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Letter to the Editor

Analysing mammalian fertilisation: reservations and potential pitfalls with an *in vitro* approach

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Introduction

There can be little doubt that one of the major technical advances in the discipline of mammalian reproductive physiology in recent years has been the development and refinement of procedures of cell maintenance and culture outside the body. Such *in vitro* systems have been applied, for example, to questions of (1) ovarian follicular physiology, especially the respective hormonal contributions and interactions of granulosa and theca interna cells, (2) oviduct and uterine activity, especially the novel secretions of endosalpinx and endometrium at different stages of the reproductive cycle, and (3) maturation of gametes, especially endocrine support of the resumption of meiosis in artificially liberated oocytes and the requirements for final maturation (capacitation) of epididymal or ejaculated spermatozoa, either fresh or cryopreserved. Establishment of *in vitro* systems has been assisted and encouraged by the ready availability of chemically-defined culture media, disposable plastic dishes and tubes, and computer-controlled incubators set to predetermined gas phase, humidity and temperature. Laminair flow-hoods for tissue preparation and culture laboratories with filtered air and ultraviolet irradiation have together reduced problems of contamination and infection. Overall, the technical standards are impressive and inspection of modern tissue culture facilities invariably generates confidence in the quality of studies being pursued. In a phrase, such aesthetically pleasing and excellent laboratory facilities must surely be producing excellent science.

There is an equally persuasive aspect of *in vitro* technology linked to modern human medicine and hospital practice. Infertility as defined by a long-term inability to conceive influences at least 10% of couples in a stable relationship. One of the most successful approaches to overcoming problems of human infertility has involved laparoscopic aspiration of pre-ovulatory oocytes followed by *in vitro* fertilisation, culture of embryos through the early cleavage stages, and then simple non-surgical transplantation into the uterus via the cervix. Clinics undertaking these procedures have been established widely throughout the Western world, and many of them have at least 10 years of experience and yet the global success of this technology in generating full-term pregnancies is less than 20%, more commonly closer to 15%.

Although the technology is again impressive and there remains intense interest in procedures of *in vitro* fertilisation in human medicine, one judgement would be that this very approach has narrowed our view of physiological events occurring within the maturing Graafian follicle and Fallopian tubes. In fact, reference to physiological events brings this essay to its principal point, one best expressed as a question. Whilst appreciating the many advantages of *in vitro* procedures, should there not be greater caution in extrapolating from *in vitro* observations to *in vivo* dogma, not least now that reproductive science is moving into the sphere of genomics and proteomics? Conditions applied fruitfully *in vitro* may not be closely representative of those found *in vivo* and levels of success *in vitro* seldom match those found in *vivo* – assuming that stepwise selection of figures is not imposed on the calculation by elimination of unwanted samples. Levels of *in vitro* success may be relatively high when judged on limited numbers of observations in the short term but rarely compare with *in vivo* figures derived from stable breeding populations.

Quite apart from the context of human reproductive medicine, pressures to apply *in vitro* approaches to fundamental problems have never been stronger. First, there is the powerful attraction of convenience since tis-

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sue specimens obtained at autopsy are relatively easy and inexpensive to come by, even with the current increase in veterinary surveillance at abattoirs. Second, in the light of a greater emphasis on ethical considerations in animal experimentation and in the light of protests from anti-vivisectionists, an *in vitro* approach to scientific questions has become ever more prominent. Nonetheless, veterinary schools remain a notable exception in this regard, for there are clearly legitimate circumstances linked to carefully regulated procedures for conducting scientific experiments on domestic animals, not least those concerned with alleviating pain. However if, for the purposes of argument, it is accepted that the trend in university departments and research institutes pursuing projects in mammalian reproduction is more and more towards *in vitro* studies, what special considerations should be brought into focus? It is appropriate at this point to indicate that the pages that follow represent the current views of two reproductive physiologists working in university veterinary schools.

General reservations: pre-laboratory

A body of general reservations can be mounted with respect to an *in vitro* approach to reproductive experimentation. Some or all of these may apply in particular instances.

1. Tissues sampled at an abattoir are frequently taken from animals of unknown reproductive history and, in some cases, unknown health status. Previous episodes of infertility and/or gross pathology may have occurred. For example, there may have been a history of endometritis, unrecognised if only ovarian or oviduct tissues are sampled and yet which could influence experimental results.
2. The stage of the reproductive cycle is not usually known with precision and yet the stage of follicular maturity or oviduct tissue development in relation to ovulation may have a critical influence on the observations to be made. Prepuberal tissues may be the only ones available, frequently a limiting factor in porcine studies.
3. The procedure of slaughter itself may have strongly influenced the condition of the tissues, in fact it may have imposed major perturbations on their presumed physiological status. Slaughter by electrocution or captive-bolt shooting leads to dramatic contractile activity in the viscera, especially in the reproductive tract, and overdosing with chloroform, ether or barbiturates will likewise have wayward influences. Local products of the nervous system and muscular tissues will have modified the condition of material being sampled, and bladder contents may have refluxed deeper into the reproductive tract.
4. There is usually a period of temperature fluctuation before reproductive tissues can be sampled, at least in the large domestic species. Cattle and sheep carcases cool during the procedures of bleeding and skinning, whilst pig carcases are routinely immersed in a hot water bath before bristles can be scraped from the skin. Not only are there significant temperature shifts during these steps, but the tissues would become anoxic and degradation products would begin to exert deleterious influences.
5. Finally, removal of reproductive tissues and transport to the laboratory will involve further hazards. Procedures on the floor of a commercial abattoir can rarely be viewed as aseptic, and the risk of contamination is always present. Actual removal of tissues accentuates temperature fluctuations, and transport to the laboratory either on ice or in heated containers is not ideal. Chilling of tissues or organs prior to human transplant surgery is a well-accepted procedure, but derivation of the tissues is not in a comparable *post mortem* situation, nor does the subsequent restoration of a blood supply correspond to laboratory routines of cell or tissue culture.

Even though the list is far from exhaustive, this body of reservations taken together and applicable even before commencing *in vitro* studies should sound a note of caution.

Considerations in the laboratory

Moving on from considerations in the abattoir to those in the laboratory, initial procedures invariably involve dissection of tissues and/or scraping of cell layers with modification or loss of structural integrity. Coordinated contractile activity, for example of the myosalpinx or myometrium, will be in abeyance. Diverse forms of cell interface, junction and communication will be modified as will the basement membrane and extracellular matrix. Cell surfaces may have been further compromised by enzymatic treatment for disaggregation of tissue. There are major deprivations involving loss of a vascular supply, lymphatic drainage and nervous regulation, together with an absence of paracrine and autocrine regulation. Removal of the vascular and nervous systems may have imposed more subtle influences than generally appreciated, for there will be local deprivation of nerve growth factors, vascular endothelial growth factors and other peptide regulators. The normal regulatory influences of white cell traffic and their cytokine products will have been denied.

Tissues set up in organ culture, even in agitated systems of roller or rocker tubes, and cells set up as proliferating monolayers in Petri dishes, are almost certainly exposed to inappropriate gas tensions beneath the

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fluid surface. This point would concern not only oxygen and carbon dioxide, but also the local influence of nitric oxide in its anti-oxidant capacity. Levels of free radicals might become a serious problem. Moreover, recent surgical studies suggest that incubator temperatures commonly used for *in vitro* experiments should not correspond to accepted deep body temperature but rather may need to be specific for the stage of the reproductive cycle under consideration and the particular reproductive tissue being examined. Even such a simple point as opening and closing the incubator door may produce minor fluctuations of temperature in culture dishes or tubes which, if repeated, may have a cumulative deleterious influence.

Use of a chemically-defined medium in culture studies has generally been considered a significant step forward, conferring specific advantages of control and uniformity. Less frequently considered, however, is the fact that a chemically-defined medium rapidly becomes an undefined medium due to the accumulation of degradation products from dead and dying cells. Many of these could become harmful or even toxic in appropriate concentrations. And what happens to such toxic products? Seldom have culture systems been designed to displace them continuously from the vicinity of cell surfaces, and thus they may reach potent concentrations around groups of dead or dying cells. In fact, the composition of an initially chemically-defined medium near the cell surface may differ dramatically from that in the bulk of a medium, with no appropriate mechanisms readily available for restoration of a more suitable composition. Furthermore, normal features of cell death in the form of coordinated apoptosis could be thrown into disarray by damaging feedback influences from degradation products. Dedifferentiation of viable cells may be widespread, especially when long-term culture is performed.

In one sense, perhaps most serious of all, supplementation of a culture medium with a spectrum of hormones, for example steroids and gonadotrophins for tissues taken from Graafian follicles, can never accurately mimic the physiological programming of cells by a functional endocrine system. Not only is the latter dynamic with ever-changing hormone concentrations reaching individual cells and regulated by sensitive feedback mechanisms, but the molecular composition of the endogenous spectrum of hormones will invariably differ from that added to a culture system. Moreover, hormones exert their influences via specific receptors at the cell surface, whereas systems of culture may frequently act to modify or down-regulate the receptor molecules. Indeed, receptor molecules may be progressively leached from the plasma membrane as the system of culture degenerates with time.

Supplementation of the culture medium with antibiotic preparations may also have consequences far removed from the physiological situation.

In the light of all that has been written so far, the reader may reasonably be surprised that *in vitro* systems have been so extensively applied, have proved so valuable and, in a reproductive context, have been so fruitful. Nonetheless, it is perhaps time to take stock and consider the possibility of inappropriate conclusions being drawn from *in vitro* studies. A good example here might be to discuss systems of *in vitro* fertilisation. This choice is made in part because of the research orientation and experience of the two authors, in part because procedures of *in vitro* fertilisation – frequently preceded by *in vitro* maturation of aspirated oocytes – are widely used in both animal experimentation and, as noted, in human clinical practice.

Cautions from *in vitro* fertilisation

Limitations that must be borne in mind before drawing conclusions from *in vitro* fertilisation experiments concern the source of gametes and especially the nature of somatic cells added to any system of co-culture. Overall, *in vitro* experiments tend to divert attention away from the integrated actions of a complete genital tract in a live animal, that is to say the progressive sets of gamete and embryonic interactions with successive regional and microenvironments in the female reproductive tract.

As to the gametes, spermatozoa may be of epididymal origin, or washed-ejaculated, or frozen-thawed still with residual quantities of diluent and/or cryoprotectants. They are seldom, if ever, derived from the female tract of mated animals, and yet selection within the population of ejaculated spermatozoa is a well-established fact as spermatozoa progress towards and into the oviducts and down a gradient of steeply diminishing sperm numbers. *In vitro* selection by 'swim-up' procedures in an artificial medium is unlikely to correspond in a number of regards to events occurring *in vivo*, with coordinated modification and then removal of male tract macromolecules from the sperm surface leading to remodelling of the plasma membrane. This stepwise modification of viable spermatozoa, frequently taking hours or even more than a day in the female tract, culminates in the arrival of a discrete number of competent spermatozoa at the site of fertilisation – the ampullary-isthmic junction – close to the time of ovulation. Such spermatozoa have been prepared for penetrating the egg investments by progressive interaction with successive fluid compartments and epithelial surfaces, themselves specifically regulated by changing concentrations of ovarian steroid hormones and inherent auto- and paracrine mechanisms. Initial sperm:egg ratios at the

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site of fertilisation may be close to unity in a spontaneously mating animal.

This sequence of subtle and highly coordinated events will scarcely be mimicked in a microdrop of culture medium with a population of spermatozoa simply added to the immediate vicinity of an oocyte. For example, the use of *in vitro* fertilisation droplets containing 15–20 oocytes and huge numbers of spermatozoa ($0.5–1.0 \times 10^6$) in monovular species such as cows and humans is clearly far removed from the *in vivo* situation. Capacitation achieved under *in vitro* conditions may enable sperm penetration of the zona pellucida and fusion with the vitelline membrane but may not always promote formation of a correspondingly viable zygote.

Turning to the female gamete, procedures of *in vitro* maturation frequently employ oocytes that would not have been selected during the events of follicular maturation and then ovulated spontaneously. Indeed, a proportion of primary oocytes liberated artificially from the ovary *post mortem* or *in vivo* and taken through presumptive nuclear maturation *in vitro* may have been obtained from prepuberal animals and/or already destined for atresia. Even if not, it is difficult to imagine that systems of culture for oocytes liberated from the ovary by aspiration or dissection confer the same molecular messages during presumptive maturation *in vitro* as those operating in the intact follicle *in vivo*. The microenvironment of the oocyte within its cumulus cell investment will almost certainly be significantly different, and the specific junctional connections between the various populations of granulosa cells and between corona cells and oocyte will have been modified or even interrupted.

There is a further aspect of oocyte maturation to consider in terms of post-ovulatory interactions of the cell and somatic investment with its environment prior to sperm penetration at the site of fertilisation. A secondary oocyte is exposed to specific secretions in the form of unique oviduct glycoproteins as it progresses from the fimbriated infundibulum to the ampullary–isthmic junction. Even though this passage is relatively rapid, commonly requiring some 10–30 minutes across species, there is ample time for macromolecules to modify the oocyte surface and influence cytoplasmic and nuclear properties. Such putative modifications may find expression not during the process of fertilisation itself but rather during subsequent development – or failure of development – of an embryo.

Co-culture with oviduct epithelial cells may attempt to mimic such physiological interactions, but at the best can only be a crude copy. Preparations of endosalpinx will suffer from many of the limitations discussed earlier in this essay. As with oocytes themselves, they may have been taken from immature (prepuberal) animals devoid of suitable hormone priming and, if not, they will seldom be at the requisite post-ovulatory stage. Not only will they not have been programmed by the correct backcloth of systemic ovarian hormones, but they will not have received the further local programming influence of (1) the oocyte and its secretions, (2) the cumulus cell investment synthesising steroid hormones and peptides, and (3) the follicular antral fluid containing high titres of prostaglandins. Whilst reservations here primarily concern epithelial cells prepared from the oviduct ampulla, corresponding reservations would apply to epithelial cells from the isthmus. Under physiological conditions of pre-ovulatory mating, a population of viable spermatozoa bathed in intra-luminal fluid binds to endosalpingeal organelles in the caudal isthmus and reprogrammes secretions from that portion of the duct. This may not be matched *in vitro*. Worse still, cultured oviduct epithelial cells do de-differentiate and may then re-differentiate with a modified spectrum of surface characteristics and a modified form of secretion.

Taking all the above points into consideration, caution must clearly be applied to interpretation and extrapolation from co-culture, for one cannot be certain what is representative of the physiological situation. This will be discussed in a final section of this essay by reference to successful *in vitro* fertilisation, embryo culture and transplantation culminating, however, in a clinical problem of current interest and considerable practical significance.

Modulation of gene expression

Modulation of gene expression in reproductive tissues requires specific physiological and biochemical conditions brought about under a closely regulated and changing pattern of endocrine control. Gonadal steroid hormones themselves can act to influence gene expression. The endocrine backcloth found *in vivo* is not mimicked or even approached closely *in vitro*. Accordingly, the sequence and pattern of gene expression in both gametes and embryo and the extent of gene products would rarely if ever match precisely those found under *in vivo* conditions. Hence, it is not surprising that the viability of embryos generated *in vitro* does not correspond with results obtained from those generated in the live animal. *In vitro* fertilisation itself may not be seriously compromised nor indeed the relatively straightforward development of an embryo up to the stage of early blastocyst. However, the subsequent differentiation clearly requires precise chronological and quantitative expression of an unfolding spectrum of genes. When viewed at this level, *in vitro* procedures leading to only minor perturbations from physiological conditions could have serious influences on a coordinated expression of the gene programme

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resulting in modifications to the protein chemistry and specific protein folding that regulate development.

A prominent example of current concern is seen in the Large Offspring Syndrome, to date reported extensively in calves and lambs. As suggested, ruminant fetuses are conspicuously overweight at term and suffer from a variety of developmental problems resulting in a high incidence of peri- and post-natal mortality. Such animals have been derived from procedures of *in vitro* maturation of oocytes, *in vitro* fertilisation and embryo culture, followed by surgical or non-surgical transfer to the uterus of suitable recipients. However, neither the gametes nor the embryos have been exposed to the environment of the oviduct and have therefore been denied the influence of specific constituents of oviduct luminal fluid, not least the modulating influence of unique glycoprotein secretions. One hypothesis is that such macromolecules have a vital input in coordinating the sequence and extent of expression of the embryonic genome as it unfolds in the earliest stages of development. This critical modulation as an embryo progresses along an oviduct would clearly not be found *in vitro* – compromising the viability of embryos or fetuses so generated.

The above reservations would apply not only to standard procedures of *in vitro* maturation, *in vitro* fertilisation and embryo culture, but also in a context of transgenic manipulation by injection of gene constructs into pronucleate eggs and in procedures of cloning.

Concluding thoughts

When taken together, the body of reservations presented in this essay should sound a note of caution. The use of simple *in vitro* systems to analyse components of the complex process of fertilisation suffers from serious limitations, despite the fact that *in vitro* models have proved valuable for molecular studies and yielded much new information. Straightforward extrapolation from *in vitro* experiments to the integrated physiology of a whole animal can seldom be warranted. A meaningful interpretation of *in vitro* studies must depend on appropriate corroboration *in vivo*. This is especially so now that functions are being assigned to individual genes after manipulation of gametes and embryos under diverse experimental circumstances. The relevance of the procedures to physiological events should always be questioned before generating dogma.

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