Follicular fluid rheology and the duration of the ovulatory process

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The fluid dynamics of ovulation were investigated to understand the mechanical role of follicular fluid in oocyte release. A set of equations describing the flow of fluid from an evacuating follicle was derived from basic principles. These equations demonstrate that, subject to assumptions about the available pressure differential and the source of the expulsive force, the size and shape of the ovulatory orifice have the largest influences on the rate of fluid loss, although the viscosity of the fluid is also an important variable. A thorough rheological examination of pig, bovine and human follicular fluids, performed using a cone-plate viscometer, demonstrated that these fluids have complex, non-Newtonian characteristics. The fluids also undergo time-dependent and spontaneous changes in viscosity at constant shear rates; some fluids were subject to coagulation-like events. Viscosity characteristics were unrelated to broad parameters of follicle development. The models used representative viscosity values to demonstrate that variations in the rate and duration of follicle evacuation, as observed by ultrasonography, could be explained largely by variations in fluid viscosity and the characteristics of the ovulatory orifice.

Introduction

Recent studies using ultrasonography have provided non-invasive visualization of the ovulatory process (DeCrespigny et al., 1981; Townson and Ginther, 1989a,b; Hanna et al., 1994; Kot and Ginther, 1999). These studies used quantitative analyses of follicle size and indicate that there is much inter-animal variation in the rate at which ovulating follicles collapse and that evacuation of follicular contents occurs in a complex non-linear manner. Such results have greater reliability than those obtained by direct observation under surgical conditions (Blandau, 1955; Hill et al., 1935; Kraus, 1947; McKenzie and Terrill, 1937; Espey and Lipner, 1965; Lofman et al., 1982; Talbot, 1983; Zackrissken, 1997), as the peritoneal environment remains intact and disturbances to physical (pressure, temperature and humidity) or other variables which may influence fluid and tissue behaviour, are avoided. Despite advances in our understanding of the tissue processes that lead to follicle rupture (McIntush and Smith, 1998; Murdoch, 1998), little is understood about the mechanisms that cause follicular fluid to be discharged from the peritoneal space, or about the importance of flow patterns in liberating the oocyte to the tubal fimbriae.

Evacuation of a follicle at ovulation may be considered as a problem of fluid dynamics. Successful release of an oocyte requires fluid movement and, therefore, depends on the viscometric properties of the fluid, the dimensions of the follicle and any pressure gradient that is generated. This dependence will be particularly strong in humans and domestic species in which follicle volumes are large and a great deal of fluid is released. The aim of the present study was to understand ovulation from a physical perspective and to define the likely determinants of the flow patterns observed by ultrasonography. Therefore, the studies comprised: (i) a theoretical evaluation of the ovulatory flow of follicular fluid, in which the dominant flow parameters were identified; (ii) a rheological investigation of the fluid from three species (cows, pigs and humans); and (iii) the use of mathematical models incorporating these rheological data to account for the flow patterns revealed in published ultrasonographic descriptions.

Previous attempts to describe the flow of fluid from ovarian follicles mathematically have been based on a pressure-release model, driven by elasticity in the follicular wall (Rodbard, 1968; Lipner, 1993). It is now generally agreed that immediate pre-ovulatory follicles have little or no elastic tension and that the pressure differential needed for final rupture of the tissues results from hydrostatic pressure within the intact capillary bed (Espey and Lipner, 1963, 1965, 1994; Parr, 1975; Lofman et al., 1982; Zackrissken, 1997; Zackrissken et al., 2000). The role of capillary-derived pressure in driving the subsequent evacuation of the fluid is not clear, although a pressure differential between the inside and outside of the follicle must pertain throughout the period of fluid loss. It is possible that pressure is generated subsequent

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to rupture, either from conformational changes within the wall tissue itself or from an actively generated hydrostatic gradient across the follicle wall. Muscle fibres have been reported in the follicle tissues of some species and wall contraction has been induced pharmacologically but the identification of the structures involved is uncertain and there is little physiological evidence that such structures play a role in follicle evacuation (Edwards and Steptoe, 1975; Löfman et al., 1982; Yoshimura and Wallach, 1987; Janson et al., 1988; Talbot, 1991; Espey and Lipner, 1994). There is no information on fluid movements through the follicle wall during ovulation or any evidence that suitable transport mechanisms exist. The magnitude of the pressure differential needed to produce an appropriate flow of fluid for oocyte release is unknown.

The rheological properties of follicular fluid have not been investigated previously in detail. Fisch et al. (1996) reported values for the viscosity of human fluid samples obtained during IVF treatment and proposed that such fluid behaves in a non-Newtonian manner (viscosity decreases with shear rate). Other reports make anecdotal or unquantified references to changes in viscosity during the periovulatory period, some referring to primary and secondary fluids of different viscosities (Zachariae, 1959; Edwards and Steptoe, 1975; Parr, 1975; Hunter, 1984; Hunter et al., 1989; Zackrisson, 1997).

Materials and Methods

Mathematical models of fluid flow

Initially, simple fluid flow models may be constructed based on the idealized dominant attributes of the physical system. The pre-ovulatory follicle was taken to be a sphere filled with fluid from which evacuation takes place after rupture of the external membrane. In developing the models the following parameters were identified: Q: flow rate of fluid; V: volume of fluid expelled; p: pressure inside (p_i) and outside (p_a) the follicle; a: radius of follicle at the start (a_i) and end (a_e) of evacuation; µ: viscosity of follicular fluid; ρ: density of follicular fluid; c: radius of orifice formed at the rupture site; t: length of nozzle; and τ: duration of flow.

Several conjectures surround the nature of fluid release and consequently models for a number of scenarios have been formulated. These models are based on the following idealizations and assumptions: (i) that follicular fluid has a Newtonian or near-Newtonian viscosity over the period of evacuation (data which test this assumption are presented below); (ii) that the flow of fluid is relatively slow and dominated by viscous rather than inertial forces (an estimate of the Reynolds number (Qρ/µρc) for the flow, based on the aperture diameter, is 0 (10^-5–10^-3), indicating that fluid theory based on low Reynolds number hydrodynamics is appropriate); (iii) that the follicle collapses as a sphere of uniformly decreasing diameter; and (iv) that fluid movement to the extra-follicular space is not constrained mechanically and that evacuation is complete.

Preliminary investigations indicated that fluid would be affected by the shape of the orifice formed at the site of rupture. Accordingly, separate models were derived for flow from a simple circular aperture (model 1) and through a short uniform nozzle (model 2).

Model formulation

The problem of low Reynolds number Newtonian flow through a circular hole in a plane wall is discussed by Happel and Brenner (1986). Of particular interest is the pressure drop experienced by the fluid flowing through the aperture. This is given by:

\[ p_2 - p_1 = \frac{3\mu}{c^3} Q \]  

(Eqn 1)

Although theoretically valid for a wall of negligible thickness, good experimental agreement has been reported for thin walls. Furthermore, provided that the aperture radius c is not large compared with the radius of curvature of a shaped wall, then general agreement is maintained.

The behaviour of a Newtonian fluid within a circular pipe is given by the Poiseuille Expression (Happel and Brenner, 1986), which links the pressure differential between the ends of a pipe of length l with the volumetric discharge according to the relationship:

\[ p_2 - p_1 = \frac{8\mu}{\pi c^4} Q \]  

(Eqn 2)

This result is based on a steady flow; in the present study, fluid inertia effects are negligible (low Reynolds number), and so the result is expected to be valid throughout evacuation.

Models 1 and 2 are considered in some detail for the purposes of evaluation. The data to be presented indicate that follicular fluid shows some shear thinning and, therefore, strictly speaking, it is non-Newtonian. Nevertheless, Eqs 1 and 2 provide reasonable approximations and form the basis for calculations. The behaviour of a near-Newtonian fluid can be included in the models if the fluid is assumed to follow a power law. Under these conditions the fluid viscosity is given by:

\[ \mu = \mu_0 |w|^{n-1} \]  

(Eqn 3)

in which \( \mu_0 \) is the kinematic viscosity and \( w \) is the rate of strain experienced by the fluid. The expression corresponding to model 2 for the volumetric discharge (Deen, 1998) is:

\[ p_2 - p_1 = \frac{2\mu_0}{c^3} \left( \frac{3n+1}{n^2} \right) Q^n \]  

(Eqn 4)

In these equations, \( n = 1 \) for a Newtonian fluid and \( n < 1 \) for shear-thinning fluids.

Estimates of \( n \) for bovine, pig and human fluids will be presented, to justify the Newtonian simplification.

The simplest mathematical assumption (case 1) is that the pressure differential across the follicle wall remains constant throughout evacuation of fluid. However, as the mechanisms that generate the pressure differential are uncertain, models 1 and 2 are considered further (case 2) on the basis that evacuation is driven by elastic, contractile, hydrostatic or other mechanical forces within the wall of the collapsing follicle. Case 2 assumes, by analogy with the surface tension...
forces acting on a bubble, that the fluid is expelled by a pressure that increases as the follicle gets smaller.

\[ p_2 - p_1 = \frac{V}{a} \]  

(Eqn 5)

This scenario (Batchelor, 1967) uses a coefficient (with dimensions MT\(^{-2}\)) that is a measure of the ‘elastic’ force exerted by the membrane. Invoking case 2 is not intended to imply that elastic forces necessarily pertain; the resulting equations apply to any situation in which the pressure differential is inversely proportional to follicle radius.

Separate models in which flow is generated by muscular contraction have not been considered because, in addition to the physiological uncertainty, the manner in which such forces would be applied is unclear. It would be necessary to know whether the expulsive force is exerted for the full duration of the evacuation, whether it is constant or variable (for example, related to the presence of hormones or neurotransmitters, or dependent on the length–tension relationship of contracting fibres), and whether it applies uniformly to the follicle surface; no such information is available.

**Follicular fluid samples and viscometry**

All pig and bovine material was obtained from a commercial abattoir and was derived from animals of unknown reproductive history. Healthy maturing follicles were selected on the basis of visual criteria (shiny surface, thinning of a presumptive rupture site, clear fluid and good development of peripheral capillaries). Ovaries with cystic follicles or other obvious abnormalities were excluded. For reasons of volume limitation, the fluids from all large follicles on each single pig ovary were pooled; an average size was recorded. Bovine follicles were treated individually. Details of follicle size distribution are shown (Table 1). Follicular fluid was obtained by puncture of the follicles at room temperature, usually within 2 h after the animals were killed. Shear stress on the samples was minimized by gentle aspiration (syringe and 21 gauge needle) and avoiding centrifugation. Follicles that were contaminated with blood or significant amounts of cellular debris were discarded.

Human follicular fluid was collected from patients \((n = 35)\) undergoing oocyte recovery during IVF treatment at the NURTURE unit (Queen’s Medical Centre, Nottingham). The local ethical committee approved the investigation and written consent was obtained from each patient. Oocyte recovery took place 36 h after administration of hCG and a period of ovarian stimulation by hMG in the presence of pituitary downregulation by GnRH agonist. The fluid of the first follicle \(\geqslant 17\) mm in diameter aspirated from each patient was stored at \(-20^\circ\)C (the viscosity of follicular fluid is unaffected by a single freeze–thaw cycle; M. R. Luck and H. Almislimani, unpublished). Samples were rejected if they contained red blood cells or if, in the opinion of the operator carrying out the aspiration, they may have been contaminated with flushing medium.

Viscosity was investigated using a computer-driven Cone/Plate viscometer (model DV-III; Brookfield Viscometers, Harlow) fitted with a CP40 cone spindle. The plate was a waterjacket cup, supplied from an adjacent waterbath and housing an integral temperature probe. Sample volume was 500 \(\mu\)L. Measurements of viscosity against increasing shear stress (programmed speed increments at 30 s time intervals) or over time (at constant shear stress) were made at temperatures between 37.4\(^\circ\)C and 37.7\(^\circ\)C. The recorded cup temperature was stable to \(\pm 0.1^\circ\)C over a single run. A further set of studies examined the effects of temperature on fluid viscosity at constant shear stress. After viscometric evaluation, samples were stored at \(-20^\circ\)C until steroid hormone concentrations were measured.

**Hormone assays**

The concentrations of steroid hormones in bovine and pig follicular fluids were measured by radioimmunoassay, using extracted or unextracted procedures as described by the original authors (Table 2). Samples from each species were assayed in a single batch. Oestradiol and progesterone concentrations in human fluid were assayed routinely using kits (oestradiol: Microparticle Enzyme Immunoassay; Abbott

### Table 1. Summary of follicle data and viscosity measurements

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean volume (ml)</th>
<th>Mean viscosity (cP)</th>
<th>Viscometry statistics, based on all measurements made at all spindle speeds for each species (cP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(range) of follicles (approximate shear rate)</td>
<td>(range) at three different spindle speeds (cP)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(cows, humans)</td>
<td>20 rpm</td>
<td>60 rpm</td>
</tr>
<tr>
<td>Pigs</td>
<td>(cows, humans)</td>
<td>(150 s(^{-1}))</td>
<td>(450 s(^{-1}))</td>
</tr>
<tr>
<td>59 (48)*</td>
<td>(2–30)</td>
<td>(0.27 \pm 0.04)</td>
<td>(1.74 \pm 0.10)</td>
</tr>
<tr>
<td>34</td>
<td>1</td>
<td>(1.99 \pm 0.20)</td>
<td>(1.18–8.22)</td>
</tr>
<tr>
<td>Humans</td>
<td>34</td>
<td>(2.5^b)</td>
<td>(1.42 \pm 0.04)</td>
</tr>
</tbody>
</table>

*Eleven samples were measured at 20 rpm only.

*Follicle diameter \(\geqslant 17\) mm.

Mode: most frequently occurring value.

Values are mean \(\pm\) SEM where indicated.
Table 2. Steroid hormone assay characteristics for bovine and pig samples

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Procedure</th>
<th>Recovery (mean %)</th>
<th>Assay sensitivity (100 μl sample; pmol l−1)</th>
<th>Coefficient of variation (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>Extracted</td>
<td>94</td>
<td>0.50</td>
<td>5.6</td>
<td>Hunter et al. (1986)</td>
</tr>
<tr>
<td></td>
<td>(bovine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unextracted</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(pig)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestradiol</td>
<td>Unextracted</td>
<td>–</td>
<td>0.94</td>
<td>4.8</td>
<td>Foxcroft et al. (1984)</td>
</tr>
<tr>
<td></td>
<td>(bovine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unextracted</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(pig)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>Extracted</td>
<td>102</td>
<td>0.07</td>
<td>4.6</td>
<td>Purvis et al. (1974)</td>
</tr>
<tr>
<td></td>
<td>(bovine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unextracted</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Laboratories, Abbott Park, IL; progesterone: Serozyme; Serono Diagnostics, Fleet, Hants). Quoted ranges for intra- and inter-assay coefficients of variation were 3.8–10.4 and 4.3–16%, respectively (oestradiol assay), and 3.7–3.9 and 6.4–10.8%, respectively (progesterone assay).

Results

Models of fluid flow

The following models evaluate the functional relationships between the key parameters affecting the flow rate of fluid, $Q$, and the duration of evacuation, $\tau$, associated with possible scenarios for rupture and subsequent expulsion mechanisms for the follicular fluid.

Model 1: evacuation through a simple circular orifice. In case 1, a constant pressure gradient is generated from an unspecified mechanism, leading to a constant value for the flow rate $Q$. The corresponding duration of evacuation, $\tau = V/Q$, is given by rearranging Eqn 1 as:

$$\tau = \frac{3 \mu}{c^3(p_2 - p_1)} V$$

(Eqn 6)

This expression indicates direct dependence on the fluid viscosity and follicular volume and inverse proportionality to the pressure difference. The most significant feature is the sensitivity of the duration time to the radius of the rupture aperture. In case 2, elastic forces, such as those described by Eqn 5, generate the pressure differential. The rate of discharge will vary over time due to the decrease in the internal volume of the follicle $V = \frac{4}{3} \pi a^3$ (instantaneous volume) according to:

$$Q = -\frac{4 \pi a^2}{3} \frac{da}{dt}$$

If the flow rate given by Eqn 1 and the pressure differential given by Eqn 5 are used, the prediction for the changing flow rate is:

$$Q = \frac{c^3 \gamma}{3 \mu} \left[ a_0^3 - c^3 \gamma t / (3 \pi \mu) \right]^{1/2}$$

and the duration of evacuation becomes:

$$\tau = \frac{3 \pi \mu (a_0^3 - a_t^3)}{c^3 \gamma}$$

(Eqn 7)

Here, the radius of the follicle decreases from $a_0$ to $a_t$ non-uniformly with time and the rate of discharge increases until the final radius $a_t$ is reached.

Model 2. This model was developed as for model 1, but with the membrane tearing at the rupture site to form a short nozzle for the evacuating fluid. For case 1, this situation is described by Eqn 2, which allows a prediction of duration time according to:

$$\tau = \frac{8 \mu l}{\pi c^4 (p_2 - p_1)} V$$

(Eqn 8)

Here, the duration of evacuation is increased by the length of the nozzle ($l$), which is formed by the disrupted tissues, but it is also increasingly sensitive to the rupture diameter with inverse quartic proportionality. For case 2, the corresponding flow rate and duration of evacuation are:

$$Q = \frac{\pi c^4 l}{8 \mu} \left( a_0^3 - (c \gamma t / 8 \mu) \right)^{1/2}$$

and

$$\tau = \frac{8 \mu (a_0^3 - a_t^3)}{c^4 \gamma}$$

(Eqn 9)

It can be seen from Eqs 6 and 8 that in case 1 the relationship between volume and duration of evacuation is direct and linear for both models. Replacing $V$ with $\frac{4}{3} \pi a_0^3$ these Eqs become:

$$\tau = \frac{4 \pi \mu}{\left( \frac{c}{a_0} \right)^3 (p_2 - p_1)}$$

and

$$\tau = \frac{32 \mu l a_0}{3 \left( \frac{c}{a_0} \right)^4 (p_2 - p_1)},$$

respectively.

These equations can now be compared more directly with Eqs 7 and 9, and show that the duration of flow is inversely proportional to the third and fourth powers of the ratio of orifice radius to follicle radius ($c/a_0$), respectively. For case 2, the duration of flow has a direct relationship with the dimensions of the nozzle ($l/c$), reflecting the local geometrical effect imposed by this structure.

Viscometry: effect of shear

Viscosity measurements were made over the speed range 2–100 r.p.m. (shear rate 15–750 s−1) but measurements below 10 r.p.m. (75 s−1) were considered to be unreliable at the programmed time interval (Brookfield Viscometer instruction manual) and were excluded. A summary of all viscosity measurements obtained for each species is shown.
Table 1. In the follicle samples from pigs (Fig. 1), mean viscosity showed a complex variation with shear rate, increasing (‘shear thickening’) at low spindle speeds (< 20 r.p.m., 150 s⁻¹) and decreasing sigmoidally (‘shear thinning’) to a plateau beyond 70 r.p.m. (525 s⁻¹). The bovine samples showed similar mean viscosities at low speeds but a lack of shear thickening and a greater degree of thinning with shear rate. The bovine data (Fig. 1) exclude those from one sample (V133) which behaved differently from the rest of the cohort by undergoing an increase in viscosity from 30 r.p.m., a major but transient peak at 46 r.p.m. (345 s⁻¹) and subsequent irregularities (Fig. 2). In two further samples of bovine fluid (data not shown), viscosity recordings had to be aborted (at 18 and 42 r.p.m., respectively) due to sudden torque overload in the viscometer. Inspection of the sample chamber revealed that these samples had coagulated, leaving a fibrous precipitate that prevented the spindle from rotating.

Human follicular fluid also demonstrated shear thinning, although the range of viscosity over which this occurred was greater than that in the other two species (Fig. 1). In general, the viscosity of human follicular fluid was about 20% lower that that of the other species.

Fig. 1. Viscosity of pig (n = 48), bovine (n = 33) and human (n = 35) follicular fluids at different spindle speeds. Data are mean ± SEM.

Viscometry: effects of time and temperature

A number of experiments were performed in which pig (n = 10 samples) and bovine (n = 8 samples) fluids, unrelated to those used in the main study described above, were exposed to constant shear for up to 45 min. Various shear rates were used. All showed evidence of thixotropy (decreasing viscosity over time) or rheopexy (increasing viscosity over time), with some of the changes observed occurring quite suddenly. The intensity, duration and reversibility of the effects varied considerably, with some samples showing a pronounced but transient response. In other cases, an increase in viscosity was followed by a period of intense instability, which prevented further recording. Examples of three pig and three bovine profiles are shown (Fig. 3). Owing to their unpredictable nature, it was not possible to generalize about the nature of these effects or to perform a meaningful statistical analysis.

A similar investigation was carried out on all of the human samples described above, using a constant spindle speed of 40 r.p.m. (shear rate 300 s⁻¹; Fig. 4). These samples showed no spontaneous changes in viscosity of the type observed with pig and bovine samples but a small and highly uniform thixotropy was observed (approximately 13 μP min⁻¹) over 1 h. Treatment of water samples (n = 5) in this manner produced a virtually horizontal line at 0.8 centipoise (cP). The evidence of slight sine wave variation in the data is attributable to vibration in the viscometer.

The temperature dependence of pig and bovine follicular fluid viscosity was examined over the range 35.5–39.9°C, at a constant spindle speed of 50 r.p.m. Fluid viscosity decreased by 0.04 (pig) and 0.07 (bovine) cP °C⁻¹. Similar experiments with water produced a decrease of 0.01–0.02 cP °C⁻¹.

Data modelling

Within each species, values for the modal, maximum and minimum viscosities recorded during the speed range experiments (Table 1) were used for modelling (Table 3). It was necessary to back-model on the basis of a specified duration of evacuation to estimate the effects of variations in
viscosity on the projected duration of evacuation, without knowing details of orifice size or nozzle length and in the absence of values for the pressure differential or elastic coefficient. For each species, the steps in the modelling process were as follows: (i) orifice radii were calculated for models 1 and 2, case 1, such that complete evacuation of a large preovulatory follicle would occur in a representative time (diameters and times taken from published data; see below) at the modal viscosity value recorded in the present study; an arbitrary pressure differential of 1 and a nozzle length of 1 mm were assumed; (ii) model 1, case 2, was evaluated to obtain a value for the elastic force coefficient which would result in the same representative evacuation time in the presence of the orifice radius calculated for case 1 in (i); (iii) model 2, case 2, was evaluated using the elastic force coefficient value obtained in (ii) but allowing the orifice radius to vary until the representative evacuation time was obtained; the coherence of the models results in a similar value for the duration of evacuation to that obtained for model 2 in step 1; and (iv) all calculations were reworked to determine the duration of evacuation at the extremes of the measured viscosity range.

For modelling of bovine data, the value for follicle diameter was based on the mean volume described by Kot and Ginther (1999); the representative evacuation time of 4.2 min was the median of the average times for their two groups of data (rapid and slow evacuation profiles). The follicle diameter for modelling pig data was obtained from Grant et al. (1989). The duration of evacuation of pig follicles in vivo is unknown; a hypothetical duration of 2.92 min was obtained by extrapolation from the values for humans and cows, assuming a cross-species linear relationship between volume and duration (the validity of this assumption is unknown).

As expected from the models, the duration of evacuation was proportional to viscosity (Table 3). Increasing the viscosity from the smallest recorded value to the largest.

Fig. 3. Examples of pig and bovine time–follicular fluid viscosity profiles showing spontaneous thixotropy and rheopexy. (a) Pig sample 1: 0.5 cm (mean follicle diameter), 37.1°C, 100 r.p.m. (b) Bovine sample 1: 3.1 ml (follicle volume), 37.9°C, 60 r.p.m. (c) Pig sample 2: 0.3 cm (mean follicle diameter), 37.2°C, 100 r.p.m. (d) Bovine sample 2: 1.1 ml (follicle volume), 37.6°C, 50 r.p.m. (e) Pig sample 3: 0.3 cm (mean follicle diameter), 37.8°C, 30 r.p.m. (f) Bovine sample 3: 2.0 ml (follicle volume), 37.7°C, 50 r.p.m. *Point at which data became too unstable to record.

Fig. 4. Time–viscosity profiles for human follicular fluid (black; \( n = 35 \)) and water (grey; \( n = 5 \)).
increased the evacuation time by 6.5 (pigs), 17.6 (cows; 59.1 times if the extreme value from V113 was used) or 2.9 (humans) times. Adding a 1 mm nozzle reduced radius of the required orifice by 15 (pigs), 6 (cows) or 4% (humans). Across the three species, the size of the elastic modulus in case 2 was proportional to the diameter of the follicle.

Parameters of follicle maturation

Owing to the non-Newtonian nature of the fluid, further analysis of data was performed using values obtained at shear rates representative of different parts of the speed range (20, 60 and 100 r.p.m.; Table 1).

The viscosity of pig fluids showed no simple relationship (non-linear regression analyses with up to three parameters; not shown) to either follicle volume or steroid hormone concentrations (Table 4). Similarly, there was no simple relationship between viscosity and volume for bovine fluids, although the highest values (sample V113) came from the smallest follicle. Comparisons of viscosity with oestradiol, progesterone and testosterone concentrations by linear regression (not shown) revealed no significant relationships. It became clear during this analysis that the steroid data fell into sub-groups based on the molar concentration ratio of oestradiol to progesterone (oestradiol:progesterone ratio; < 1 and > 1) and on the concentration of testosterone (< 35 and > 35 nmol l−1). There was also a further natural sub-grouping of the low oestradiol:progesterone group by testosterone concentration (Fig. 5). There was a tendency for viscosity to be higher in follicles with oestradiol:progesterone < 1 or with

Table 3. Calculated duration of evacuation, orifice radii and elastic coefficients for representative pig, bovine and human follicles using modelling

<table>
<thead>
<tr>
<th>Species</th>
<th>Representative follicle diameter (mm)</th>
<th>Orifice radius (mm)</th>
<th>Elastic coefficient (Nm−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calculated duration (s)</td>
<td>Minimuma</td>
<td>Modema</td>
</tr>
<tr>
<td>Pigs</td>
<td>9</td>
<td>175</td>
<td>119</td>
</tr>
<tr>
<td>Cows</td>
<td>14.6</td>
<td>252</td>
<td>23</td>
</tr>
<tr>
<td>Humans</td>
<td>18.8</td>
<td>366</td>
<td>271</td>
</tr>
</tbody>
</table>

Table 4. Concentrations of steroid hormones in follicular fluid

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of samples</th>
<th>Oestradiol (range) (μmol l−1)</th>
<th>Progesterone (μmol l−1)</th>
<th>Testosterone (nmol l−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigs</td>
<td>59</td>
<td>0.7 ± 0.1 (0.01–2.3)</td>
<td>1.7 ± 0.4 (0.1–16.7)</td>
<td>332.5 ± 38.1 (2.1–1088.6)</td>
</tr>
<tr>
<td>Cows</td>
<td>34</td>
<td>1.2 ± 0.3 (0.004–6.1)</td>
<td>0.4 ± 0.1 (0.05–2.9)</td>
<td>73.94 ± 22.31 (0.85–424.45)</td>
</tr>
<tr>
<td>Humans</td>
<td>35</td>
<td>13.1 ± 2.2 (1.6–54.5)</td>
<td>4.3 ± 0.5 (0.0005–11.3)a</td>
<td>nd</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM (n > 3).
aFor three samples which were below the sensitivity of the assay, the minimum detectable concentration of 0.5 nmol l−1 was used.
nd: not determined.

Fig. 5. (a) Viscosity of bovine follicular fluid samples measured at 20 ( ), 60 ( ) or 100 ( ) r.p.m. (b) Volume of bovine follicular fluids according to oestradiol (O):progesterone (P) molar ratio (O:P < 1 or O:P > 1) and testosterone (T) concentration (T < 35 or > 35 nmol l−1). Data are mean ± SEM. Values in the same category with different letters are significantly different.
the lower testosterone content. The data for viscosity and steroid concentrations were not distributed normally and so comparisons between groups were performed using the Wilcoxon non-parametric test. There were significantly higher mean viscosities at 60 and 100 r.p.m. in the low oestradiol:progesterone ratio group. Other comparisons of viscosity between the groups failed to show significant differences and there were no significant differences in mean follicle volumes among the follicle categories.

The viscosity of human follicular fluid showed no relationship with either progesterone or oestradiol concentration (not shown). The nature of the collection procedure at the IVF clinic made it inappropriate to try to relate viscosity to follicle volume.

### Discussion

These data demonstrate that bovine, pig and human follicular fluids have complex rheological characteristics. Some degree of shear thinning was observed in all three fluids but the relationships between viscosity and shear were non-linear. Pig fluid showed a slight shear thickening at low speeds but there was greater variance than in the fluids of the other two species. The degree of shear thinning was greatest in the human fluids, confirming an earlier observation that human follicular fluid is non-Newtonian (Fisch et al., 1996). Fisch et al. (1996) used a larger cohort of IVF-derived fluids and observed similar mean viscosity values (1.48 and 1.38 cP) to those observed in the present study at 115 and 230 s⁻¹. These authors also reported values of up to 3 cP for viscosity at shear rates < 75 s⁻¹ (equivalent to 10 r.p.m. in the present study). The shapes of our non-Newtonian curves indicate that at low shear stresses the viscosity would be similar to (pig) or slightly higher than (bovine, human) the highest values measured in the present study. The shear stress imposed on follicular fluid during follicle evacuation in vivo is unknown.

In addition to being non-Newtonian, significant thixotropy and rheopexy (changes in viscosity with time at constant shear rate) were observed in the fluids in the present study, only some of which was reversible. With the human samples, this was a linear thixotropic effect, presumably representing the rheological influence of non-variant constituents of the fluid. In contrast, unpredictable thixotropy and rheopexy was observed in the fluid from the two domestic species. This finding is indicative of the presence of rheologically influential constituents, the composition and concentration of which vary widely and which may have a propensity for conformational or structural change when subjected to continuous shear stress. Although the nature of the constituents responsible for these rheological characteristics is unknown, some may be related to those involved in coagulation. As described, some fluid samples underwent coagulation during measurement, whereas others showed sudden increases in viscosity to unstable levels. Bovine follicular fluid tends to coagulate in other circumstances (unless Ca²⁺ ions are excluded; M. R. Luck, unpublished) and this is consistent with the presence of fibrinogen (Shalgi et al., 1973; Parr, 1974) and proteins of the extrinsic coagulation cascade (Gulamali-Majid et al., 1987; Yamada and Gentry, 1995) within the fluid. Zackrisson (1997) reported the occurrence of a gelatinous mass at the stigmatic orifice of rat ovaries and subsequent coverage of the rupture site by a fibrin-like mesh. It is not known whether fluid coagulation plays a role in the normal process of ovulation, determines the subsequent behaviour of the liberated fluid or contributes to the wound repair processes of luteinization (Silvester and Luck, 1999). Nevertheless, premature coagulation of incompletely expelled fluid and the formation of a thrombus within the ruptured follicle would hinder successful release of the oocyte. Evidence of anticoagulant activity has been found in pig and rat follicles (Andrade-Gordon et al., 1992; Hosseini et al., 1996).

It is clear that such variations in viscosity over time confound attempts to measure viscosity at different speeds. This finding was well illustrated by the bovine data, where one sample could be excluded from the variable shear data set on the basis of its unusual behaviour. It is likely that in each of the three sets of variable shear data the general variance encompasses other, less dramatic, time-related variations in individual samples. Indeed, it is difficult to envisage how investigations might be designed to separate these variations. It is also likely that the process of sampling from follicles, which was performed with a needle and syringe, actually influences subsequent measurements of viscosity. In vivo, follicular fluid is subjected to zero shear until the moment of follicle rupture; the necessity for sampling, however gentle, makes it impossible to reproduce this condition when carrying out mechanical measurements. In this context, it is important to note that the observed changes in viscosity with time occurred within time scales appropriate to the reported duration of follicle evacuation. Such changes, whether predictable and uniform as in humans or chaotic as in the other species, have the potential to affect the progress of follicle evacuation independently of pressure differential and shear stress.

These data indicate that follicular fluid viscosity has a temperature sensitivity that is about 2–3 times that of water. This conclusion has significance in the light of observations that pre-ovulatory follicles may exist in vivo at a temperature up to 2°C cooler than that of the surrounding stroma and peritoneum (Hunter et al., 1997, 2000). On this basis, ovulated fluid would be subjected to a sudden increase in temperature as it reaches the peritoneal space, reducing its viscosity and increasing its rate of flow.

None of the viscometric properties described in the present study had a simple relationship with the indices of follicle maturation (size and steroid concentration). There was a tendency for bovine and pig viscosity to be higher in smaller follicles and in those with lower oestradiol contents. However, these effects did not form part of an obvious pattern of development and it is concluded that viscosity is not a strongly maturation-dependent variable, at least over the range of the stages of maturation represented in the present study. The bovine and pig follicles came from animals of unknown reproductive status and will have included representatives of several developmental stages. In the human samples, the measurements represent the preovulatory condition, in as much as the follicles of women undergoing
preparation for IVF are highly stimulated and oestrogenic. However, these follicles showed wide variations in oestriadiol and progesterone concentrations and it is possible that a range of conditions, in including cystic structures, was represented in the cohort. Fluid flow and the variables that determine it become relevant only at the time of follicle rupture; now that the basic rheological characteristics have been established it may be possible to undertake a more detailed study of fluids from the last few hours or minutes before rupture.

The basic models derived in the present study establish the variables that determine the movement of fluid from an ovulating ovarian follicle. The dimensions of the orifice are quantitatively dominant, as they operate to power derivatives. Model 1 assumes the orifice to be a two-dimensional circular tear, the diameter of which is maximized instantaneously at the start of the evacuation process. Morphological evidence is lacking but this is unlikely to be the case in vivo; it is more reasonable to expect that the orifice acquires an irregular shape and expands gradually over the course of evacuation. Model 2, which is based on a tubular nozzle, indicates what might happen if flaps of tissue formed around the orifice and impeded the flow of fluid. Again, in reality such flaps are more likely to be irregular than tubular in conformation. Thus, a model that takes accurate account of the effects of orifice shape is likely to fall between the extremes considered here. Nevertheless, small variations in orifice dimensions will have the greatest quantitative effects on fluid flow.

Both models make the implicit assumption that there is no physical or physiological relationship between orifice dimensions and viscosity (for example, a more viscous fluid does not cause a larger ‘tear’ in the follicle wall, and orifice size and viscosity do not share a common determinant). These models also assume that follicles are spherical and remain so throughout the evacuation, which may not be realistic (Townson and Ginther, 1989a). As discussed above, it is also unclear whether the measurements made in the present study represent the range of viscosities applicable in each species at the moment of ovulation. The bovine and human predictions are likely to be more realistic than those for pigs because they are modelled around observations of the duration of evacuation made by ultrasonography (Hanna et al., 1994; Kot and Ginther, 1999).

Despite these qualifications, and the quantitative importance of the orifice, the analyses presented indicate that fluid viscosity has a significant influence on flow during ovulation. In the bovine models, the measured range of viscosity varied the duration of evacuation from 23 s to nearly 7 min (or 23 min if one extreme value is included). Kot and Ginther (1999) reported a range of overall evacuation times in cattle from 6 s to 14.5 min. In humans, the models provide a range of 4.5–13.0 min compared with observed times of between 6 s and 18.5 min (Hanna et al., 1994), 2–5 min (Brännström et al., 1996), or from ‘a few seconds’ to 35 min (DeCrespigny et al., 1981). In pigs, using a hypothetical evacuation time, the measured viscosity range permits a six-fold range for the duration of evacuation. Thus, it appears that our models encompass much of the observed variation if only slight modifications were made to orifice dimensions.

These models operated on the assumption that the fluid is Newtonian, or nearly so, whereas the data indicate that this is not the case. With a Newtonian fluid, a single value for viscosity would apply irrespective of the shear stress, whereas the speed of evacuation and the size and shape of the orifice actually influence the viscosity of follicular fluid. The extent to which this deviation might influence the predictions of the models can be indicated by calculating parameter $n$ in the power-law equations (Eqsns 3 and 4). It $n = 1$ represents the slope of a log–log plot of viscosity against shear. The data for each species fit this linearization well (Table 5) and the corresponding values of $n$, particularly for the pig and bovine data, are very small. Therefore, the Newtonian assumption can be taken as a reasonable approximation to the real state.

Ultrasound evidence from cows (Kot and Ginther, 1999), humans (Hanna et al., 1994) and horses (Townson and Ginther, 1989a,b), and other observational data (rats: Zackrissen, 1997) indicates that the rate of follicle evacuation is non-linear. Follicles may collapse in two or more phases, with a rapid initial extrusion followed by a slower phase. Several potential scenarios may be invoked to account for this observation, including the existence of sub-fluids of differing viscosity, progressive changes in orifice conformation and alterations in wall ‘tension’. The theopexy observed in the present study, together with chaotic thickening events and coagulation, would also contribute. The present models can be used to examine the theoretical validity of some of these possibilities. The variations in orifice radius that would be needed to account for the observations that: (i) human follicles reach, on average, 70% evacuation within 0.9 min (Hanna et al., 1994); and (ii) that a rapidly evacuating subgroup of bovine follicles reaches 89%
evacuation within 4 s (Kot and Ginther, 1999) are shown (Table 6). In both species, the lowest viscosity value permits rapid evacuation if the orifice radius is increased by only 40–80%. In humans, the mid- to high range viscosities require an orifice radius approximately twice that predicted previously. In cows, the higher viscosities require an orifice that is 5–7 times larger, which is probably unreasonable. These outcomes indicate strongly that viscosity is an important determinant of evacuation rate. The outcomes are consistent with the presence of sub-fluids of differing viscosities (rapid release of a low viscosity fluid, followed by slower release of a higher viscosity fluid) and with a progressive change in viscosity as the fluid moves.

There is uncertainty about the nature of the pressure gradient during follicle evacuation. Previous attempts to model processes associated with ovulation (Rodbard, 1968; Lipner, 1993) predicted the limiting pressure attainable in a follicle during the final stages of growth. However, in these models, it was assumed that the follicle wall has significant elasticity in the face of increasing intrafollicular pressure and that the elastically stored energy is released catastrophically at the time of rupture; there is no evidence for such a mechanism (Espey and Lipner, 1994). In fact, it has been demonstrated that follicles tend to become flaccid in the hours immediately before rupture (Lenz, 1985; Murdoch, 1985; Espey et al., 1994; Hanna et al., 1994; Fisch et al., 1996), although intact follicles in vitro may experience significant capillary pressure (Espey and Lipner, 1963, 1965; Zackrisson et al., 2000). Fluid flow is described as gentle rather than spurting (Edwards and Steptoe, 1975; Van Blerkom and Motta, 1979). Our models are consistent with this observation in that they operate with low coefficients of elasticity (where this represents any wall-related pressure factor). It remains possible that the pressure gradient increases after rupture of the tissue, either due to tissue contraction or to hydrostatic pressure. Such effects might give rise to a pressure gradient which acted as if it were based on previously generated elastic forces (a function of the decreasing diameter of the collapsing follicle), rendering model 2 potentially instructive at least as far as the pattern of flow is concerned. Taken together, these models provide a basis for predicting how great the pressure would need to be for ovulation in a particular species and for predicting where it might originate. Unfortunately, complete information on all variables is unavailable for any species. In particular, details of orifice size and shape are very limited. Evacuation of rabbit follicles perfused in vitro (Löfman et al., 1982) occurred through orifices that were small (compared with the diameter of the follicle) and of minimal tubularity. Orifices of hamster follicles ovulated in vitro (Talbot, 1983) varied greatly in size; most were 'large' or 'very large' and facilitated free movement of the cumulus–oocyte complex, whereas other orifices took a considerable time to enlarge to a sufficient diameter. Talbot (1983) proposed that hamster oocytes are extruded largely independently of free-moving fluid, implying that the extrusion rate is determined mostly by the extent to which the orifice diameter exceeds that of the cumulus–oocyte complex. In species with larger follicles (domestic species and humans), a large volume of fluid is released and flow will be influenced by the orifice in the manner indicated by our models.

In conclusion, a set of equations to describe the flow of fluid from an evacuating ovarian follicle has been derived. These equations demonstrate that, subject to assumptions about the mechanisms of fluid expulsion, the size and shape of the ovulatory orifice have the largest effects in determining the rate of follicle evacuation but that the viscosity of the fluid is also important. Rheological examination of pig, bovine and human follicular fluid indicates that these fluids have complex, non-Newtonian characteristics, unrelated to broad parameters of follicle development. The fluids also show spontaneous changes in viscosity at constant shear rate and may be subject to coagulation-like events. Known variations in the duration of ovulatory follicle evacuation can be explained largely by variations in fluid viscosity. The characteristics of follicular

### Table 6. Calculated orifice radii (mm) required to facilitate partial evacuation of bovine and human follicles under models (i) and (ii), using published times for the initial phase of follicle collapse

<table>
<thead>
<tr>
<th>Bovine</th>
<th>Model 1 Case 1</th>
<th>Model 2 Case 1</th>
<th>Model 1 Case 2</th>
<th>Model 2 Case 2</th>
<th>100% in 4.2 min</th>
<th>100% in 6.1 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu = 0.14$ cP</td>
<td>$\mu = 0.53$ cP</td>
<td>$\mu = 8.25$ cP</td>
<td>$\mu = 2.45$ cP</td>
<td>$\mu = 1.53$ cP (from Table 3)</td>
<td></td>
</tr>
<tr>
<td>Model 1 Case 1</td>
<td>1.19</td>
<td>2.65</td>
<td>4.65</td>
<td>3.10</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Model 1 Case 2</td>
<td>1.17</td>
<td>2.61</td>
<td>4.57</td>
<td>3.05</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Model 2 Case 1</td>
<td>1.08</td>
<td>1.96</td>
<td>3.00</td>
<td>2.20</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Model 2 Case 2</td>
<td>1.05</td>
<td>1.13</td>
<td>1.36</td>
<td>0.74</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Human</th>
<th>Model 1 Case 1</th>
<th>Model 2 Case 1</th>
<th>Model 1 Case 2</th>
<th>Model 2 Case 2</th>
<th>100% in 4.2 min</th>
<th>100% in 6.1 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu = 0.91$ cP</td>
<td>$\mu = 1.23$ cP</td>
<td>$\mu = 2.64$ cP</td>
<td>$\mu = 1.23$ cP</td>
<td>$\mu = 1.23$ cP</td>
<td></td>
</tr>
<tr>
<td>Model 1 Case 1</td>
<td>1.21</td>
<td>1.33</td>
<td>1.72</td>
<td>0.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 1 Case 2</td>
<td>1.11</td>
<td>1.19</td>
<td>1.44</td>
<td>0.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 2 Case 1</td>
<td>1.12</td>
<td>1.24</td>
<td>1.60</td>
<td>0.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 2 Case 2</td>
<td>1.05</td>
<td>1.13</td>
<td>1.36</td>
<td>0.74</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bovine: 89% evacuation in 4 s from a follicle 14.6 mm in diameter, based on ‘Pattern 1’ (Kot and Ginther, 1999).
Human: 70% evacuation in 0.9 min from a follicle 18.8 mm in diameter, based on ‘Pattern 1’ (Kot and Ginther, 1999).
fluid flow may determine the success of oocyte liberation at ovulation.

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