]. Concentrations of lactate exceed those of blood; evidently it accumulates during follicular growth under the control of gonadotrophins and provides an important source of energy for the oocyte [14].

The results appear to be in conflict with chemical analysis of porcine follicular fluids in which hypoxanthine concentrations were estimated to be 1–2 $mmol\,l^{-1}$ by other methods [15]. At comparable concentrations in vitro, this base inhibits meiotic maturation of mouse oocytes, but the present results indicated that physiological concentrations in 3 species are much lower.

The variable concentrations of some of the follicular fluid constituents are difficult to explain. Those of lactate and ketone bodies could be due to differences in depth of anaesthesia or resting position during surgery or to the composition and timing of the last feed. The presence of ethanol is particularly puzzling. While it may represent uptake into the circulation from the skin after swabbing the site of incision, this explanation cannot hold for animals that were slaughtered. The samples were shown to have remained sterile during storage by seeding fractions on bacteriological blood culture plates. The possibility of having introduced ethanol in the diet could not be ruled out, nor the possibility that it had arisen from fermentation by gut flora, although ethanol is not recognised as a major intermediate metabolite [16]. While ethanol can adversely affect embryo development [17] the concentrations measured in the follicles may be too low to account for the substantial prenatal mortality in farm species [18, 19].

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1 Edwards, R. G., J. Reprod. Fert. *37* (1974) 189.
2 McNatty, K. P., in: The Vertebrate Ovary, p. 215. Ed. R. E. Jones. Plenum, New York 1978.
3 Gosden, R. G., Hunter, R. H. F., Telfer, E., Torrance, C., and Brown, N., J. Reprod. Fert. *82* (1988) 813.
4 Szollosi, D., Gerard, M., Menezo, Y., and Thibault, C., Annals Biol. anim. Biochem. Biophys. *18* (1978) 511.
5 Haseltine, F. P., Arias-Mendoza, F., Kaye, A. M., and Degani, H., Magn. Reson. Med. *3* (1986) 796.
6 Dawson, M. J., McFarlane, D. K., McFarlin, B. L., Noyszewski, E. A., and Trupin, S. R., Biol. Reprod. *38* (1988) 31.
7 Gosden, R. G., and Hunter, R. H. F., Experientia *44* (1988) 212.
8 Bales, J. R., Higham, D. P., Howe, I., Nicholson, J. K., and Sadler, P. J., Clin. Chem. *30* (1984) 426.
9 Nicholson, J. K., O'Flynn, M. P., Sadler, P. J., Macleod, A. F., Juul, S. M., and Sonksen, P. H., Biochem. J. *217* (1984) 365.
10 Bell, J. D., Brown, J. C. C., Nicholson, J. K., and Sadler, P. J., FEBS Lett. *215* (1987) 311.
11 Diem, K. (Ed.), in: Documentia Geigy. Scientific Tables, 6th edn, p. 557. Geigy Pharmaceutical Co., Ltd., Manchester 1962.
12 Altman, P. L., and Dittmer, D. S., in: Biology Data Book, vol. 3, p. 1805. 2nd Edn. Federation of American Societies for Experimental Biology, Bethesda, Maryland 1974.
13 Hunter, R. H. F., Cook, B., and Baker, T. G., Nature *260* (1976) 156.
14 Harlow, C. R., Winston, R. M. L., Margara, R. A., and Hillier, S. G., Hum. Reprod. *2* (1987) 649.
15 Downs, S. M., Coleman, D. L., Ward-Bailey, P. F., and Eppig, J. J., Proc. natl Acad. Sci. *82* (1985) 454.
16 Hungate, R. E. in: The Rumen and its Microbes, p. 278. Academic Press, New York, London 1966.
17 O'Neill, G. T., and Kaufman, M. H., Development *100* (1987) 441.
18 Wilmut, I., Sales, D. I., and Ashworth, C. J., J. Reprod. Fert. *76* (1986) 851.
19 Udomprasert, P., and Williamson, N. B., Theriogenology *28* (1987) 323.

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