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GAMETE BIOLOGY

Considerations of viscosity in the preliminaries to mammalian fertilisation

Ronald H. F. Hunter · P. Coy · J. Gadea · D. Rath

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Abstract Migration of spermatozoa in the female genital tract will be strongly influenced by the viscosity of the fluids encountered, yet little systematic analysis has been given to such a consideration. This essay reviews the series of milieux confronting a fertilising sperm during its progression to the oviduct ampulla. Two groups are discussed, first those in which ejaculation is into the vagina, second those in which semen enters the uterus during a protracted mating. Viscous glycoprotein secretions that accumulate in the oviduct isthmus of both groups before ovulation are highlighted, as is the environment generated in the ampulla by the post-ovulatory suspension of oocyte(s), cumulus cells and spermatozoa; follicular and peritoneal fluids may also be present. The viscosity of all female tract fluids responds to cyclical variations in temperature, and these exist within the oviduct near the time of ovulation. Gradations in viscosity influence the pattern and strength of sperm flagellar activity and the rate of forward movement. Measurements of sperm motility are currently made in a physiological medium of constant viscosity and temperature, thereby overlooking changes in the female genital tract. A more sophisticated approach might reveal an adequate fertilising potential in a proportion of putatively poor semen samples.

Keywords Semen · Sperm motility · Cervix · Uterus · Oviduct · Ovarian steroid hormones · Temperature gradients

Capsule Little systematic analysis has been given to the viscosity of female tract fluids, despite their influence on sperm movements and relevance to assessment of sperm fertilising potential.

R. H. F. Hunter
Institute of Reproductive Medicine,
Hannover Veterinary University,
Bünteweg 15,
30559 Hannover, Germany

P. Coy · J. Gadea
Department of Physiology, Faculty of Veterinary Medicine,
University of Murcia,
Murcia, Spain

D. Rath
Institute for Animal Breeding,
Mariensee, Germany

R. H. F. Hunter (✉)
Ladfield,
Oxnam,
Jedburgh TD8 6RJ, Scotland, UK
e-mail: frances521@btinternet.com

Introduction

Modern estimates of sperm motility in mammals, made for the purposes of quality control or fertility prediction, use optical or laser-tracking technology to analyse cell movement within a semen sample diluted in a physiological medium [1–3]. Although such an in vitro approach is valuable in both a human and veterinary context, it fails to take account of the varied conditions encountered by a sperm cell during passage along the female tract. The changing fluid environments between the vagina or cervix and the ampullary portion of the oviduct will have an important influence on sperm motility and on ad-ovarian progression, and the successive regions of the tract are worth systematic consideration. Furthermore, conditions within the female genital tract are themselves changing with time as programmed by the secretion of ovarian steroid hormones, and due also to an influx of fluids from the gonads and peritoneum close to the time of ovulation.

The complex series of fluids within the female genital tract and their changing viscosities will influence diverse

components of the movement of a sperm cell. In particular, the viscosity or viscoelasticity of the suspending fluid will act to influence lateral movement of the sperm head and the range and pattern of movement of the sperm flagellum. There will be related influences on sperm metabolism and there may be subtle effects on the sperm surface membranes. Mechanical inhibition and biochemical stimulation may both be involved. Somewhat surprisingly, therefore, viscosity appears to have received little consideration in the preceding context. In fact, the word viscosity does not feature in the index of most major reference works on mammalian reproduction [*eg*. 4–7], nor in many of the specialist books dealing with gametes and fertilisation. There are various research papers that touch on the problem [eg. 8–10], but not with reference to conditions in the living animal.

The present essay will first consider the different regional environments encountered by a spermatozoon along the length of the female tract, and will then refer to more specific microenvironments. Influences of the stage of the oestrous or menstrual cycle will be noted in these paragraphs and brief reference will also be made to temperature gradients which may change in relation to the time of ovulation [11]. The underlying focus on viscosity could suggest modifications to procedures of sperm evaluation and/or to the composition of media employed for in vitro fertilisation.

Site of semen deposition

Environments within the female tract encountered by a sperm suspension will depend on the site of semen deposition at ejaculation. It needs to be born in mind, too, that the viscosity of female genital fluids may be modified by contact with an ejaculate, and that this influence will change with time after mating.

In a first group of animals such as rabbits, ruminants and primates, semen is deposited in the vagina during coitus. The volume of male fluids is relatively small and the sperm suspension is highly concentrated. The interface between male and female fluids is initially between male secretions and vaginal fluid or, more typically, between semen and cervical mucus. This mucus exudes from the external cervical os into the anterior vagina. The physical nature of the vaginal pool of semen may change markedly due to coagulation—decoagulation reactions, as in primates [12] or formation of a copulatory plug, as in rodents [13]. Seminal plasma may also influence the condition of the semen-mucus interface [14].

Highly motile spermatozoa penetrate rapidly into the cervical mucus and begin to colonise the cervical crypts. However, events within the cervical tissues depend on the properties of so-called cervical mucus which change according to the stage of the menstrual or oestrous cycle. Such mucus is not a true fluid or homogeneous substance and its viscosity and viscoelasticity cannot be measured with any degree of accuracy. Anomalous viscous behaviour is the general term applied to secretions such as cervical mucus [15], whose composition is strongly influenced by the ovarian endocrine status. The mucus may develop a more watery central channel if retrograde flow of uterine fluid occurs [16, 17].

Although most spermatozoa entering the cervical canal will colonise the crypts due to orientation by flow lines or micelles in the mucus [18], a vanguard of spermatozoa may pass rapidly through the less viscous central channel into the uterus [16]. An alternative model proposes a thinner mucus close to the epithelial surface, and this may favour sperm progression towards the uterus [19]. Confronting different fluid interfaces within the cervical region will assist in liberating spermatozoa from the bulk of seminal plasma and will also influence the rate of sperm passage. Diverse forms of screening and/or selection may be imposed on sperm populations within the cervical mucus [20, 21].

In a second group of animals represented by pigs, horses, dogs and various rodents, semen accumulates in the uterus during a relatively prolonged mating. This is not to suggest that the glans penis penetrates as far as the inner cervical os. Indeed, only in pigs does the glans penis fully engage with the muscular folds of the cervix. In this second group, the volume of semen is relatively large whereas the sperm concentration is much lower than in rabbits, ruminants and primates. The ejaculate mixes with uterine fluids, a process facilitated by myometrial contractions which themselves are enhanced in response to seminal plasma constituents; a local uterine secretion of prostaglandins has been noted [22, 23].

Despite the influence of contractile activity, there are regional fluid environments along the length of the uterus and close to the endometrial surface. The latter surface is not smooth, as usually depicted in line drawings, but is strongly corrugated with ridges, folds and grooves [24–26]. Fluids close to the endometrial surface may differ markedly in composition and viscosity from the bulk of fluid in the lumen of the uterus. Apart from these considerations, assessments of viscosity will be made more complex by *(1)* infiltration of polymorphonuclear leukocytes and T-cells from the mucosa to form a dense suspension, this occurring in response to PSP-I/PSP-II in seminal plasma [27] and *(2)* changing transudation activity in the wall of the uterus; the latter is regulated by ovarian steroid secretion. In addition, spermatozoa and seminal plasma can act to stimulate modified secretions from the endometrium and a modified pattern of transudation from the uterine vascular bed. The

changing ratios of seminal plasma volume to that of uterine secretions will be critical.

The influence of a gelatinous portion of the ejaculate also needs to be considered. In pigs, for example, the globules of gelatinous secretion from the bulbo-urethral glands [28, 29] can enter the uterus and change the physical condition of the uterine fluids: droplets of gel will absorb water, making the fluid remaining in the lumen more resistant to passage of spermatozoa. Boars also emit different concentrations of spermatozoa during the prolonged ejaculation, referred to as the pre-sperm, sperm-rich and post-sperm fractions. This sequence enables a high concentration of spermatozoa to bathe the utero-tubal junction [30].

In rabbits, ruminants and primates, spermatozoa will be exposed to fluids in the uterine lumen that differ significantly in composition and consistency from cervical mucus. It would be valuable to try to model what impact such a change in environment might have on flagellar movement, lateral head movement and progressive sperm motility, and to examine its influence on the sperm surface coating. For example, does the change in milieu prompt an increased leaching of seminal plasma molecules from the sperm surface?

In both groups of animals referred to above, a suspension of spermatozoa gains the utero-tubal junction, although usually at different times after completion of mating and at different concentrations. The concentration of spermatozoa bathing the uterine side of the utero-tubal junction will invariably be higher in species in which whole semen enters the uterus at mating, especially if there should be preferential transport of a sperm-rich portion of the ejaculate to this junction, as indicated above for pigs.

The utero-tubal junction

After passage through uterine cornua of varying length, the next prominent region of challenge to progression of spermatozoa is the utero-tubal junction. Ad-ovarian waves of myometrial contraction will have displaced suspensions of spermatozoa and of polymorphonuclear leukocytes to this region. Due to their continuing infiltration, the suspension of polymorphonuclear leukocytes will have increased in density with time elapsing from mating. Together, these twin suspensions will alter the physical nature of the luminal fluids. In addition, viscosity will be influenced by glandular secretions in this junctional region of the tract and also by a changing transudation activity associated with distended vascular and lymphatic beds [see Figs. in 31, 32]. Not to be overlooked, there can be seepage or reflux of accumulated glycoprotein secretions from the caudal portion of the oviduct into the tip of the uterine horn [33, 34]. Overall, the junctional region between the uterus and oviduct should be regarded as an important fluid interface between two specialised portions of the Müllerian ducts.

Because of its prominent and distended lymphatics close to the time of ovulation, in addition to various histological specialisations, the utero-tubal junction and its secretions may present a selective challenge to spermatozoa attempting to enter the oviduct [35]. Although as yet there is no rigorous experimental evidence, the utero-tubal junction may perform some degree of immunological monitoring of sperm surface antigens as a sequel to interactions with leukocytes in the uterine lumen [36]. Such a system of dual surveillance could have a major influence on the population of spermatozoa that becomes established in the oviduct.

Oviduct fluids

Within the oviduct, a distinction can be drawn between the fluid milieu of the isthmus and that of the ampulla. Viscous glycoprotein secretions accumulate as a mucus-like substance in the restricted lumen of the isthmus [37] and, at the pre-ovulatory stage, may contribute to the suppression of sperm motility and formation of a sperm reservoir in the caudal portion of the isthmus [35]. Parallels have been suggested between this viscous secretion in the female duct and a viscous secretion in the cauda epididymidis [35, 38]. A critical function of the viscous secretions would be to isolate spermatozoa stored with suppressed motility before ovulation from the metabolic stimulation caused by contact with uterine or ampullary fluids [33, 35].

Samples of mucus have been obtained at surgery by passing flame-polished glass micropipettes through the utero-tubal junction and into the isthmus lumen of oestrous pigs. The aspirated material is creamish-white in colour and extremely viscous before ovulation. In fact, such mucus is invariably pulled back into the isthmus during sampling due to its tenacious viscoelastic properties [34, 35]. Primarily because of an influence of ovarian steroid hormones, the mucous secretions become progressively less viscous after ovulation, in step with a reduction in the tonicity of the myosalpinx. This post-ovulatory reduction in viscosity represents in part an influence of enhanced transudation into the oviduct lumen. Together with an increased patency of the duct, this would facilitate an accentuated flagellar beat and onwards progression of spermatozoa towards the ampulla.

Fluids at the ampullary-isthmic junction are less viscous than those in the caudal isthmus and can be freely sampled with fine glass pipettes. However, after ovulation, the fluid milieu in the ampulla becomes especially complex due to diverse contributions from the ovulating follicle(s) and

potentially also from peritoneal fluids. The follicular contribution will be principally antral fluid plus the depolymerising and expanding cumulus matrix, that is the viscoelastic products generated as a form of mucification. There will also be novel secretions from this cell mass and from the liberated follicular cells. A majority of these cells does not die promptly but remains viable in suspension in the vicinity of the egg(s) after liberation from the surface of the zona pellucida. There they act as a potent paracrine tissue [39–42]. They will continue to secrete a wide spectrum of molecules, although not necessarily those secreted by granulosa cells within a Graafian follicle. Thus, there will be a microenvironment in and around this cell suspension which changes in viscosity and molecular composition with time after ovulation. Georgiou et al. [43] demonstrated that both male and female gametes can activate a cell type-specific signal transduction pathway within the oviduct, and viscosity of luminal fluids could be modified by such changes. In addition, developing zygotes may themselves stimulate the endosalpinx to alter both its transudation activity and the nature of its secretions [42] as, for example, is the case for tissue-plasminogen activator [44].

Fluid in the ampullary portion of the oviduct should be viewed as a dynamic complex, and notably so around the time of ovulation. Overall, there are regional fluid environments along the length of the oviduct which exist despite the influence of contractile activity and cilial beat. The complex microarchitecture of the epithelial surface is thought to stabilise differing fluid compositions [33, 45].

The zona pellucida itself has not yet been specifically discussed, but its viscoelastic nature should be taken into account in any logical extension of the above paragraphs. Indeed, the changing nature of the zona surface places stringent requirements on sperm flagellar forces for penetration of the entire substance of this prominent egg coat. Its viscoelastic properties are modified according to the time elapsing from ovulation. This has been reported for cow eggs [46], although these authors assumed an increased resistance of the zona pellucida to proteolytic digestion as a consequence of fertilisation. However, this does not happen in cow eggs. On the contrary, the resistance of the zona increases due to addition of oviduct fluid molecules [47].

Perivitelline fluid

Although seldom commented on, the final fluid encountered by a spermatozoon before its fusion with the egg plasma membrane is that in the perivitelline ‘space’. The volume of this fluid increases after completion of the first meiotic division and extrusion of the first polar body. There is a further contraction of the vitellus upon sperm fusion and activation of the secondary oocyte. Now that experimental studies are entering the era of nanotechnology, attention should be given to the perivitelline fluid, for it is the true microenvironment of fertilisation and the early cleavage stages of the embryo. If sufficiently sensitive measurements could be made, then the viscosity of perivitelline fluid may be noted to change due to secretory activity of the oocyte and of any subsequent zygote. This is not to suggest that constituents of oviduct fluid do not enter the perivitelline space, but their influence on the composition of the perivitelline fluid might be less than that of vitelline secretions.

Influence of temperature

Not mentioned so far is the fact that there may be variations in temperature within the female genital tract. In particular, there may be temperature gradients within the oviduct of oestrous animals [11, 48–50]. Values in the literature suggest that these can be of the order of 1°–2°C or more between the caudal portion of the isthmus and the cranial portion of the ampulla in the hours before ovulation. This was proposed to contribute towards reduced sperm motility and a sperm storage function of the caudal isthmus [49]. The magnitude of the temperature gradient may change according to the stage of the cycle and especially according to the time of ovulation [49]. Therefore, a possible influence of temperature on the viscosity and viscoelasticity of female tract fluids and on the zona pellucida needs to be considered, and this might be most significant at the time when viable spermatozoa would be expected in the oviduct. A peri- and post-ovulatory increase in oviduct temperature would influence sperm metabolism and motility.

Specific observations related to viscosity

The condition of hyper-viscoelasticity in human semen is associated with a reduced percentage of motile spermatozoa [51, 52]. Moreover, there is a change in motility characteristics, as revealed in the motion parameters of curvilinear velocity, average path length and amplitude of lateral head displacement [53]. Hyper-viscosity of human semen is associated with male infertility [54] and with a poor outcome of assisted reproductive techniques [52].

The flagellar movements of human spermatozoa migrating in solutions of low or high viscosity have been characterised [55]. The rolling rate, planarity, torsion, waveform, trajectory and progression per flagellar beat are significantly altered by viscosity, but not the progressive velocity. This study therefore strongly suggests that exposure to viscosities within the physiological range for

a given species will become essential in future research on sperm motility.

Moving from observations in primates to South American Camelids, the seminal plasma of llamas has a high structural viscosity and permits sperm oscillatory movements but not progressive motility [56]. Enzymatic treatment of the seminal plasma facilitates progressive sperm motility. Parallel findings are typical across the family Camelidae, the so-called sperm plug dissolving with time after ovulation.

Despite the value of these observations, a strong limitation is imposed by the microscopic systems available for evaluation of sperm movements. The use of a low volume sperm sample could influence sperm motility by either restricting displacement in the z-axis or interaction with the sample chamber walls [1]. Use of higher volumes makes the analysis of all cells difficult as they will be moving in different focal planes. To overcome these limitations, new microscopes are currently being developed which should permit super-resolution imaging of three-dimensional samples by numerical refocusing using digital holographic microscopy [57]. Even so, such sophisticated approaches may have little overall value for prediction of eventual fertility.

Overall perspective and conclusions

A primary objective of this essay is to emphasize that after mating of mammals, those spermatozoa that gain the site of fertilisation at the ampullary-isthmic junction of the oviduct have been exposed to a series of regional and microenvironments within the lumen of the female genital tract. Differences in the regional environments will include differences in viscosity and viscoelasticity of the luminal fluids and within the oocyte investments. Gradations in viscosity could have a profound influence on the pattern and strength of flagellar activity and on the rate of forward movement of an individual sperm cell, as suggested by [58]. Evidence from in vitro observations indicates that prior interactions with viscous fluids may enhance the ability of boar spermatozoa to progress and to penetrate the zona pellucida of oocytes in vitro [59]. Suarez & Dai [60] noted that the mouse sperm flagellum may generate a stronger propulsive movement when confronted by increasing viscosity and viscoelasticity in the suspending medium. It certainly seems possible that spermatozoa escaping from the constraints of a viscous or viscoelastic milieu may rebound into hyperactive motility.

In conventional laboratory practice, computer-assisted measurements of progressive sperm motility, lateral head displacement and other descriptive features are made in a standard physiological medium of constant viscosity; they thus overlook the complex nature of the luminal fluids in the female genital tract. The critical question must therefore be asked as to whether motility measurements made in a uniform fluid and at a single temperature in vitro provide a sufficient estimate of a sperm cell's swimming potential during progression within the female tract towards the oocyte(s). More specifically, might a proportion of sperm samples with ultimately a satisfactory fertilising ability be rejected as a consequence of the current oversimplified approach? As a corollary, is it possible that for some males, sequential exposure of their sperm suspensions to differing viscosities and temperatures within the female tract might have a beneficial influence on specific motility characteristics of putatively poor samples as determined in vitro?

Appropriate background data derived from observations on sperm swimming characteristics in a series of culture media of differing viscosity and temperature should enable a more sophisticated computer analysis programme to be developed. The consequences of such an approach that focuses on changes in viscosity might prove invaluable, not least in human fertility clinics. It could also be of value in an agricultural context, especially for supposedly poorly-fertile males with desirable production traits (*eg.* growth rate, food conversion efficiency), as discussed in another context [61].

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