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REVIEW

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Physicochemical Properties of Follicular Fluid and Their Relation to in Vitro Fertilization (IVF) Outcome

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INTRODUCTION

Follicular fluid (FF) creates a relatively isolated compartment which provides both the granulosa cells and the oocyte with the highly specific milieu required for their maturation. The organic composition of this microenvironment, including steroid and pituitary hormones, follicular proteins, and glycosaminoglycans, has been studied extensively over the last decade (1–4). Moreover, some of these components have been found to serve as potential predictors of IVF outcome (5). In contrast, information about the physical properties of FF in the preovulatory follicle is scant and confusing. This is due largely to technical difficulties as well as the paucity of experimental equipment suitable for assessing parameters such as FF pressure and temperature or electric potentials across the follicle wall, which require intact follicles for measurements *in situ*. The aim of this paper is to summarize the available data concerning the physicochemical properties of FF obtained during natural and stimulated cycles. It is important to assess whether iatrogenic changes in this well-defined set of physiological conditions adversely affect the outcome of IVF treatment and, also, to identify those properties of FF which correlate positively with successful treatment and therefore could be used as markers for IVF outcome.

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FACTORS AFFECTING FF PHYSICOCHEMICAL PROPERTIES

Physical characteristics of any biological fluid are the product of its chemical composition and reflect the degree of its isolation from the surrounding environment. In the preovulatory follicle, these factors are influenced primarily by the properties of the tissues which separate the FF from the blood: follicular wall permeability to water (6) and to substances that derive from the blood (7,8), on one hand, and granulosa cell secretory function (9,10), on the other hand, would affect physical parameters such as volume, viscosity, specific gravity, osmolarity, color, and pH of the liquor accumulating within the follicle; the distance between the antral cavity and the blood vessels coupled with the specific thermal conductivity of the follicle wall would determine FF temperature; the rate of FF formation and the tensile strength of the follicle wall would contribute to the intrafollicular pressure, and these are just a few examples.

Before ovulation, vasculature is confined to the theca-interna and the so-called "blood–follicle barrier" is thought to prevent the high molecular weight proteins from traversing the lamina basalis to enter the avascular granulosa cell layer (8,11,12). Interestingly, it appears that this barrier is only functional, since follicles do not possess a structural barrier comparable to that which exists in the testis (13). In their elegant paper, Gosden and colleagues (14) discussed the physiological factors underlying FF formation and provided evidence to support the theory that the granulosa cell layers which line the follicular cavity behave like a "leaky" epithelium according to morphological, electrical, and chemical criteria. However, the question of whether an

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active mechanism of ion transport is involved has yet to be resolved.

FOLLICULAR FLUID PROPERTIES

Temperature

The effect of high temperature on the female ovary was reviewed by Waites (15). Interpolating from the male testes, which require relatively low temperatures for the process of spermatogenesis, the theory that similar conditions are necessary for folliculogenesis seemed attractive. Initial tests failed to show differences between ovarian and core temperatures (16). Later, Grinsted and associates (17), using both microthermoelectrodes and thermovision techniques showed that, although actual follicular temperature in rabbits varied from animal to animal, there was a clear difference between FF and core temperatures; follicular temperature was lower by 2.8 ± 0.2°C. These results were impressively uniform despite the fact that the experiments were conducted in three different microenvironments. Also, when follicular and ovarian stroma temperatures were compared, that of the follicle was found to be lower by 1.4°C. This difference was similar for large and small follicles. Benoit and colleagues reported results from studies in ovulating ewes (18). Using ovarian thermistors, they observed that ovarian temperature was higher than body temperature by 0.14°C. However, in this study no attempt was made to measure follicular temperature. The study design by Grinsted and colleagues (17) excluded the possibility that the temperature difference between the follicles and both the core body and the ovarian stroma was due to heat conduction, heat convection, heat radiation, or evaporation. The putative mechanism that they suggested for the formation of this temperature gradient was an endothermic chemical reaction, due to changes in concentrations of FF macromolecules such as mucopolysaccharides and proteins during follicular growth. Nevertheless, it is difficult to explain how such a temperature gradient is kept stable despite the open channels to the surrounding ovarian capillary network. The physiological significance of this temperature gradient has still to be carefully evaluated with regard to follicular development and oocyte maturation.

Volume

Attention has been paid lately to this parameter, since it became clear that with IVF, outcome relates directly to follicular size. Like most other FF parameters, its volume should be plotted along a time axis, since this variable changes as the follicular phase progresses. The diameter of the dominant follicle, averaging 9.8 mm 5 days before ovulation, undergoes an exponential growth, reaching a mean value of 21 mm prior to ovulation (19). Gosden and Telfer (20) compared ovarian follicles of 29 mammalian species in nine orders and showed that the diameters of primordial follicles correlated significantly with body size. Their systematic work substantiated the conclusion reached by Parkes (21) about half a century earlier. They also observed that in all species studied, FF started to accumulate at the time that follicular diameter was 0.2–0.4 mm. The size of the mature follicle appears to be genetically determined (14). The accumulation of antral fluid is hormonally regulated (22), and the midcycle luteinizing hormone (LH) surge seems to be the final note in a series of events that cause the rapid preovulatory increase in FF (2,23). The three common methods for estimating FF volume were ultrasonic evaluation, fluid aspiration, and measurements of ovarian histological cross sections. O'Herlihy *et al.* (24) showed that the mean follicular size in spontaneous cycles was 21 mm, which corresponded to a mean volume of 5.1 ml. He noted an excellent correlation between the calculated FF volume according to ultrasonic measurements (using the formula for sphere volume, $\frac{4}{3}\pi r^3$) and the actual fluid volume recovered at laparoscopy. He concluded that ultrasound was an accurate tool for estimating follicular volume. The correlation between follicular volume and IVF results was evaluated by several authors. Bayer and associates (25) found FF volume to be a poor predictor of fertilizing capacity. However, Quigley and colleagues (26) described a direct relationship between follicular size (above 20 mm) and oocyte recovery, fertilization, and cleavage rates. These findings were supported by Scott and colleagues (27), who, based on data from 412 aspirated follicles, reported that follicles of diameters greater than 14 mm have the highest chance of containing mature oocytes. Simonetti *et al.* (28), reviewing 547 cycles stimulated by human menopausal gonadotropin (hMG) found that mature oocytes were associated with larger follicles (over 2.7 ml) and, more importantly, that the incidence of spontaneous abortion was high with conceptuses derived from smaller follicles. In conclusion, despite some contradictory data, the association between FF volume and successful IVF outcome is well established.

Color and Spectrophotometric Absorbance

FF is described as "a straw-colored liquid" (2,23). Bayer and colleagues (25), examining FF obtained during IVF treatment cycles, noted that the intensity of its yellow color varied. To allow a quantitative determination of the pigments present, they analyzed the spectrophotometric absorbance pattern of human FF in the visible spectrum. In this study, two peaks were identified within the yellow spectrum at 415 and 455 nm, but the amount of absorbance varied among individual follicles. It was also shown by the authors that oocytes which subsequently fertilized originated from follicles containing FF that had significantly higher absorbance at 455 nm compared to those that failed to fertilize. Further studies (29) confirmed a highly significant association between fertilization and absorbance at Δ optic density of 455 nm as well as 360 nm, but no similar relationship was revealed with embryo cleavage. These researchers previously postulated that the 455-nm absorbance, which correlated with the FF bilirubin content, could be proportional to the degree of follicular vascularization. However, results from their more recent study (29) did not demonstrate any correlation between the 455-nm absorbance and FF protein or hormone concentration and therefore were not consistent with this hypothesis. Further studies are required to clarify these observations.

Viscosity and Refractive Index

Systematic studies of FF viscosity have not been described. It appears that this physical parameter changes during follicular growth (2), although differently, in various species; in the cow (10) and the rat (30) it decreases, whereas in primates (31–33) it increases with follicular maturation. These changes have been attributed to alterations in glycosaminoglycan concentration prior to ovulation. Limited data are available, however, concerning viscosity and specific gravity of human FF from preovulatory follicles in spontaneous cycles. In contrast to FF, blood viscosity has been investigated extensively and may serve as a model for investigating this physical property of FF. In pregnant women, plasma viscosity was found to increase with advanced gestation and to correlate positively with increasing fibrinogen content but not with circulating hormone levels (34). Factors known to influence whole-blood viscosity include hematocrit, plasma viscosity (34), shear rate (34,35), red cell deformability, pH, P_aO_2, temperature (35), and plasma protein concentration (35,36). Increased concentrations of certain plasma proteins, especially fibrinogen and macroglobulins, enhance cell aggregation and may greatly increase whole-blood viscosity at low shear rates. Therefore it appears that, of all the factors which influence the viscosity of body fluids, FF viscosity will be determined mainly by its protein content. In a recent study, standard values of FF viscosity and refractive index (which reflects FF specific gravity) were set using FF samples collected at the time of oocyte retrieval for IVF (37). Viscosity was measured at 37°C in a Wells–Brookfield microviscometer equipped with a CP-7 spindle (cone 1565°). Refractive index was determined by the Goldberg refractometer (American Optical Company) for both fresh and frozen specimens. Viscosity determinations (centipoise; mean ± SD) for shear rates of 23, 46, 115, and 230 sec^{-1} were 2.04 ± 0.86, 1.84 ± 0.49, 1.48 ± 0.27, and 1.38 ± 0.22, respectively. The average (±SD) refractive index was 1.030 ± 0.002. There was no significant difference between the values for thawed frozen fluids and those for fresh samples of the same specimens. Whole blood behaves as a non-Newtonian fluid at low shear rates, reflecting the effect of reversible adhesion of red cells via fibrinogen (38,39). These results suggest that FF also behaves as a non-Newtonian fluid at the shear rates studies (its viscosity changes at different shear rates), possibly reflecting the interaction between fibrinogen and cells present in the fluid (erythrocytes and granulosa cells). Of follicular proteins studied, only alpha-1-antitrypsin and fibrinogen levels were found to correlate positively with fertilization and pregnancy (40). As FF fibrinogen is a major determinant of FF viscosity, a positive correlation between FF viscosity and fertilization could be expected. However, data from the above-mentioned study showed no correlation between FF viscosity or refractive index and the presence of oocytes, their maturation grade, or their fertilizing capacity (unpublished data).

Electrolyte Content and Osmolarity

Osmolarity of human FF equals that of the plasma (1,11,14,41) in both natural and clomiphene-induced cycles (42). Similarly, no concentration gradients (FF vs plasma) were identified with regard to the principal electrolytes: sodium, potas-

sium (1,11,41), chloride (11,41), magnesium, and calcium (41). Several mechanisms have been proposed as being involved in the regulation of FF inorganic component concentration. Current concepts favor simple diffusion as the major determinant of FF electrolyte and gas composition. Early experiments showed that tritium-labeled water reached equilibrium with FF within 15–25 min, suggesting that the follicular blood barrier is highly permeable to water (6). Similar conclusions were drawn using ^{131}I (43). David *et al.* (44) found that sodium and chloride concentrations as well as osmolarity in rabbit FF were identical to those in the ovarian vein and artery, thus supporting the transudation theory. But they also found significantly elevated FF levels of K^+ and assumed that it was a granulosa cell contribution. However, evidence for a possible role for local follicular secretion or active mechanism of ion transport remains inconclusive (14). It should also be taken into consideration that different techniques of FF collection and analysis may lead to contradictory results. This was clearly demonstrated in several studies on porcine FF. Shuetz and Anisowicz (45), using slaughterhouse material, showed that the sow FF is "K^+ rich and Na^+ poor." Chang *et al.* (46) found that freshly collected porcine FF had higher K^+ levels than the plasma. They also observed an obvious decrease in FF K^+ when stored in ice. Knudsen and colleagues (47) reported that Na^+, K^+, and osmolarity were similar to plasma levels in cyclic pigs, with small changes in K^+ concentration during the cycle, in contrast to slaughterhouse material, which had higher K^+ and osmolarity as well as lower Na^+. Slightly different results were provided by Gosden and Hunter (48), who studied porcine FF samples from anaesthesized animals. They observed that the elemental composition of FF and plasma was identical, with two exceptions: concentrations of Na^+ in FF were higher, whereas those of bicarbonate were lower. It appears that the precautions taken by Shalgi *et al.* (11), who used only fresh tissue collected during surgery and delivered it to analysis within 5 min, are mandatory in order to achieve reliable results. Apart from effects induced by experimental design, FF electrolyte content may vary because of differences in follicle size. This was demonstrated by Knudsen and associates (47), who found that K^+ concentration was higher in small as opposed to medium or large follicles in cyclic sows. Unlike animal data, little is known about factors affecting changes in electrolyte concentrations in human FF. Likewise, there is no information linking FF electrolyte measurements and IVF outcome.

pH and Gases

The pH of human FF is similar to that of the serum (49) and mostly controlled by pCO_2 (23). In their basic work, Shalgi and associates (11) revealed that the mean partial oxygen tension (±SE) in freshly obtained human FF was 54.3 ± 3.54 mm Hg. The authors noted a significant variation (over a ninefold range) in these results, which could not be related to follicular size or histological differences. FF was also found to be distinctly acid (pH 7.267 ± 0.013; mean ± SE) with pCO_2 ranging slightly lower than venous values (35.1 ± 1.5 mm Hg). An acid excess of 10.1 ± 0.39 mEq/liter was calculated using the Siggaard Andersen alignment normogram. Hence it was concluded that although pCO_2 is a major determinant of FF pH, the fluid must contain a large excess of nonvolatile acids. It seems probable from observations on bovine FF (49) that local formation of acid mucopolysaccharides might provide an adequate explanation for this excess. More information was added by Fraser and colleagues (50), who, using freshly collected FF from ovarian specimens, noted a high range of pO_2 values (53–151 mm Hg). The authors suggested that the pO_2 levels might be even lower than calculated due to technical difficulties associated with sampling small follicles and that this relatively low oxygen tension may influence the metabolism of the oocyte and the granulosa cells. These findings correlated well with Gosden and Byatt-Smith's prediction, based on a mathematical model, of a steeply descending inward oxygen concentration gradient (51). Another observation by Fraser *et al.* was a relatively high pO_2 in the ovarian vein as compared to peripheral vein levels. These investigators assumed that arterial venous shunting exists in the ovary. In their study pCO_2 approximated that of peripheral venous blood, while the pH, averaging 7.32, was higher than peripheral vein, ovarian vein, and arterial blood mean values.

The apparent discrepancy between the above findings may be attributed to difficulties in measuring pH of freshly aspirated FF. Once FF is removed and laced in air, its pH will begin to rise to very high levels (52). Conversely, it was thought that the use of carbon dioxide to achieve pneumoperitoneum might induce acidosis in FF. The gas could dissolve

in extracellular and intracellular fluids, releasing carbonic acid and altering the pH. However, it appears that when using 100% CO_2 for periods of up to 1 hr, no change will occur in FF pH, providing that the follicle remains intact. This is due to follicular capacity to buffer any CO_2 which diffuses through its wall (52). When the effect of CO_2 pneumoperitoneum on rabbit oocytes and subsequent embryos was assessed, it was shown that despite a rise in arterial pCO_2 and pO_2, as compared to the air-treated animals, there was no significant difference in embryo cleavage rates between the two groups (53). Nevertheless, the introduction of the transvaginal aspiration method, which obviates the need for intraperitoneal insufflation, eliminates any possible effect of exogenous gas on FF pH.

Electrophysiological Properties of the Follicle Wall

Evidence for the existence of a transmural potential difference can support the theory that FF is formed by a flow of fluid that follows an active ion transport through the follicle wall. To test this hypothesis, McCaig (54) applied probing microelectrodes for voltage recording across the epithelial cell layer of superfused mouse follicles, isolated at various stages of the ovarian cycle. Measurements revealed a steady potential when the antrum was entered, which was maintained as the electrode continued to move through the FF. This transfollicle wall potential difference measured was small (1.2 ± 0.3 mV; antrum positive) in cycling animals and became more positive as ovulation approached. Furthermore, metabolic inhibitors, which would block any active ion transport, caused an increase in the internal positivity of the potential difference. More recently, Gosden and Hunter (48) have added information concerning the electrophysical properties of the follicle wall. Mounting explants of porcine follicles in a Ussing chamber, they found that the potential difference across the pig follicle wall was small (0.2 ± 0.1 mV), a finding which was in agreement with the previous results. The specific electrical resistance of these explants was calculated to be very low (59 $\Omega \cdot cm^2$). These observations were interpreted as evidence that the follicle wall is an electrically "leaky" epithelium (14) and that the current is conducted paracellularly rather than transcellularly. Therefore, it is probable that the major pathway for entry of water into the follicle is paracellular.

Intrafollicular Pressure

Important studies regarding changes in intrafollicular pressure were conducted more than 20 years ago. Earlier works (12,49) noted an increase in pressure prior to ovulation as part of the suggested "egg expulsion" mechanism. In the rat, intrafollicular pressure as measured in 250 graafian follicles was found to average 23.1, 21.9, and 19 cm of water in large, intermediate, and small follicles, respectively (55). Simultaneously, parallel changes were observed in the mesenteric capillary pressure. Also, no preovulatory surge in intrafollicular pressure was noted and ovulation was concluded to result from a sequence of morphologic changes around the stigma, which in turn appeared to be hormonally controlled. Espey and Lipner (56), by direct cannulation of rabbit graafian follicles with micropipettes, found that the average pressure was 17 mm Hg, whereas in the preatretic follicle it was 50 mm Hg. The intrafollicular pressure was proportional to the blood pressure, suggesting that colloid osmotic pressure has a negligible contribution to the total hydrostatic pressure in the follicle. The authors found a decrease in pressure to 5 mm Hg at the time of rupture (ovulation) with no preovulatory surge. They viewed ovulation as an event occurring under the force of steady antral pressure. Using exteriorized rabbit ovaries Rondell (57) introduced two micropipettes into follicles. He used one pipette to measure the hydrostatic pressures and the other for saline injection. Two interesting observations resulted from this experiment: impending ovulation did not change intrafollicular pressure and an increase in intrafollicular pressure could not be induced by fluid injection. Tension–length diagrams in these experiments indicated an increased follicular wall extensibility prior to rupture. Such a change in the elastic properties of the follicle wall would explain why the rapid preovulatory rise in FF volume is not accompanied by a concomitant increase in intrafollicular pressure.

SUMMARY

Despite the limited data that are available concerning FF physicochemical properties, the following conclusions can be drawn.

(1) FF temperature is lower than ovarian stroma and body temperatures. The physiological significance of this gradient is unknown.

(2) Follicular size increases exponentially prior to ovulation. The relationship between FF volume and successful IVF outcome is well established.

(3) A highly significant association exists between fertilization (but not embryo cleavage) and FF spectrophotometric absorbance at Δ optic density of 455 nm.

(4) FF behaves as a non-Newtonian fluid—its viscosity changes at different shear rates. Neither FF viscosity nor its refractive index was found to correlate with the presence of oocytes, their maturation grade, or their fertilizing capacity.

(5) FF osmolarity is similar to that of the plasma. There is no information linking variations in FF osmolarity to IVF outcome.

(6) FF pH is acidic, probably due to acid mucopolysaccharides. It appears that the intact follicle is capable of buffering any carbon dioxide which diffuses through its wall at the time of intraperitoneal insufflation. The transvaginal aspiration technique eliminates any possible effect of exogenous gas on FF pH.

(7) Regarding the intact follicle, it was shown that (a) there is a small potential difference across the follicle wall, and (b) intrafollicular pressure remains steady prior to ovulation.

This information may shed some light on mechanisms underlying FF formation and ovulation. No experiments relating these properties to IVF outcome have been performed.

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