

In-vitro fertilization of ruminants

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Introduction

The first ruminant offspring from in-vitro fertilization was a bull calf born in 1981 (Brackett *et al.*, 1982). Since then, fertilization has been accomplished *in vitro* for the three domestic ruminants—cattle, sheep and goats. These developments are largely the result of recent advances in our understanding of oocyte maturation, sperm capacitation and embryonic development. It is the intent of this manuscript to address four questions concerning in-vitro fertilization with primary emphasis on cattle. The questions to be addressed are: (1) what are the uses or reasons for performing IVF, (2) can it be done, (3) what are the relative efficiencies of each step and which steps need further development and (4) what are the physiological mechanisms through which the normal in-vivo or in-vitro fertilization process is accomplished.

Potential use for in-vitro fertilization

The strongest motivation for development of systems for fertilization and development of embryos *in vitro* comes from the realization that development of the biotechnologies for producing transgenic offspring or for multiplication *in vitro* of lines of superior offspring are dependent on volume production of precisely staged embryos and on the ability to develop these unique embryos to a stage compatible with transfer to recipient mothers. In commercial use it is not likely that cows of high genetic value will be subjected to slaughter or surgery in order to obtain pronuclear stage eggs for gene microinjection or tubal stage embryos for use in embryo multiplication. However, a procedure based on in-vitro fertilization of laparoscopic recovered follicular oocytes would be a useful and much needed alternative. Similarly, commercial multiplication of identical embryos is expected to be dependent on the use of abattoir-obtained oocytes as recipients for transfer of nuclei from valuable multicellular stage embryos and on the ability to develop the resulting embryos to a transferable stage. Important to the study of reproductive physiology is that fertilization *in vitro* of oocytes recovered in large numbers from abattoir ovaries could provide abundant numbers of embryos for research. The ability to mature and fertilize oocytes *in vitro* would also provide a means for production of a few calves from a dead or dying cow of great genetic value. Lastly, there is considerable hope that in-vitro fertilization tests will be useful for predicting fertility *in vivo*.

In-vitro fertilization, can it be done?

The production of calves from fertilization *in vitro* of oocytes matured *in vivo* (Brackett *et al.*, 1982, 1984; Sirard & Lambert, 1985, Sirard *et al.*, 1985) or *in vitro* (Critser *et al.*, 1986) and lambs from oocytes matured *in vitro* (Cheng *et al.*, 1986) attest to the fact that in-vitro fertilization can be

successfully accomplished. The efficiency of producing offspring is less than achieved by natural processes (Tables 1 and 2). The overall efficiency is reduced by the sequential multiplicative effects of a series of seemingly efficient steps. This results in slightly more than 10% of recovered oocytes producing offspring (Table 3). The most limiting step at present appears to be the inefficiency of development of embryos *in vitro* from the pronuclear to the blastocyst stage (Table 3). For immature cow oocytes recovered from small follicles of 1–5 mm in diameter, an additional limitation is the failure to acquire competence for development from the pronuclear to blastocyst stage (Leibfried-Rutledge *et al.*, 1986a; Table 4). This deficiency by immature cow oocytes also occurs for immature oocytes of sheep after fertilization *in vivo* (Staigmiller & Moor, 1984). Embryo developmental competence can be acquired by immature oocytes through coculture with abundant numbers of preovulatory granulosa or cumulus cells which are receiving stimulation from FSH, LH and oestradiol (Staigmiller & Moor, 1984; Critser *et al.*, 1986). This developmental problem is but one manifestation of a need to understand the steps and mechanisms regulating oocyte maturation.

Table 1. Efficiency of in-vitro fertilization of in-vivo matured cow oocytes

	Brackett <i>et al.</i> (1982, 1984)	Lambert <i>et al.</i> (1986)	Leibfried- Rutledge <i>et al.</i> (1986a)*
Sperm capacitation method	High ionic strength medium	High ionic strength medium	Heparin
Oocyte maturation		67%*	43%* 78%†
Fertilization‡			
Follicular	14%	41%	57%
Oviducal	12%		32%
Embryo development§		15–20%	45%
Pregnancies	5/19 (21%)	6/13 (46%)	2/10 (20%)
Calves	3/19 (16%)	6/13 (46%)	0/10 (0%)

*Oocytes recovered from follicles with expanded cumulus.

†Oocytes recovered from oviducts with an evenly granulated cytoplasm and no evidence of fragmentation or degeneration.

‡Oocytes were recovered from follicles or oviducts. Oocytes were fertilized when two pronuclei, not polyspermic, or cleavage to the 2-cell stage occurred.

§Only oocytes obtained from follicles.

Table 2. Efficiency of in-vitro fertilization of in-vitro matured bovine oocytes

	% oocytes matured*	Type of spermatozoa	% Fertilized†
Ball <i>et al.</i> (1983)	> 70	Epididymal	70
Iritani <i>et al.</i> (1984)	80	Ejaculated	44, 63
Hensleigh & Hunter (1985)	56	Ejaculated	15
Parrish <i>et al.</i> (1985a)	> 70	Ejaculated	72
Fukui <i>et al.</i> (1983)	64	Ejaculated	28
Parrish <i>et al.</i> (1986b)	> 70	Ejaculated, frozen–thawed	81
Leibfried-Rutledge <i>et al.</i> (1986a)	70‡ 73	Ejaculated, frozen–thawed	94‡ 91

*Oocytes with expanded cumulus and/or at metaphase II of meiosis.

†No. of ova penetrated or cleaved to 2 cell/total no. oocytes.

‡During maturation, oocytes cocultured with granulosa cells.

Table 3. Efficiency of each step involved in successful in-vitro fertilization of cow oocytes

	Efficiency (%)	% of original 100 oocytes
Oocyte maturation	80	80
Sperm penetration	80	64
2 Pronuclei	80	51
Sheep oviduct—recovery	80	41
1 Cell—blastocyst	55	23
Transfer pregnancy	60	14
Calves	80	11

Table 4. Development in sheep oviducts of in-vitro fertilized cow oocytes matured *in vitro* or *in vivo** (from Leibfried-Rutledge *et al.*, 1986a)

Maturation of oocytes	Fertilization			Development*	
	No. of ova penetrated/total no. of ova (%)	No. of ova with 2 pronuclei/no. of penetrated ova (%)	No. of normally fertilized ova*/total no. of ova (%)	No. of cleaved embryos/no. recovered (%)	No. of morulae or blastocysts/no. recovered (%)
<i>In vitro</i>	158/207 (76)	115/158 (73)	81/207 (39)	15/33 (45)	0/33 (0)
<i>In vivo</i>					
Follicular	22/ 24 (96)	18/ 22 (82)	19/ 24 (80)	66/80 (82)	36/80 (45)
Oviducal	24/ 28 (86)	20/ 24 (83)	15/ 24 (53)	38/56 (68)	14/56 (25)

*No. of ova with only two pronuclei, not polyspermic.

Mechanisms involved in fertilization

Fertilization *in vitro* requires the following: (1) a system for harvesting the oocyte which is efficient and does not damage the oocyte or donor, (2) nuclear, cytoplasmic and cumulus cell maturation of the oocyte/cumulus complex, (3) non-damaging culture systems, (4) a system for capacitating the spermatozoa and (5) efficient systems and conditions for accomplishing fertilization and development of the embryo from fertilization to a stage transferable to recipients. Accomplishment of each step is greatly aided by understanding the natural process as well as the mechanisms involved.

Harvesting of the oocyte

In-vivo matured oocytes are obtained from ovaries of superovulated cows by recovery from preovulatory follicles near the moment of ovulation or from the oviduct soon after ovulation. For research purposes, the ovaries or oviducts have usually been retrieved after slaughter of the donor. The cumulus-enclosed oocytes are then aspirated from the preovulatory follicles or flushed from the oviduct (Brackett *et al.*, 1982). Oocytes from both sources appear to have completed maturation and after fertilization, normal embryo development occurs (Brackett *et al.*, 1982; Leibfried-Rutledge *et al.*, 1986a; Table 4). However, the tubal oocytes must be recovered and placed in culture soon after ovulation. Bovine oocytes allowed to remain in excised oviducts for as little as 0.5–2 h will undergo a high frequency of parthenogenetic activation (L. Leibfried, unpublished). A more acceptable procedure for repeated oocyte recovery from cows of continued reproductive

value is with the aid of a laparoscope. This procedure has been used most successfully by Sirard & Lambert (1985), Sirard *et al.* (1985) and Lambert *et al.* (1986). The efficiency of oocyte recovery by the laparoscopic method (Lambert *et al.*, 1986) appears to be equivalent to recovery from the excised ovary. Before cumulus expansion, oocytes remain firmly attached in small and medium-sized follicles and cannot be aspirated *in vivo* as they can from small follicles of excised ovaries (Ball *et al.*, 1983). Because a large proportion of the follicles in cows are in various stages of atresia (Choudary *et al.*, 1968; Leibfried & First, 1979) oocytes are commonly screened by selecting only those oocytes which have intact, expanded and complete cumulus cell enclosure and have uniformly granulated cytoplasm (Leibfried & First, 1979; Leibfried-Rutledge *et al.*, 1986a).

A major limitation to use of *in-vivo* matured oocytes is the number of oocytes obtained per female. Even when cows are stimulated with FSH, the usable number of oocytes/donor ranges from 5 to 10 (Lambert *et al.*, 1986; Leibfried-Rutledge *et al.*, 1986a). Whenever large numbers of embryos are required, *in-vivo* matured oocytes provide an expensive supply. Collection of immature oocytes from ovaries obtained at the abattoir would provide a much cheaper source.

Immature oocytes cannot be obtained by laparoscopy and must therefore come from ovaries at ovariectomy or at the abattoir. Commonly oocytes are aspirated from 1–5 mm follicles in these ovaries. Careful monitoring of temperature is important as oocytes recovered are incapable of maturing past metaphase I if cooled below 30°C (L. Leibfried-Rutledge, personal communication; A. G. Hunter, personal communication). Because of the large percentage of atretic follicles, only non-atretic follicles with intact and compact cumulus-enclosed oocytes are selected. This differs from procedures for *in-vivo* matured oocytes for which only cumulus–oocyte complexes responding to gonadotrophins *in vivo* with expanded cumulus are used. The other criteria for selection are the same as previously described (Leibfried & First, 1979; Leibfried-Rutledge *et al.*, 1986a).

Maturation of oocytes and completion of development competence

After spermatozoa are capacitated, successful fertilization *in vitro* is dependent on the correct maturity of the oocytes to be fertilized. The mechanisms regulating maturation of ruminant oocytes are discussed by Moor & Gandolfi (1987). Oocyte maturation is commonly considered to be the progression of nuclear maturation from the appearance of the germinal vesicle of the arrested dictyate stage, through meiosis I to metaphase II and extrusion of the first polar body. For successful fertilization and embryonic development, oocyte maturation must be considered in a much broader sense. Especially important are the development of proteins in the cytoplasm which regulate meiotic events, sperm decondensation, formation of the male pronuclear envelope, initiation of cleavage, completion of cleavage-stage embryo development and ability to produce a viable offspring (First & Haseltine, 1987; Moor & Gandolfi, 1987). The development of these regulatory signals can be affected by the stage of maturity of the follicle from which the oocyte is removed, by the duration of the *in-vitro* or *in-vivo* maturation period and by conditions of the *in-vitro* maturation culture.

Common defects resulting from fertilization of immature oocytes include failure of male pronuclear development (Thibault *et al.*, 1975; Thibault, 1977) and failure to progress to organized blastocyst-stage embryos (Staigmiller & Moor, 1984; Leibfried-Rutledge *et al.*, 1986a; Table 4). The latter has been corrected by co-culture *in vitro* of immature oocytes with granulosa or abundant cumulus cells under stimulation of gonadotrophins and oestrogen (sheep: Staigmiller & Moor, 1984; cow: Critser *et al.*, 1986). Some of these defects, such as failure of male pronuclear development, have been produced experimentally by shortening the maturation period such that a given regulatory protein is not yet produced. This was accomplished in the hamster to produce oocytes that were penetrated by spermatozoa but failed to form male pronuclei (Leibfried & Bavister, 1983).

An additional aspect of oocyte maturation is the full expansion of the cumulus cells surrounding the oocyte. While cumulus cells may impede the contact of spermatozoa with the egg and delay

the time of fertilization (J. J. Parrish, unpublished), there is also evidence that, if expanded, their presence may enhance sperm capacitation and fertilization rate (Ball *et al.*, 1983). Additionally, a product of cumulus cells stimulated by FSH enhances motility of bovine spermatozoa (Bradley & Garbers, 1983). Bovine cumulus cells undergo expansion in response to FSH (Ball *et al.*, 1983) either *in vitro* or after a small FSH rise accompanying the preovulatory surge of LH *in vivo*. The principal secretory product of FSH-stimulated bovine cumulus cells is hyaluronic acid which forms a mucus matrix between and around cumulus cells (Ball *et al.*, 1983). In the hamster, hyaluronic acid will induce an acrosome reaction but only in capacitated epididymal spermatozoa. While hyaluronic acid is capable of promoting the acrosome reaction of epididymal bovine spermatozoa (Handrow *et al.*, 1982) it does not appear to do so in ejaculated bovine spermatozoa (Handrow *et al.*, 1986a). The principal function of hyaluronic acid in cattle may be similar to one of its functions in the hamster, which is to provide a large mass capable of being picked up and transported by the fimbria of the oviduct (Mahi-Brown & Yanagimachi, 1983). If so the transport function of the bovine cumulus mass is of short duration because the oviduct causes its rapid removal (Lorton & First, 1979).

Bovine oocytes cultured from immature follicles (1–5 mm; Leibfried & First, 1979) or ovine oocytes from non-atretic follicles (3–6 mm; Moor & Trounson, 1977; Staigmiller & Moor, 1984) require 24–27 h in culture to develop from the germinal vesicle stage to metaphase II. This progression in nuclear development is paralleled by a progression of cumulus expansion (Leibfried & First, 1979). While fertilization is highly temperature-dependent, nuclear maturation of bovine oocytes occurs over a temperature range of 35–41°C (Lenz *et al.*, 1983a; Katska & Smorag, 1985). Cooling oocytes below 30°C, however, can damage nuclear maturation ability by an apparent disruption of the spindle apparatus (Moor & Crosby, 1985).

Several different media have been used for successfully maturing cow or sheep oocytes *in vitro*. These include: Medium 199 (Moor & Trounson, 1977; Staigmiller & Moor, 1984; Leibfried-Rutledge *et al.*, 1986a; Katska & Smorag, 1985), Hams F-12 (Fukushima & Fukui, 1985) and TALP (Ball *et al.*, 1983; Lenz *et al.*, 1983a; Critser *et al.*, 1984; Leibfried-Rutledge *et al.*, 1986b).

There is as yet no satisfactory completely defined medium for ruminant oocyte cultures. For full development and subsequent fertilization the medium must contain a blood serum or serum extract. For the cow, whole sera such as fetal calf serum are superior to albumin both in supporting completion of nuclear maturation and viability of associated cumulus cells (Leibfried-Rutledge *et al.*, 1986b).

Capacitation and the acrosome reaction

Mammalian spermatozoa are not immediately capable of fertilizing oocytes, rather they must undergo a period of preparation, which normally occurs in the female reproductive tract, termed capacitation (Austin, 1951; Chang, 1951). Capacitation consists of at least two components, an initial sperm membrane alteration (Yanagimachi, 1981; O'Rand, 1982; Ahuja, 1984; Langlais & Roberts, 1985; Wolf *et al.*, 1986) which allows the spermatozoa to undergo the second phase, the fusion of the plasma membrane and outer acrosomal membrane (Yanagimachi & Usui, 1974). The first phase is now referred to as capacitation while the second phase is referred to as the acrosome reaction. The site of sperm capacitation, agents causing capacitation *in vivo* and mechanisms by which capacitation is manifest are not well understood for most mammalian species.

The site of capacitation within the female reproductive tract is uncertain. While sperm capacitation can take place within the uterus (Austin, 1951; Bedford, 1969, 1970; Barros, 1974) capacitation is accelerated by exposure of spermatozoa to the uterus and then the oviduct (Adams & Chang, 1962; Bedford, 1969; Hunter, 1969; Hunter & Hall, 1974). Events of sperm transport further complicate determination of the site of sperm capacitation. In the sheep (Hunter *et al.*, 1980, 1982; Hunter & Nichol, 1983) and the cow (Hunter & Wilmut, 1982, 1984; Wilmut & Hunter, 1984) adequate sperm numbers for fertilization require 6–12 h before being established in the lower

isthmus of the oviduct, the sperm reservoir for these species. Spermatozoa involved in fertilization then move up to the ampullary-isthmic junction of the oviduct only at the time of ovulation. When spermatozoa are deposited in the female at the start of oestrus, they may reside in the isthmus for as long as 18–20 h in the cow (Hunter & Wilmot, 1984) and 17–18 h in the ewe (Hunter & Nichol, 1983) before fertilization. It is unknown how sperm transport and accumulation in the lower isthmus are affected by insemination later in oestrus. Similar situations occur in the rabbit (Overstreet *et al.*, 1978; Overstreet, 1982) and pig (Hunter, 1984) in which spermatozoa are held in the lower isthmus before ovulation and move up the oviduct at or near the time of ovulation. Whether spermatozoa are normally capacitated by the time they enter the oviduct is unknown. However, sperm fragility increases during capacitation (Hunter, 1980) and with the long time spermatozoa must reside in the lower isthmus before ovulation in the cow or ewe, it is most likely that capacitation is completed in the oviduct. For cattle, we have shown that spermatozoa undergo the acrosome reaction almost exclusively in the ampulla of the oviduct ipsilateral to the side of ovulation and only near and immediately after ovulation (Herz *et al.*, 1985). The results suggest that capacitation does occur in the oviduct but primarily in the oviduct ipsilateral to the side of ovulation. Additionally, the recovered contents of the oviducts of oestrous, but not luteal-phase, ewes caused bovine spermatozoa to be capacitated and undergo an acrosome reaction (Lee *et al.*, 1986). In the hamster, capacitation of spermatozoa *in vivo* is completed in the oviduct (Bedford, 1972) and the ability of the oviduct to promote capacitation is prevented by progesterone (Viriyapanich & Bedford, 1981). Oviduct fluid from the follicular stage of the oestrous cycle therefore appears capable of causing capacitation.

In vitro, glycosaminoglycans will induce an acrosome reaction or capacitate bovine epididymal spermatozoa (Lenz *et al.*, 1982, 1983b; Handrow *et al.*, 1982; Ball *et al.*, 1983; Parrish *et al.*, 1985a). Assay of the glycosaminoglycans in bovine (Lee & Ax, 1984) and ovine (Lee *et al.*, 1986) oviduct flushings revealed a high concentration of heparin-like material. *In vitro* heparin was shown to be the most potent glycosaminoglycan in its ability to induce the acrosome reaction in bovine epididymal spermatozoa (Handrow *et al.*, 1982) and to capacitate ejaculated bovine spermatozoa (Parrish *et al.*, 1985b). A characteristic of bovine sperm capacitation by heparin is that it is inhibited by 5 mM-glucose (Parrish *et al.*, 1985a; Susko-Parrish *et al.*, 1985). Capacitation of bovine spermatozoa by oviduct fluid is also inhibited by glucose (Parrish *et al.*, 1986a). In cows, glucose is present in very low levels in the oviduct, < 1 mM (Carlson *et al.*, 1970), levels which do not inhibit sperm capacitation by heparin. In an experiment to identify the capacitating agent of oviduct fluid in which the treatments were applied sequentially, the capacitating factor was found to be not sensitive to protease digestion, soluble in trichloroacetic acid, precipitated by ethanol and inactivated by nitrous acid, a reagent which degrades the heparin-like glycosaminoglycans, heparin or heparan sulphate (R. R. Handrow and J. J. Parrish, unpublished). This suggests a heparin-like glycosaminoglycan is responsible for the capacitating activity of oviduct fluid from oestrous cows but does not as yet identify it as heparin or heparan sulphate.

After capacitation, final preparation of spermatozoa for fertilization in cows and other mammals may occur at the zona pellucida as in the mouse (Florman & Storey, 1982; Bleil & Wassarman, 1983). There is support for the idea that mammalian spermatozoa may be capacitated by materials, perhaps glycosaminoglycans, in the fluids of the female reproductive tract or cumulus cells, but the fertilizing spermatozoa complete preparation for and undergo the acrosome reaction only at the zona pellucida. In cows, spermatozoa bound on the zona pellucida were all acrosome-reacted (Crozet, 1984). Furthermore, electron microscopy revealed that bovine spermatozoa recovered around and in the cumulus mass in the oviduct had acrosomes of a fluffy appearance but only spermatozoa bound to the zona pellucida were acrosome-reacted (Crozet, 1984). Collectively these findings suggest that bovine spermatozoa are capacitated in the oviduct and that fertilizing spermatozoa complete the acrosome reaction at or near the zona pellucida.

Precise molecular events in capacitation and the acrosome reaction are not well understood. It has been suggested that an alteration of membrane proteins occurs (Gordon *et al.*, 1975;

Koehler, 1978) and that subsequent sterol depletion may be a key event (Davis, 1981, 1982; Langlais & Roberts, 1985). A molecular membrane model describing changes in sperm membranes accompanying capacitation has been proposed by Langlais & Roberts (1985). By this model, capacitation is a reversible phenomenon which upon completion results in a modification of the sperm membrane surface, causing an efflux of membrane cholesterol which alters the membrane sterol/phospholipid ratio and results in an influx of Ca^{2+} . The Ca^{2+} activates phospholipase- A_2 which catalyses the synthesis and accumulation of fusogenic lysophospholipids such as lysophosphatidylcholine. In the presence of Ca^{2+} and reduced levels of cholesterol, such fusogenic compounds would induce the acrosome reaction. The critical component of this model is the sterol depletion of sperm membranes. The remaining changes are postulated to occur as a cascade from this event. The Langlais & Roberts (1985) model, however, does not address the role of a heparin-like glycosaminoglycan from oviduct fluid in capacitation of bovine spermatozoa. Under physiological conditions, a heparin-like glycosaminoglycan appears essential for efficient capacitation of ejaculated bovine spermatozoa (Parrish *et al.*, 1985a, b, 1986a, b). While the oviduct heparin-like glycosaminoglycan may be heparan sulphate or heparin, heparin can displace specifically bound heparan sulphate from cells (Kjellen *et al.*, 1980) and exert biological effects on cells similar to those of heparan sulphate (Lattera *et al.*, 1983). Based upon known effects of heparin on spermatozoa and other cell types we will propose several possible roles of an oviduct heparin-like glycosaminoglycan during capacitation.

There are two ways in which heparin-like glycosaminoglycans could modify the sperm plasma membrane resulting in the alterations suggested by Langlais & Roberts (1985) to be the first step in capacitation. The first method is by displacing a decapacitation protein (Oliphant *et al.*, 1985) from the sperm surface similar to heparin's ability to displace lipoproteinlipase from cells (Casu, 1985). The second method of altering the sperm plasma membrane could be a direct modification of membrane domains. Heparin-like glycosaminoglycans can bind to proteins, phospholipids and themselves through Ca^{2+} bridges (Srinivasan *et al.*, 1970). A network of heparin-like glycosaminoglycan molecules and membrane components could induce reorganization of the membrane by restricting or causing movement of membrane components. In support of a direct effect on membrane domain formation, heparin binding affinity to spermatozoa (Handrow *et al.*, 1984) and ability to capacitate spermatozoa is Ca^{2+} -dependent (J. J. Parrish, unpublished).

The second major event of capacitation proposed by Langlais & Roberts (1985) was an uptake of Ca^{2+} by spermatozoa. Heparin induces a linear increase in $^{45}\text{Ca}^{2+}$ uptake during capacitation of bovine spermatozoa that does not occur when spermatozoa are incubated under noncapacitating conditions (Handrow *et al.*, 1986b). It is, however, unknown whether the $^{45}\text{Ca}^{2+}$ uptake was a result of heparin acting as an ionophore, opening a Ca^{2+} channel in the membrane or the result of the membrane changes previously proposed. Heparin-like glycosaminoglycans may therefore have direct effects on Ca^{2+} uptake into spermatozoa during capacitation.

The third major step in capacitation proposed by Langlais & Roberts (1985) is the activation of phospholipase- A_2 by Ca^{2+} or acrosin. Heparin-like glycosaminoglycans may be able to activate phospholipase- A_2 through their ability to chelate Zn^{2+} (Casu, 1985), an inhibitor of phospholipase- A_2 (Thakkar *et al.*, 1984), or stimulate conversion of proacrosin to acrosin (Parrish *et al.*, 1980).

Our studies suggest an additional effect of heparin-like glycosaminoglycan on capacitation may be the activation of a cAMP-dependent protein kinase. We have shown that exogenously added 8-bromo-cAMP will reverse the inhibition by glucose on sperm capacitation with heparin (Susko-Parrish *et al.*, 1985). Potential elevation of cAMP levels in spermatozoa cannot be the only action of heparin on spermatozoa as 8-bromo-cAMP will not be itself capacitate bovine spermatozoa (Susko-Parrish *et al.*, 1985).

The complex potential effects of an oviducal heparin-like glycosaminoglycan on spermatozoa preclude assigning it a single role in capacitation. It is likely that further research will demonstrate multiple effects of heparin-like glycosaminoglycans regulating sperm capacitation and the acrosome reaction.

Methods to capacitate ruminant spermatozoa *in vitro* rely primarily on modification of techniques that have proven useful with rodent spermatozoa, since these species are the most studied in relation to sperm capacitation. In rodents, capacitation *in vitro* is easily accomplished by a variety of media manipulations. The functions of these manipulations have been to remove surface proteins that are inhibiting capacitation and/or the acrosome reaction (Wolf, 1979; Aonuma *et al.*, 1982; Fraser, 1983) and to stimulate sterol efflux from the sperm plasma membrane (Go & Wolf, 1985). Application of these techniques to ruminant spermatozoa has not always been successful. One difference is the use of ejaculated spermatozoa for in-vitro studies with ruminants and spermatozoa removed from the cauda epididymidis for rodents. It must be emphasized that ejaculated spermatozoa are responsible for in-vivo fertilization in all species. Differences in capacitating ability of ejaculated and epididymal spermatozoa do exist. Bovine epididymal spermatozoa can capacitate in simple salt solutions (Ball *et al.*, 1983; J. J. Parrish, unpublished) but ejaculated bovine spermatozoa do not capacitate well unless capacitating agents are added (Parrish *et al.*, 1985b, 1986a, b, c). Decapacitation factors (Chang, 1951) of seminal plasma are most probably responsible for these effects.

Despite the limitations of applying results from capacitation studies of rodent spermatozoa to ruminant spermatozoa, several procedures have been developed for capacitation of ruminant ejaculated spermatozoa. A medium of high ionic strength has been used to displace proteins from the surface of bovine spermatozoa which might inhibit sperm capacitation (Brackett *et al.*, 1982; Bousquet & Brackett, 1982; Lambert *et al.*, 1986). While this technique has been proved successful by the birth of calves, results have been variable among bulls (Brackett *et al.*, 1982; Sirard & Lambert, 1985). Long incubations of bovine and caprine spermatozoa, 18–24 h, which allows time for surface proteins to dissociate, have proved useful if spermatozoa remain viable for this period (Wheeler & Seidel, 1986; Song & Iritani, 1985). Sheep spermatozoa have also been successfully capacitated by incubation at elevated pH, 7.8–8.0 (Cheng *et al.*, 1986). This treatment presumably not only dissociates proteins from the sperm surface but also induces Ca^{2+} uptake in cells. In the goat, Ca^{2+} ionophore A23187 has been used to bypass capacitation and by directly increasing Ca^{2+} to induce the acrosome reaction (Shorgan, 1984). The last technique is the use of the glycosaminoglycan heparin with bovine spermatozoa (Parrish *et al.*, 1985a, 1986b). Capacitation of spermatozoa by heparin requires 4 h and can result in high rates of fertilization (Parrish *et al.*, 1985b, 1986b). An advantage to the use of heparin is that, while bull differences to a set dose exist (Parrish *et al.*, 1986b), the dose can be varied to increase or decrease the efficiency of capacitation (Parrish *et al.*, 1986c). Heparin may be effective at several points in proposed events of capacitation, as previously discussed, but the exact mechanism of its effect on spermatozoa remains to be elucidated. In earlier studies of in-vitro fertilization, particularly in cattle (Brackett *et al.*, 1982; Fukui *et al.*, 1983; Lambert *et al.*, 1986), techniques for sperm capacitation have been confounded by collection of oocytes in heparinized media. Although high-ionic strength medium or another treatment was intended to capacitate the spermatozoa, heparin carried into the gamete co-culture with cumulus–oocyte complexes may have actually been responsible for sperm capacitation.

Despite success in capacitating ruminant spermatozoa *in vitro*, procedures are not as efficient as *in vivo*. *In vivo*, fertilization most likely occurs when sperm and egg ratios are close to unity as in the hamster (Cummins & Yanagimachi, 1982). In support of this, Crozet (1984) found very few supernumerary spermatozoa present in cow eggs collected near the time of fertilization. Sperm–egg ratios *in vitro* for ruminants have generally been 10 000–200 000:1 (Brackett *et al.*, 1982; Parrish *et al.*, 1985a, 1986b; Lambert *et al.*, 1986). Using heparin to capacitate bovine spermatozoa (Parrish *et al.*, 1986b), we have been able to reduce the ratio for frozen–thawed bovine spermatozoa to sperm–egg ratios of 2000:1 and for some bulls to as low as 500:1 (J. J. Parrish, unpublished). Development of culture media able to maintain viability of spermatozoa at dilutions sufficient to give even a 10:1 sperm–egg ratio are lacking. This is an inherent difficulty in using sperm fertilizing ability to evaluate efficiency of capacitation. An alternative method has been developed using the fusogenic lipid lysophosphatidylcholine (Parrish *et al.*, 1985b). At 100 $\mu\text{g/ml}$, lysophosphatidyl-

choline induces an acrosome reaction only in capacitated bovine spermatozoa. This test has only been applied to the heparin capacitation system but should prove useful in comparing the efficiencies of the different capacitation systems to each other.

Fertilization

The success of fertilization *in vitro* relies upon completion of both oocyte maturation and sperm capacitation. Failure of either of these steps will result in failure of fertilization. In addition, the sperm concentration, time of sperm-egg interaction, medium utilized and temperature play a role in successful fertilization which will result in developmentally competent zygotes. Except when using fertilization as a means of detecting sperm capacitation or perhaps in-vitro fertilization as a predictor of in-vivo fertility, the intended outcome of in-vitro fertilization is to produce a viable embryo. In an in-vitro fertilization system one of the prime reasons for failure of the resulting zygotes to develop is the occurrence of polyspermy. *In vivo*, an oocyte encounters very few spermatozoa at the time of fertilization and blocks to polyspermy have time to be expressed. *In vitro*, large numbers of spermatozoa are placed with oocytes. If sperm capacitation is not efficient, few spermatozoa will fertilize oocytes and polyspermy is not a problem. However, as capacitation systems become more efficient, larger numbers of spermatozoa surrounding the oocyte are capable of fertilizing that oocyte and the probability of many spermatozoa penetrating the zona pellucida and vitelline membrane increases. The time that spermatozoa and oocytes are allowed to interact becomes important because early blocks to polyspermy in mammalian oocytes may not occur as rapidly as in invertebrates (reviewed by Wolf, 1981; Shapiro, 1981) and the block to polyspermy is reduced in aged oocytes (Hunter, 1967). Additionally, in-vitro matured oocytes may not develop the block to polyspermy to the same effectiveness as in-vivo matured oocytes (Leibfried-Rutledge *et al.*, 1986a). Sperm concentrations and time of sperm-oocyte interaction should therefore both be controlled to minimize polyspermy.

Table 5. In-vitro fertilization in goats

	Sperm capacitation treatment	Oocyte source	Fertilization (%)
Shorgan (1984)	A23187	In-vivo matured	49
Song & Iritani (1985)	18 h at 20°C	In-vitro matured	57

Medium composition can influence fertilization success. We have demonstrated that glucose blocks bovine sperm capacitation (Parrish *et al.*, 1985a; Susko-Parrish *et al.*, 1985). In other ruminants, unique compounds might also be important in inhibiting or aiding fertilization.

The effect of temperature cannot be overly emphasized. *In vivo*, fertilization occurs at core body temperature which is 38–39°C for cattle, 39°C for sheep and 38–39°C for goats. In cattle, fertilization frequencies *in vitro* are very dependent on temperature and highest frequencies occur at 39°C (Lenz *et al.*, 1983a). Temperature not only controls efficiency of capacitation but probably also the ability to undergo a physiological acrosome reaction in response to interaction with the zona pellucida.

While optimization of fertilization conditions for cattle have been reported (Brackett *et al.*, 1982; Sirard *et al.*, 1985; Parrish *et al.*, 1986b) differences between bulls still persist. Optimal conditions may need to be established for each male and possibly each ejaculate. Ruminants have a distinct advantage over other species in that their semen can be successfully frozen (Mann, 1964). Only a small portion of an ejaculate need then be utilized to determine fertilization conditions for

each ejaculate or male. In-vitro matured oocytes are ideal for testing the fertilization ability of bull ejaculates. These oocytes appear to yield fertilization frequencies comparable to those with in-vivo matured oocytes (Leibfried-Rutledge *et al.*, 1986a) and large numbers can be obtained from ovaries collected at the abattoir.

Comparative results from in-vitro fertilization studies published since the review of Wright & Bondioli (1981) are shown for cattle in Tables 1 and 2. For sheep, 80% of in-vivo matured oocytes mature fully *in vitro* and 80% of these are penetrated by spermatozoa; 7 of 16 (44%) recipients of such fertilized eggs became pregnant (Cheng *et al.*, 1986). Fertilization results for goat gametes have also been good (Table 5). Overall, while fertilization frequencies have differed, rates of fertilization, >80%, with low rates of polyspermy (<15%) have been reported.

Development of embryos to a transferable stage

The production of offspring from in-vitro fertilization is considerably restricted by the absence of suitable in-vitro culture systems capable of efficiently supporting development to the morula or blastocyst stage which can be transferred non-surgically. Indeed, for mammals, suitable in-vitro culture systems are available only for embryos from a few strains of mice (Goddard & Pratt, 1983) and from women (Purdy, 1982). Cattle and sheep embryos cultured *in vitro* from the 1-4-cell stages rarely cleave beyond 8-16 cells, whereas embryos cultured from the 8-16-cell stage frequently develop into morulae or blastocysts (Thibault, 1966; Newcomb, 1982; Eyestone & First, 1986; reviewed by Wright & Bondioli, 1981). These observations suggest the existence of a block to in-vitro development at the 8-16-cell stage for cattle (Thibault, 1966; Camous *et al.*, 1984; Eyestone & First, 1986) and sheep (Bondioli & Wright, 1980) embryos. These observations also suggest that (1) the oviduct but not culture medium contains factors or conditions conducive to early embryonic development and (2) that certain developmental events occurring between the 1- and 16-cell stage require specific environmental factors or conditions normally provided by the oviduct. The time of blocked development occurs at a time of prolonged cell cycle, DNA synthesis and a transition from maternal to zygotic control of development for the mouse (Goddard & Pratt, 1983), cow (King *et al.*, 1985), sheep (Calarco & McLaren, 1976) and pig (Norberg, 1973). It is known that cells of the embryo remain alive during the period of blocked development (W. H. Eyestone, unpublished) and that they cannot be rescued once the block is initiated (Eyestone & First, 1986). In mice this period of transition from maternal to zygotic control of development is accompanied by the production of a class of proteins known as heat-shock proteins. More specifically, heat-shock proteins 68 and 70 have been shown to be the first products of the mouse embryonic genome (Flach *et al.*, 1982; Bensaude *et al.*, 1983; Bolton *et al.*, 1984). In mouse embryos which pass through this critical 2-cell period *in vitro*, heat-shock protein 70 returns to a low concentration by the 4-cell stage (Barnes *et al.*, 1987). Production of heat-shock proteins 68 and 70 at this transition period and under in-vitro culture conditions may reflect the inadequacy of the culture. Whether the culture medium fails to provide critical substrates for synthesis of essential proteins, cell cycle controlling signals, or a proper environment for transcription or translation is unknown. In spite of the absence of an adequate in-vitro embryo culture system, cattle and sheep embryos resulting from in-vitro fertilization have been developed to morula and blastocyst stages. This has been accomplished by direct surgical transfer of zygotes or early embryos to the oviduct of the cow (Brackett *et al.*, 1982, 1984). The oviducts of sheep *in situ* (Eyestone *et al.*, 1985) and rabbits (Sirard *et al.*, 1985) have also been used as temporary surrogate in-vivo incubators for development of cattle zygotes or early embryos to a transferable morula or blastocyst stage. A start to the development of an adequate in-vitro embryo culture system has been provided by the co-culture of embryos on a feeder layer of oviduct epithelial cells (Rexroad & Powell, 1986; F. Gandolfi, T. A. L. Brevini & R. M. Moor, unpublished). Development was limited for the experiments of Rexroad & Powell (1986) by culturing 1-cell sheep embryos for 24 h before transfer to recipient ewes. However, 43.8% of the sheep embryos reached the blastocyst stage after a 6-day culture period (F. Gandolfi, T. A. L. Brevini &

R. M. Moor, unpublished). The successful culture of cattle embryos on a layer of feeder cells has not been reported, but there is evidence that bovine trophoblast cells are competent to carry bovine oocytes through this period of blocked development (Camous *et al.*, 1984). It is also unknown whether cell types from other tissues or from tissues of animals not in oestrus can support embryo development. Since species differ in the length of time during which the oviduct will support development of an embryo (i.e. rabbit 3–4 days, sheep 5–6 days; Boland, 1984) it would be interesting to know whether epithelial cell cultures from these respective species also support embryo development for the same species specific times. It is hoped that well designed hypotheses and experiments utilizing these co-culture systems will identify the requirements for culture of embryos from fertilization to the blastocyst stage such that totally in-vitro systems for embryo development can be accomplished.

Use of in-vitro fertilization to predict fertility

As fertilization is one of the key events which determines in-vivo fertility of a male, it has been hoped that development of in-vitro fertilization will result in a procedure to predict fertility *in vivo*. However, fertility is complex and composed of multiple events such as sperm transport, capacitation, oocyte maturation, ovulation, female endocrine status, normality of fertilization and embryo development. The frequency of sperm penetration of zona-free hamster oocytes by bovine spermatozoa has been shown to be related to bull fertility (Bousquet & Brackett, 1982). Graham & Foote (1984) also found that penetration of zona-free hamster oocytes by bovine spermatozoa treated with acrosome reaction-inducing liposomes was correlated with bull fertility when the amount of liposome required to achieve maximum penetration of oocytes was used. However, in-vivo fertility has yet to be completely predicted by in-vitro fertilization tests. Because of the complexity of in-vivo fertility this is not unexpected. There is as yet no report on the correlation of in-vivo fertility of ruminants with results of homologous in-vitro fertilization tests. However, bull effects on embryonic development that were not related to sperm penetration frequency or normality of fertilization have been reported (Leibfried-Rutledge *et al.*, 1986a). A better prediction of fertility should therefore be obtained by including homologous fertilization data in a multiple regression model along with measures of semen quality and ability of embryos to develop.

Conclusions

It is apparent that our understanding of fertilization in ruminants has progressed considerably in recent years and that this understanding has resulted in workable systems for fertilizing oocytes of cattle, sheep and goats *in vitro*. In cattle in-vitro fertilization has resulted in birth of live offspring. There is also some evidence that in-vitro fertilization may provide a useful adjunct to present methods for evaluating the fertility of bulls. It is also apparent there is much yet to be learned about the processes of sperm capacitation and the acrosome reaction, the development of maturational competence by oocytes and the specific signals within the oocyte imparting the ability to complete maturation, to be fertilized, to initiate and complete maturation and, ultimately, result in normal embryo development.

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