Magnetic resonance image attributes of the bovine ovarian follicle antrum during development and regression

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The magnetic resonance images and maps of bovine ovaries acquired at defined phases of follicular development and regression were studied to determine whether magnetic resonance image attributes of the follicular antrum reflect the physiological status of dominant and subordinate ovarian follicles. Ovariectomies were performed at day 3 of wave one, day 6 of wave one, day 1 of wave two and at $\geq$ day 17 after ovulation. The timings of ovariectomies were selected to acquire growing, early static, late static and regressing follicles of the first wave and preovulatory follicles of the ovulatory wave. Pre-selection and subordinate follicles were also available for analysis. Serum samples were taken on the day of ovariectomy and follicular fluid samples were taken after imaging. Numerical pixel value and pixel heterogeneity in a spot representing approximately 95% of the follicular antrum were quantified in T_1 - and T_2 -weighted images. T_1 and T_2 relaxation rates (T_1 and T_2), proton density, apparent diffusion coefficients and their heterogeneities were determined from the computed magnetic resonance maps. The antra of early atretic dominant follicles showed higher T_2 -weighted mean pixel value ($P < 0.008$) and heterogeneity ($P < 0.01$) and lower T_2 heterogeneity ($P < 0.008$) than growing follicles. Subordinate follicles in the presence of a preovulatory dominant follicle had higher T_1 , T_1 heterogeneity, proton density, proton density heterogeneity, and lower mean pixel value in T_1 -weighted images than subordinate follicles of the anovulatory wave ($P < 0.04$). T_1 relaxation rate heterogeneity and proton density heterogeneity were positively correlated with follicular fluid oestradiol concentration ($r = 0.4$ and 0.3 ; $P < 0.04$). T_2 relaxation rate heterogeneity was positively correlated with follicular fluid progesterone concentration ($r = 0.4$; $P < 0.008$). Quantitative differences in magnetic resonance image attributes of the antrum observed among phases of follicular development and regression coincided with changes in the ability of the dominant follicle to produce steroid hormones and ovulate, and thus were indicative of physiological status and follicular health.

Introduction

Magnetic resonance imaging (MRI) is a relatively new imaging technique for studying ovarian function. Imaging of bovine ovaries *in vitro* has shown that MRI has excellent potential for use in assessing the physiological status of ovarian follicles because it offers many advantages over traditional imaging methods (Sarty *et al.*, 1996). MRI has a high signal:noise ratio and very high contrast resolution of soft tissues (Occhipinti *et al.*, 1993), which together give excellent anatomical resolution compared with ultrasonography (Sarty *et al.*, 1996). MRI is also non-invasive, non-ionizing (Smith and McCarthy, 1992) and can provide images in multiple planes. Furthermore, MRI can be used for

direct measurement of physiologically important properties of fluids and tissues such as nuclear relaxation times, proton densities and diffusion coefficients (Sarty *et al.*, 1996).

Follicular fluid is a straw coloured viscous fluid composed mainly of mucopolysaccharides secreted by granulosa cells and serum transudate (McNatty, 1978). Steroid and protein hormones are also present in follicular fluid (McNatty, 1978). The composition of follicular fluid depends on the physiological status of the follicle. Non-atretic follicles have higher oestradiol and total immunoreactive inhibin and lower progesterone and mucopolysaccharide concentrations than atretic follicles (Ireland and Roche, 1983; Bellin and Ax, 1984; Bushmeyer *et al.*, 1985; Price *et al.*, 1995). Histological, biochemical and morphological indicators of atresia have been identified (Ireland and Roche, 1983; Bellin and Ax, 1984; Grimes *et al.*, 1987; Guilbault *et al.*, 1993; Sunderland *et al.*, 1994; Price *et al.*, 1995). However, histological indices of

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atresia do not correlate well with endocrine indices of functional status (Price *et al.*, 1995).

Bovine follicular kinetics are well documented from ultrasound studies, and image analysis studies based on ultrasonography have been directly applicable to human research and clinical medicine, thus the bovine model provides an excellent research method for studying ovarian function (Adams and Pierson, 1995). In cattle, follicular development occurs in two or three waves per oestrous cycle (Pierson and Ginther, 1987a,b; Sirois and Fortune, 1988; Ginther *et al.*, 1989a,b; Knopf *et al.*, 1989; Adams and Pierson, 1995). As a cohort of follicles from a follicular wave grows, one follicle is physiologically selected to continue growing preferentially and becomes dominant. The rest of the recruited cohort of follicles do not grow to the same degree and become subordinate follicles destined to regress. The dominant follicle of the first wave is anovulatory because production of progesterone by the corpus luteum inhibits ovulation. A new wave emerges as the anovulatory dominant follicle regresses and a new dominant follicle is selected for eventual ovulation. The corpus luteum regresses at approximately day 17 (day 0 = ovulation) of the bovine ovarian cycle in concert with increasing oestradiol concentrations from the dominant follicle that together allow for a surge of LH on about day 20 to induce ovulation of the dominant follicle of the second or third wave. Anovulatory follicles can be identified by ultrasonography through growing (increasing in diameter), early static, late static (no longer increasing in diameter) and regressing phases (decreasing in diameter), whereas ovulatory follicles have only a growing phase (Ginther *et al.*, 1989a). Phases of development and regression defined by ultrasonography reflect follicular function (steroid and protein hormone production) and health (viable, atretic; Badinga *et al.*, 1992; Guilbault *et al.*, 1993; Price *et al.*, 1995; Sunderland *et al.*, 1996; Singh *et al.*, 1998).

Previous research using ultrasound imaging and computer-assisted image analysis has been used to show pronounced differences in image attributes among phases of follicular development in bovine ovaries (Tom *et al.*, 1998; Singh *et al.*, 1998). Predictors of ovarian follicular viability or atresia in bovine and human follicles under both normal and ovulation induction conditions have also been identified (Pierson and Adams, 1995). However, magnetic resonance image attributes of ovarian follicles have not been quantified nor have they been examined systematically at specific phases of follicular development and regression.

The present study was designed to provide information on quantitative magnetic resonance image attributes of follicles of known physiological status using MRI of bovine ovaries *in vitro*. The objective was to use a bovine model and computer-assisted analysis to study pixel values (grey scale) in T_1 and T_2 relaxation rate-weighted images and map values of relaxation rates, proton density and apparent diffusion coefficients of dominant and largest subordinate follicles obtained at four physiologically defined phases of the bovine ovarian cycle. The hypothesis was that there would be quantitative differences in magnetic resonance image and map attributes of ovarian follicular antra among dominant and subordinate follicles at different phases of development

and regression that reflect the physiological status (viability or atresia) of the follicles.

Materials and Methods

The assignment of animals to various groups, ultrasonography and ovariectomies were conducted as described by Singh *et al.* (1998), a similar study that used ultrasound image analysis.

Experimental animals: grouping and ultrasonography

Thirty-five sexually mature nulliparous heifers were used in the present study over two replicates (replicate 1 = 16 cows and replicate 2 = 19 cows). The development of ovarian follicles was monitored by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka SSD 500 ISM Inc. Edmonton, Alberta). Transrectal ultrasound examinations were performed once a day to monitor the development of follicles ≥ 4 mm in diameter commencing at least 2 days before the ovulation preceding the beginning of the oestrous cycle under investigation, and continued until the day of ovariectomy (Singh *et al.*, 1998). Retrospective analysis of hand-drawn diagrams showing topographical location and diameter of individual follicles and corpora lutea was used to determine the day of emergence of the follicular wave (day 0). The day of emergence was defined as the day on which the dominant follicle was first detected at a diameter of 4–5 mm (Ginther *et al.*, 1989a,b; Adams and Pierson, 1995; Singh *et al.*, 1998). The dominant follicle was identified as the largest follicle of a wave; the remaining follicles were identified as subordinate follicles (Ginther *et al.*, 1989a,b; Singh *et al.*, 1998). The heifers were designated randomly for ovariectomy on day 3 of wave one ($n = 10$), day 6 of wave one ($n = 9$), day 1 of wave two ($n = 9$) or in the immediate preovulatory period ≥ 17 days ($n = 8$) after ovulation (Singh *et al.*, 1998). On day 1 of wave two, the largest of the pre-selection follicles was selected to be the dominant follicle of wave two. Heifers in the $\geq$ day 17 after ovulation group were ovariectomized 1 day after detection of pro-oestrus (Singh *et al.*, 1998). Retrospective analysis of the oestrous cycles before ovariectomy revealed that all cows showed two waves of follicular development. Pro-oestrus was defined as the day when any three of four oestrus-like characteristics were detected (high uterine tone, oedematous echotexture, intrauterine fluid collection and mucous discharge; Pierson and Ginther, 1987b; Singh *et al.*, 1998). The timings of the ovariectomies were selected to represent the growing (day 3 of wave one), early static (day 6 of wave one), late static (day 1 of wave two) and regressing phases ($\geq$ day 17 after ovulation) of dominant follicles of wave one as well as the pre-selection (day 1 of wave two) phase and the post-selection growing phase ($\geq$ day 17 after ovulation) of the preovulatory dominant follicle (Fig. 1). Subordinate follicles were also available for analysis on the selected days of ovariectomy.

Ovariectomy

Ovaries were removed from the heifers with a single incision through the dorso-lateral aspect of the vaginal wall (Hudson, 1986). Surgery was conducted with cows in the standing position and under caudal epidural anaesthesia using 2% (w/v) lidocaine HCl with 0.001% (w/v) adrenaline. Clenbuterol ($0.6 \mu\text{g kg}^{-1}$ body weight; Boehringer Ingelheim Ltd, Ontario) was given i.v. 10 min before colpotomy to induce relaxation of the ovarian ligament. The ovarian ligament was compressed with lidocaine-soaked gauze immediately before the ovaries were excised using a chain ecraseur looped around the ovarian ligament. After ovariectomy, ovaries were placed immediately in warm (37°C) physiological saline, transported to the MRI suite in an insulated container, and images were taken within 60 min.

Hormone assays

Blood samples were collected on the day of ovariectomy and serum was stored at -20°C . Follicular fluid was aspirated after MRI was completed. Follicular fluid samples were collected from the dominant follicle; and follicular fluid samples from subordinate follicles were pooled. All follicles other than the morphologically dominant follicle made up the pooled fraction of subordinate follicles to ensure a large enough sample for analysis. The experimental design assumed that the composition of the pooled follicular fluid fraction would approximate that of the largest subordinate follicle. Follicular fluid was stored at -20°C . Radio-immunoassays to measure oestradiol and progesterone were performed on serum and follicular fluid samples using validated assays (Currie *et al.*, 1993; Joseph *et al.*, 1994; Bartelewski *et al.*, 1999). Follicular fluid was diluted with charcoal-stripped serum until results fell within the sensitivity of the assay (Singh *et al.*, 1998). Standards were made in charcoal-stripped serum.

Magnetic resonance imaging

MRI was performed on four ovaries (the ovaries from two heifers; $n = 31$) or two ovaries (the ovaries of one heifer: $n = 4$) at a time in a small plastic dish with a divider separating the ovaries of the two heifers. The dish contained only the ovaries and no surrounding material. MRI was carried out using a 1.5 Tesla SP Magnetom MR imager (Siemens, Erlangen) with a Helmholtz RF receiver coil of 150 mm in diameter. Images were acquired using T_1 and proton density sequences ($T_R/T_E = 480, 1000, 2000, 4000$ per 15 ms), 16-echo T_2 sequences ($T_R/T_E = 2000$ per 20–245 ms), as well as unweighted and weighted diffusion sequences ($b = 0$ and 17776 s cm^{-2} in the z direction). The T_1 sequences at $T_R/T_E = 2000, 4000$ per 15 ms and diffusion sequences were added for the second replicate (19 of the 35 heifers used in this study) to obtain more accurate T_1 maps and to add diffusion maps to the analysis (day 3 of wave one, $n = 5$; day 6 of wave one, $n = 5$; day 1 of wave two, $n = 5$; $\geq$ day 17 after ovulation, $n = 4$). The T_1 and T_2 protocols were the same as the standard

protocols found to provide opposing contrasts for the follicles and corpus luteum versus the stroma (Sarty *et al.*, 1996).

T_1 , T_2 and proton density data were acquired with a field of view of $120 \text{ mm} \times 120 \text{ mm}$ for the first replicate and $100 \text{ mm} \times 100 \text{ mm}$ for the second replicate; both replicates had an acquisition matrix size of 160×256 and thickness of each slice equal to 2 mm. Weighted and unweighted diffusion data were acquired with a field of view of $100 \text{ mm} \times 100 \text{ mm}$, an acquisition matrix size of 128×256 and a slice thickness of 2 mm. All magnetic resonance data were Fourier-transformed into 256×256 pixel images with a pixel resolution of 0.47 mm or 0.39 mm depending on the field of view.

Maps were computed by solving for T_1 , T_2 , proton density or the apparent diffusion coefficient in mono-exponential decay equations using least squares best fit analysis of the corresponding weighted image data.

Quantitative analysis of magnetic resonance images

A subset of magnetic resonance images was selected from the T_1 -weighted and 16 T_2 -weighted image sets for grey-scale analysis. Selection was based visually on the best follicle to stroma image contrast (T_1 at $T_R/T_E = 480$ per 15 ms and T_2 at $T_R/T_E = 2000$ per 50 ms) and within that set, images were selected that contained the greatest cross-sectional area of the follicle in question. The map images that corresponded to the selected weighted images were used for map image attribute analysis. The image attributes evaluated in the present study included mean numerical pixel values calculated from T_1 - and T_2 -weighted images, T_1 and T_2 relaxation rates, proton density and apparent diffusion coefficient calculated from respective maps.

Pixel value analysis of magnetic resonance-weighted images

Selected T_1 - and T_2 -weighted images were linearly converted from 12 bit to eight bit images and saved in a database. Images of the antrum of each of the dominant and largest subordinate follicles were analysed using Synergyne1 Version 2.8 (© R. A. Pierson, 1997, Saskatoon, Saskatchewan, Canada) on an Ultra 2 SunSparc Station (Sun Microsystems, Mt View, CA) computer (Pierson and Adams, 1995; Singh *et al.*, 1997; Singh *et al.*, 1998). A user-selected circular region of interest incorporating approximately 95% of the follicular antrum was analysed. The mean grey-scale value (numerical pixel value; 0 = black, 255 = white and 254 shades of grey in between) and pixel heterogeneity score (standard deviation, a measure of texture) were determined from the selected regions in the weighted images.

Nuclear relaxation rate analysis of magnetic resonance map images

Analysis of the dominant or subordinate follicle antrum in the T_1 , T_2 , proton density and diffusion maps was performed

using a custom designed program to analyse the original 12 bit map images. Again, a circular region of interest encompassing approximately 95% of the follicle antrum was selected. Mean T_1 and T_2 relaxation rates, proton density and apparent diffusion coefficient, and their heterogeneities (standard deviations) were determined from the respective maps.

Statistical analyses

Two-factor ANOVA SPSS Version 9.0 (Statistical Product and Service Solutions, Chicago, IL) were used to determine the effects of the types of follicle (dominant or largest subordinate) and phase (growing, early static, late static, regressing, pre-selection or preovulatory) on magnetic resonance image attributes and hormone concentrations. Protected LSD tests were performed to determine differences between specific groups when significance ($P \leq 0.05$) was shown for type of follicle, follicle phase or type by phase interaction (Snedecor and Cochran, 1980). Significance values for comparisons between specific groups reported in the text were taken from the protected LSD. Image attribute endpoints were analysed among dominant and largest subordinate follicles. Only the largest of the subordinate follicles was analysed because ultrasound endpoints were found to be similar between the largest and second largest subordinate follicles (Singh *et al.*, 1998). Pearson's correlation coefficients between hormone data and magnetic resonance image attributes were calculated by matching the values of individual follicles without categorization by type or phase. Only significant correlation coefficients > 0.25 are reported. Results are reported as means $\pm$ SEM.

Results

The diameter profiles of dominant and largest subordinate follicles as determined by ultrasonography are presented (Fig. 1). T_1 - and T_2 -weighted images, T_1 , T_2 , proton density and apparent diffusion coefficient maps are shown for ovaries removed at day 1 of wave two and day 6 of wave one (Fig. 2) and $\geq$ day 17 after ovulation and day 3 of wave one (Fig. 3). Serum and follicular fluid hormone concentrations are presented (Fig. 4). The image attribute data are illustrated (Figs 5–7) according to the type of follicle (dominant and subordinate) and the phase of follicular development (growing, early static, late static, pre-selection and preovulatory).

Follicle status

The follicle diameter profiles confirmed that the anatomical follicular status of the dominant and largest subordinate follicles was appropriate for the timing of ovariectomies (Fig. 1).

Fig. 1. Diameter profiles (mean $\pm$ SEM) of the dominant and largest subordinate bovine follicles of wave one (anovulatory) and wave two (ovulatory). The vertical dotted lines indicate the mean days of ovariectomy on day 3 of wave one, day 6 of wave one, day 1 of wave two, and during pro-oestrus $\geq$ 17 days after ovulation. The numbers in parentheses represent the number of follicles of each type available for image analysis for each period.

Hormone assays

Serum oestradiol tended to increase from day 6 of wave 1 to $\geq$ day 17 after ovulation, but this result was not significant (Fig. 4). Serum progesterone increased from day 3 of wave one to day 1 of wave two, which was consistent with development and maturation of the corpus luteum ($P < 0.001$). Serum progesterone decreased abruptly at $\geq$ day 17 after luteolysis ($P < 0.02$). Follicular fluid sampled at day 1 of wave two was collected from the late static phase dominant follicle of the anovulatory wave (Fig. 4). Although not statistically significant, the oestradiol concentration in anovulatory dominant follicles was maximum at day 3 of wave one (47465 ± 16813 ng ml $^{-1}$) and decreased progressively during the early (day 6 of wave one, 33526 ± 6832 ng ml $^{-1}$) and late static phases (day 1 of wave two, 16503 ± 11779 ng ml $^{-1}$). The progesterone concentrations were higher in regressing subordinate follicles of wave one (day 6) compared with early static subordinate follicles at day 3 ($P < 0.05$). The progesterone concentration in dominant follicles was constant among the phases. Follicular fluid from dominant follicles had a higher concentration of oestradiol than the pooled subordinate follicular fluid at day 3 and day 6 of wave one ($P < 0.02$). At day 3 and day 6 of wave one, dominant follicles showed a lower concentration of progesterone than subordinate follicles ($P < 0.03$). Dominant follicles had higher ratios of oestradiol:progesterone than subordinate follicles at day 3 of wave one, day 6 of wave one and $\geq$ day 17 after ovulation (ovulatory wave; $P < 0.03$).

Follicular fluid oestradiol concentration was negatively correlated with serum progesterone ($r = -0.416$; $P < 0.03$) and positively correlated with the oestradiol:progesterone ratio in follicular fluid ($r = 0.903$; $P < 0.001$). Follicular fluid progesterone concentration was negatively correlated with

Fig. 2. Magnetic resonance images (a,b) and magnetic resonance maps (c–f) of bovine ovaries at day 1 of wave two (top two ovaries in each part) and day 6 of wave one (bottom two ovaries in each part). (a) T_1 -weighted image ($T_R/T_E = 480/15$ ms), (b) T_2 -weighted image ($T_R/T_E = 2000/50$ ms), (c) T_1 , (d) T_2 , (e) proton density and (f) diffusion maps. A_1 : dominant follicle of wave one; A_2 : dominant follicle of wave two; B: largest subordinate follicle; C: corpus luteum. Pixel value analysis of the antrum was performed in both 8 bit magnetic resonance weighted images and 12 bit magnetic resonance maps by placing the measuring circle inside the antrum and adjusting the size of the circle to incorporate approximately 95% of the antrum. Mean numerical pixel value (black = 0 and white = 255) and heterogeneity of all pixels within the selected region were calculated from the weighted images. Mean relaxation rates (T_1 or T_2), proton density, and diffusion coefficients (0 = black and 4095 = white) and their heterogeneities within the selected region were calculated from the corresponding magnetic resonance map.

Fig. 3. Magnetic resonance weighted images (a,b) and magnetic resonance maps (c–f) of bovine ovaries during pro-oestrus at ≥ 17 days after ovulation (top two ovaries in each part) and at day 3 of wave one (bottom two ovaries in each part). (a) T_1 -weighted image ($T_R/T_E = 480/15$ ms), (b) T_2 -weighted image ($T_R/T_E = 2000/50$ ms), (c) T_1 , (d) T_2 , (e) proton density and (f) diffusion maps. A_1 : dominant follicle of wave one (anovulatory); A_2 : dominant follicle of wave two; C: corpus luteum.

Fig. 4. (a) Concentration (mean $\pm$ SEM) of oestradiol (pg ml⁻¹, ■) and progesterone (ng ml⁻¹, □) in bovine serum. (b–d) Concentration (mean $\pm$ SEM) of oestradiol (b) and progesterone (c) and the oestradiol:progesterone ratio (d) in bovine follicular fluid of dominant (■) and pooled subordinate (□) follicles at different phases of development and regression. Bars that do not share a common letter indicate significantly different values ($P < 0.05$). D3W1: day 3 of wave one; D6W1: day 6 of wave one; D1W2: day 1 of wave two; D $\geq$ 17: during pro-oestrus $\geq$ 17 days after ovulation.

follicular fluid oestradiol:progesterone ratio ($r = -0.288$; $P < 0.05$).

Quantitative analysis of magnetic resonance image attributes

T_2 -weighted numerical pixel values of anovulatory dominant follicles increased from day 3 of wave one to day 1 of wave two ($P < 0.008$). T_2 -weighted pixel heterogeneity of anovulatory dominant follicles increased from day 3 of wave one to day 1 of wave two ($P < 0.01$). T_2 relaxation rate heterogeneity of dominant follicles decreased from day 3 of wave one to day 1 of wave 2 ($P < 0.008$). Regressing

anovulatory follicles ($\geq$ day 17 after ovulation) tended to have higher T_2 relaxation rates than growing (day 3 of wave one) and static phase (day 6 of wave one and day 1 of wave two) anovulatory follicles (595 ± 138 versus 460 ± 14 , 425 ± 23 and 445 ± 18 ms, respectively), but this result was not significant.

T_1 relaxation rate, T_1 relaxation rate heterogeneity, proton density and proton density heterogeneity of dominant follicles of the ovulatory wave increased from the pre-selection phase (day 1 of wave two) to the preovulatory phase ($\geq$ day 17 after ovulation; $P < 0.04$). T_1 - and T_2 -weighted numerical pixel values of ovulatory dominant follicles decreased over the same times (day 1 of wave two to $\geq$ day 17 after ovulation, $P < 0.001$).

Fig. 5. T_1 magnetic resonance image attributes. Mean $\pm$ SEM (a) numerical pixel value and (b) pixel heterogeneity from T_1 -weighted images and (c) T_1 relaxation rate and (d) T_1 relaxation rate heterogeneity from T_1 maps, of dominant (■) and largest subordinate (□) bovine ovarian follicles at four phases of follicular development and regression in anovulatory and ovulatory waves. Values under the x-axis indicate the number of follicles analysed for each follicle group. Bars that do not share a common letter indicate significantly different values ($P < 0.05$). D3W1: day 3 of wave one; D6W1: day 6 of wave one; D1W2: day 1 of wave two; D $\geq$ 17: during pro-oestrus $\geq$ 17 days after ovulation.

Table 1. Correlation values for magnetic resonance attributes of bovine ovarian follicular antra

	T_1 NPV		T_2 NPV		Proton density		Proton density heterogeneity	
	r	Significance	r	Significance	r	Significance	r	Significance
T_1 NPV	–	–	0.637	$P < 0.001$	–0.888	$P < 0.001$	–0.834	$P < 0.001$
T_2 NPV	0.673	$P < 0.001$	–	–	–0.717	$P < 0.001$	–0.730	$P < 0.001$
T_1 RR	–0.937	$P < 0.001$	–0.781	$P < 0.001$	0.974	$P < 0.001$	0.923	$P < 0.001$
T_2 RR	–	NS	–	NS	–	NS	–	NS

NS: not significant.

NPV: numerical pixel value; RR: relaxation rate.

Fig. 6. T_2 magnetic resonance image attributes. Mean $\pm$ SEM (a) numerical pixel value and (b) pixel heterogeneity from T_2 -weighted images and (c) T_2 relaxation rate and (d) T_2 relaxation rate heterogeneity from T_2 maps, of dominant (■) and largest subordinate (□) bovine follicles at the four phases of follicular development and regression in anovulatory and ovulatory waves. Values under the x-axis indicate the number of follicles analysed for each follicle group. Bars that do not share a common letter indicate significantly different values ($P < 0.05$). D3W1: day 3 of wave one; D6W1: day 6 of wave one; D1W2: day 1 of wave two; D $\geq$ 17: during pro-oestrus $\geq$ 17 days after ovulation.

T_1 relaxation rate, T_1 relaxation rate heterogeneity, proton density and proton density heterogeneity of subordinate follicles in the presence of a preovulatory follicle at $\geq$ day 17 after ovulation were higher than anovulatory wave subordinate follicles at day 6 of wave one ($P < 0.04$). T_1 -weighted numerical pixel values of subordinate follicles at $\geq$ day 17 after ovulation were lower than those of anovulatory wave subordinate follicles at day 3 of wave one ($P < 0.01$). Subordinate follicles showed higher T_1 relaxation rate heterogeneity at day 6 of wave one than dominant follicles at day 6 of wave one ($P < 0.05$).

Correlation

Correlation values between different magnetic resonance image attributes are shown (Table 1).

Hormone concentrations and magnetic resonance image attributes

T_1 relaxation rate heterogeneity and proton density heterogeneity were positively correlated with follicular fluid

Fig. 7. Proton density and apparent diffusion coefficients. Mean $\pm$ SEM (a) proton density and (b) proton density heterogeneity from proton density maps and (c) apparent diffusion coefficient and (d) apparent diffusion coefficient heterogeneity from the diffusion maps, for dominant (■) and largest subordinate (□) bovine follicles at the four phases of development and regression in anovulatory and ovulatory waves. Values under the x -axis indicate the number of follicles analysed for each follicle group. Bars that do not share a common letter indicate significantly different values ($P < 0.05$). D3W1: day 3 of wave one; D6W1: day 6 of wave one; D1W2: day 1 of wave two; D $\geq$ 17: during pro-oestrus $\geq$ 17 days after ovulation.

oestradiol concentration ($r = 0.4$ and 0.3 , respectively; $P < 0.05$). T_2 relaxation rate heterogeneity was positively correlated with follicular fluid progesterone concentration ($r = 0.357$; $P < 0.01$). No magnetic resonance image attributes correlated with follicular fluid oestradiol:progesterone ratio.

Discussion

The results of the present study support the hypothesis that the fluid of dominant and subordinate follicles shows quantitative differences in magnetic resonance weighted image and magnetic resonance map image attributes among

different phases of development and regression that reflect the physiological status of the follicles. The composition of follicular fluid changes as the ovarian follicle develops through growing and static phases and then regresses. These physiological changes were detected with MRI as quantitative differences in weighted image attributes and relaxation rates specific to the phase of development and the type of follicle. Changes in magnetic resonance image attributes among the phases of development and regression were coincident with changes in ovulatory potential or functional dominance of the dominant follicle. In particular, late static phase dominant follicles of the anovulatory wave had brighter and more heterogeneous follicular fluid in

T_2 -weighted images than growing and early static dominant follicles. Subordinate follicles in the presence of a preovulatory dominant follicle showed brighter and more heterogeneous follicular fluid than subordinate follicles under the influence of dominant follicles from the anovulatory wave. Specific relationships were identified between follicular fluid hormone (oestradiol and progesterone) contents and magnetic resonance image attributes (T_1 and T_2 relaxation rate heterogeneity and proton density heterogeneity).

Growing and early static dominant follicles of the anovulatory wave can ovulate in response to a luteolytic dose of prostaglandin (Kastelic *et al.*, 1990; Savio *et al.*, 1990; Fortune *et al.*, 1991). However, after day 1 of wave two, in the late static phase, the anovulatory dominant follicle is no longer functionally dominant and does not possess the ability to ovulate (Fortune *et al.*, 1991). The dominant follicle of the anovulatory wave at day 3 and day 6 of wave one had magnetic resonance image attributes similar to those of the viable dominant follicle at $\geq$ day 17 after ovulation. However, at day 1 of wave two the dominant follicle of the anovulatory wave was brighter in T_2 -weighted images, perhaps attributable to lower steroid and protein hormone production by the atretic dominant follicle associated with loss of functional dominance. At day 1 of wave two, anovulatory dominant follicles were also more heterogeneous in T_2 -weighted images than at day 3 and day 6 of wave one, which may indicate the presence of cellular debris in the antrum producing increased heterogeneity. Granulosa cells are sloughed into the antrum (cellular debris) as follicle hormone production changes during the late static and regressing phases of dominant and subordinate follicles (Singh, 1997). Similarly, in ultrasound images, increased pixel heterogeneity of the follicle antrum and higher mean pixel value near the wall compared with near the centre of the antrum were observed at day 1 of wave two and $\geq$ day 17 after ovulation, and were attributed to cells or their degeneration products within the antrum (Singh *et al.*, 1998). In the present study, pixel heterogeneity in T_2 -weighted images did not remain high at $\geq$ day 17 after ovulation in the regressing anovulatory follicles, unlike ultrasonographic pixel heterogeneity.

Regressing follicles showed unique T_2 magnetic resonance image and map attributes. Regressed dominant follicles tended to have higher T_2 relaxation rates than growing and static dominant follicles, possibly attributable to a watery follicular fluid. At day 6 of wave one, regressing subordinate follicles of the anovulatory wave appeared brighter in T_2 -weighted images than the dominant follicles, again indicating water-like follicular fluid in regressing follicles.

Dominant follicles produce oestradiol and inhibin to suppress growth of subordinate follicles. Preovulatory dominant follicles produce more oestradiol than do dominant follicles of the anovulatory wave (Singh *et al.*, 1998), which may explain the unique characteristics of subordinate follicles in the presence of a preovulatory follicle. The preovulatory state affected the magnetic resonance image attributes of the largest subordinate follicle, resulting in higher T_1 relaxation rate and corresponding lower T_1 quantitative signal intensity, possibly indicative of a

watery follicular fluid. The fluid of subordinate follicles in the preovulatory phase was also more heterogeneous and more proton dense. Image attributes of the subordinate follicular antra at $\geq$ day 17 after ovulation were similar to those of the preovulatory dominant follicles, indicating a systemic response affecting both dominant and subordinate follicles in the preovulatory state. In ultrasound images, subordinate follicles in the presence of a preovulatory dominant follicle also showed higher grey-scale values in the antrum than the subordinate follicles of the anovulatory wave and the pre-selection follicles (Singh *et al.*, 1998).

Changes in quantitative signal intensity values in the weighted images (numerical pixel values) did not always reflect changes in relaxation rates, most likely due to combined proton density and T_1 or T_2 information in the T_1 - and T_2 -weighted images. Nonetheless, quantitative signal intensity values from the weighted images correlated with relaxation rates and proton density. There were also discrepancies between pixel heterogeneity scores and relaxation rate heterogeneity. Heterogeneity is a measure of pattern or texture of the image based on the distribution of values about the mean. Heterogeneity scores are more accurate when determined from the weighted images because of noise inherent in the map images. Noise tends to disrupt the subtle underlying image texture.

In agreement with other studies, follicles generally appeared dark in T_1 -weighted images and bright in T_2 -weighted images (Dooms *et al.*, 1986; Olson *et al.*, 1992; Occhipinti *et al.*, 1993; Outwater and Dunton, 1995; Outwater and Mitchell, 1996; Outwater *et al.*, 1996; Sarty *et al.*, 1996; Woodward and Gilfeather, 1998). Differences specific to the phase of development and the types of follicle observed in follicles of different status were not visible with the naked eye and were only apparent with quantitative computer-assisted image analysis.

The largest pools of hydrogen protons in the body are in water and fat; magnetic resonance signals can also originate from the protons in lipid molecules in addition to water. In nuclear magnetic resonance, the protons of hydrogen linked to oxygen in water behave differently from hydrogen protons linked to carbons in lipids. Given the potentially high lipid content of follicular fluid in the form of steroid hormones (oestradiol and progesterone), it is possible that during at least some phases of follicular growth a portion of the magnetic resonance signal could be the contribution of lipid protons. Comparison of conventional T_1 and T_2 images as produced in this study with fat saturation images of ovarian follicles at different phases of development and regression would help to determine whether the differences in magnetic resonance image attributes observed at different phases of follicular development or regression could be due to changes in steroid hormone content, lipid composition of follicular fluid or to changes in the water component of follicular fluid (serum transudate). Fat saturation MRI sequences destroy any signal coming from lipids and ensure that the magnetic resonance signal is only from water hydrogen protons. Differences between conventional weighted images and the fat saturation images would indicate a fat component to the magnetic resonance signal from the follicular fluid.

The onset of atresia of dominant anovulatory follicles was detected as increased quantitative signal intensity and pixel heterogeneity in T_2 -weighted images. Dominant follicles in later atresia tended to have higher T_2 relaxation rates. Regressing subordinate follicles showed higher T_2 quantitative signal intensity within the follicular fluid than healthy dominant follicles in the anovulatory wave. Subordinate follicles under the influence of a preovulatory (healthy) dominant follicle had higher T_1 relaxation rates, T_1 relaxation rate heterogeneity, proton density and proton density heterogeneity but lower T_1 quantitative signal intensity compared with subordinate follicles under the influence of the dominant follicle in the anovulatory wave. The exact nature of changes in the follicular fluid composition that contributed to differences in magnetic resonance image attributes among the phases analysed remains to be determined; however, specific relationships between hormone concentration data and image attributes were observed and the nature of the underlying mechanisms warrant further investigation.

Some of the magnetic resonance image attributes studied may be a direct reflection of the hormone content of a follicle or may reflect mucopolysaccharides or plasma transudate content within the follicle. Identification of the compounds causing the changes specific to the developmental phase and the type of follicle that were observed in the present study will allow further non-invasive study of ovarian function *in vivo*, which will provide more information than is presently available from ultrasound studies about the content of healthy and atretic follicles during development and regression or ovulation. Although it is unlikely in the near future that MRI will replace ultrasonography in the evaluation of ovarian function, the use of MRI may become justified in certain situations because of the additional physicochemical information that can be measured directly with magnetic resonance image analysis of follicular fluid with advancing MRI technology.

The status of a follicle is reflected in the composition of the follicular fluid. In the present study, follicular status was established on the basis of follicle diameter profiles preceding ovariectomy, as reported by Singh *et al.* (1997, 1998). Differences in magnetic resonance image attributes observed among phases of follicular development and regression coincided with changes in the ability of the follicles to produce steroid hormones and ovulate, indicative of follicular health. Growing dominant follicles with healthy granulosa cells characteristically had more homogeneous follicular fluid than early atretic follicles. The antra of dominant follicles became more heterogeneous as they regressed and during later regression had higher T_2 relaxation rates indicative of water-like content. Regressing subordinate follicles were differentiated from growing and early static dominant follicles of the anovulatory wave by the higher T_2 quantitative signal intensity of the follicular fluid. Finally, the preovulatory status of the dominant follicle is indicated by higher T_1 and proton density map attributes and lower T_1 -weighted signal intensity of the largest subordinate follicle in the presence of a preovulatory follicle compared with the subordinate follicles recruited with the dominant follicle of the anovulatory wave. Quantitative

signal intensity analysis of T_1 and T_2 magnetic resonance images as well as quantification of T_1 and T_2 relaxation rates and proton density were used to detect physiologically significant changes in follicular fluid of dominant and subordinate follicles during development and regression. Signal intensity analysis was more direct and provided a more accurate measurement of the pattern than the map attributes; however, analysis of map attributes provided exact physicochemical parameters more easily compared among examiners.

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