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In vitro fertilization in cattle: a review

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Summary — *In vitro* maturation and fertilization of cattle oocytes and subsequent *in vitro* culture of zygotes and embryos is discussed in the context of recent encouraging data. Mass production of embryos produced in this way in the future will have a great impact on animal production and animal breeding plans, for example the so-called MO-ET (multiple ovulation and embryo transfer) plan which aims at establishing nucleus breeding herds.

***In vitro* / cattle / oocytes / sperm / embryo**

Résumé — Fécondation *in vitro* chez les bovins (revue). *Les résultats récents et encourageants sur la maturation et la fécondation* in vitro *d'ovocytes de vache et la culture* in vitro *subséquente des zygotes sont analysés. La production en masse d'embryons aura un grand impact sur la production et la planification de l'élevage, par exemple le plan OM-TE (ovulation multiple et transfert d'embryons).*

bovin / maturation ovocytaire / sperme / fécondation* in vitro / *embryon

INTRODUCTION

The natural *in vivo* process of fertilization and early embryonic development involves innumerable factors, both well-studied and unexplored, which interact to affect each event of the process. Therefore, it is not surprising that results obtained using the techniques of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) are neither optimum nor predictable. Several groups have reported the occurrence of pregnancies and the birth of calves following IVM and IVF (Critser *et al*, 1986; Lu *et al*, 1987, 1989; Xu *et al*, 1987; Goto *et al*, 1988; Sirard *et al*, 1988; Stubbings *et al*, 1988; Utsumi *et al*, 1988; Greve *et al*, 1989a; Pavlok *et al*, 1989; Pollard *et al*, 1989; Gordon and Lu, 1990), but the total number of offspring on a worldwide basis is still limited (Polge, personal communication, 1988). All data clearly reflect the difficulty of applying *in vitro* techniques toward research and reproduction, and indicate that a much better understanding of the many processes involved in reproduction must be gained before the *in vitro* system can successfully be used at an optimal level.

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Highlighted here are some practical aspects of IVM, IVF and IVC. For a more extensive review of the state of the art of *in vitro* maturation and/or fertilization, see Thibault *et al* (1976, 1987), Wright Jr and Bondioli (1981), Moor *et al* (1983), Brackett (1983, 1985), Ball *et al* (1984), First and Parrish (1987), Le Guienne *et al* (1988), Staigmiller (1988), Leibfried-Rutledge *et al* (1989) and Gordon and Lu (1990).

IN VITRO MATURATION

Cumulus oocyte complexes

The morphological events associated with *in vitro* oocyte maturation very closely resemble those occurring *in vivo* (Hyttel *et al,* 1989c), except that the process *in vitro* proceeds more quickly than *in vivo* (King *et al,* 1986). From the onset it must be emphasized that *in vitro* maturation, by and large, yields oocytes with a lowered potential for developmental capacity in comparison to *in vivo* matured oocytes (Leibfried-Rutledge *et al,* 1986a, 1987; Pavlok *et al,* 1988).

The criteria for evaluation and assessment of the quality of follicular and even oviductal oocytes are not well established. In addition, conditions for maturation *in vitro* vary between research groups and have not been standardized. Multiple integrated factors play important roles in the completion of meiotic and cytoplasmic maturation *in vitro*, and the capacity of such an oocyte upon fertilization to develop into a viable blastocyst.

The size of the follicle from which the oocyte originates does not seem to influence its ability to undergo nuclear maturation (Leibfried and First, 1979; Fukui and Sakuma, 1980; Grimes and Ireland, 1986) or even to be fertilized *in vitro* (Leibfried-Rutledge *et al,* 1985). Recent evidence suggests, however, that oocytes which have not completed their growth, and therefore have not synthesized adequate amounts of RNA, possess a reduced capacity for early embryonic development (Crozet *et al,* 1986; Crozet, 1989). Since the effect of follicle and oocyte size on IVF and IVM has still not been sufficiently studied and understood, one should, as emphasized by Staigmiller (1988), attempt to use medium-sized follicles as the origin of oocytes to be used for maturation *in vitro.* This concept has recently been substantiated by Tan and Lu (1990).

The macroscopic appearance of the follicle may also be used to select oocytes capable of maturation and development. Oocytes originating from translucent follicles (Grimes and Ireland, 1986) have reduced potential because of advanced atresia.

Assessment of characteristics of the cumulus oocyte complexes acquired by aspiration of follicles has been used by many research groups to estimate oocyte quality. Oocytes surrounded by a tight, complete, multilayered cumulus investment and with an ooplasm void of rough granules seem to be most capable of undergoing normal maturation and fertilization *in vitro* (Leibfried and First, 1979; Fukui and Sakuma, 1980; Greve *et al,* 1984; Hensleigh and Hunter, 1985; Xu *et al,* 1987; Shioya *et al,* 1988; Greve *et al,* 1989b; Sirard, 1989; Younis *et al,* 1989; Kruip *et al,* 1990; Gordon and Lu, 1990; Yang and Lu, 1990).

The optimal time and temperature for ovary transport from the abattoir and until initiation of culture has been examined by Yang *et al* (1990). These authors found that ovaries can be stored for up to 8 h at 25 °C without a reduction in the rate of fertilization or in the capacity of early embryos to develop to blastocysts *in vitro.*

Storage for over 4 h at 37 °C or at 4 °C reduced the viability. It is known that periods of reduced temperature during maturation result in chromosomal abnormalities in sheep oocytes (Moor and Crosby, 1985). Katska and Smorag (1985) suggested that incubation temperatures of 35–37 °C assure a higher viability of bovine oocytes as judged by fluorescent staining. However, the results of Ball *et al* (1982) and Lenz *et al* (1983a) clearly indicate that the penetration rate of cattle oocytes *in vitro* is significantly correlated with the temperature during IVM; penetration rates of 36% and 58% result following IVM at 37 °C and 39 °C, respectively. Presently, virtually all groups are performing IVM/IVF at 39 °C. The time period of incubation required to complete meiotic maturation ranges from 18-27 h (King *et al,* 1986; Xu *et al,* 1986a; Süss *et al,* 1988) and is somewhat dependent on the thickness of the cumulus mass (Xu *et al,* 1986a).

It has been demonstrated that culture of follicle-enclosed oocytes leads to a higher rate of maturation and fertilization than culture of aspirated oocytes (Fukui *et al,* 1987; Staigmiller, 1988). However, most research groups use oocytes either aspirated directly from ovarian follicles or released from the follicles after dissection.

Media, supplementation and culture conditions

A variety of media have been used for IVM. Bavister (1989) emphasized the importance of proper media preparation and the use of the hamster sperm bioassay to test water, media, filters, etc for toxicity. For review articles concerning quality control in the laboratory, see Boone and Shapiro (1990) and Schiewe *et al* (1990).

Although Ham's F-10, Ham's F-12, Brinsters BMOC-3, KRB and MEM have been used successfully in many studies (Staigmiller, 1988; Leibfried-Rutledge *et al,* 1989), both TCM 199 and TALP (Parrish *et al,* 1986; Sirard *et al,* 1988) seem to emerge as the media of choice for IVM/IVF work. In our laboratory we initially used Ham's F-10, but have recently changed to TCM 199, which we found to give a nuclear maturation rate of 80%. The pH and osmolarity should be 7.2 to 7.4 and 285-300 mOsm, respectively, and in most cases the oocytes were cultured in 5% CO_2 in air.

In vivo, the LH surge triggers final maturation and ovulation of the selected oocytes. A delicate and well balanced hormonal microenvironment ensures that the signaling between the oocyte and its surrounding is kept within certain well defined limits (Callesen *et al,* 1986). It seems to be of paramount importance that the hormonal environment (particularly the steroids) is well-balanced during the maturation process (Baker and Hunter, 1978; Moor, 1978; Moor *et al,* 1980; Osborn and Moor, 1983; Moor and Seamark, 1986). In order to achieve this goal a variety of hormones, such as FSH, LH, estradiol-17β and even progesterone, were added to the medium. It is generally believed that the addition of hormones provides oocytes with higher viabiblility, *ie* oocytes which undergo normal fertilization and embryonic development at a higher rate (Fukui *et al,* 1982a, b; Fukushima and Fukui, 1985; Fukui, 1989; Younis *et al,* 1989). However it must be realized that the initial hormonal environment of the maturation medium changes during incubation if the oocytes are cultured in medium under oil (Xu *et al,* 1988a). In a recent study no beneficial effect of the addition of hormones was observed with regard to subsequent embryo development (Sirard *et al,* 1988). In our laboratory and many others, addition of hormones has been substituted by estrous cow serum (ECS), which has proven to be very efficient for attaining oocytes with high

developmental competence (Lu *et al,* 1987, 1988; Xu *et al,* 1987; Fukui and Ono, 1988, 1989; Lu *et al,* 1989; Fukui *et al,* 1989). This may be due to the presence of hormones in ECS that may support the oocyte maturation.

Oocyte maturation *in vivo* is arrested by follicular substances that are not well-defined. Follicle-enclosed oocytes remain arrested in the dictyate stage of meiosis until the endogenous or exogenous exposure to gonadotropins causes the resumption of meiosis. By contrast, oocytes removed from their follicles spontaneously resume meiosis. For *in vitro* purposes, attempts have been made to arrest or at least delay maturation of bovine aspirated oocytes. Liehman *et al* (1986) showed that addition of dbc-AMP did not prevent oocyte maturation, but had a beneficial effect on the sperm penetration rate. Sirard and First (1988) showed that dbc-AMP, IBMX and hypoxantine transiently (up to 21 h) prevent resumption of meiosis; an effect that was also observed when the oocytes were cultured with bovine follicular fluid (BFF) and NaF + BFF (Sirard, 1990). This last observation has resulted in addition of BFF to maturation medium, but so far neither of the above substances have been added to media on a routine basis.

The somatic cells surrounding an oocyte facilitate production of nutrients and their transport into an oocyte. In addition they generate signals which control and regulate oocyte metabolism, as well as nuclear and cytoplasmic maturation (Osborn and Moor, 1982; Moor and Seamark, 1986). Supplementation of maturation media with additional granulosa cells has been performed in a number of studies and has been found to be absolutely essential for achieving full developmental competence (Staigmiller and Moor, 1984; Critser *et al,* 1986; Lu *et al,* 1987; Lutterbach *et al,* 1987; Fukui and Ono, 1988; Fukui *et al,* 1988; Leibfried-Rutledge *et al,* 1989). In our system cumulus oocyte complexes were cultured without supplementary granulosa cells (Madison *et al,* 1991).

Addition of serum to maturation medium is one of the requirements for achievement of cumulus expansion and complete oocyte maturation, and attainment of normal embryonic development in cattle. Fetal calf serum (FCS) has been shown to be superior to bovine serum albumin (BSA) (Leibfried-Rutledge *et al,* 1986b). As mentioned earlier, ECS is now the serum of choice and seems to increase cleavage rate in comparison to FCS (Fukui, 1989), probably because of the content of hormones. Serum collected around the time of onset of estrus improves *in vitro* development after IVF (Sanbuissho and Threlfall, 1989; Younis *et al,* 1989).

The size of the droplets in which the oocytes are cultured and the number of oocytes per droplet varies from lab to lab. In our laboratory 10 oocytes were cultured in 100-μl droplets under paraffin oil.

IN VITRO FERTILIZATION

Semen and semen treatment

For practical purposes ejaculated semen is used for bovine IVF and seems to give rise to more normal eggs than epididymal spermatozoa (Pavlok *et al,* 1988).

The first calf born following *in vitro* fertilization of cattle oocytes matured *in vivo* was the result of using fresh semen treated with high ionic strength solution (HIS) (Brackett *et al,* 1982). Since then, this technique has also been used successfully by other groups (Greve *et al,* 1984; Sirard and Lambert, 1985, 1986), and also for frozen/thawed semen (Bondioli and Wright, 1983).

Later, Fukui *et al* (1983) performed studies which revealed that semen treatment with bovine follicular fluid (BFF) resulted in a higher fertilization rate than treatment with HIS, probably due to a proteoglycan in the BFF known to enhance the acrosome reaction (Lenz *et al,* 1982).

Subsequent detailed studies have clearly shown that 1) certain glycosaminoglycans (GAGs) which are present in the female genital tract will induce the capacitation/acrosome reaction (Lenz *et al,* 1982, 1983b) and 2) the GAG, heparin, appears to be superior to chondroitin sulphate (Parrish *et al,* 1985) in terms of fertilization rates. The proportion of oocytes penetrated by sperm is greatest when fresh semen is incubated with heparin for at least 4 h (Parrish *et al,* 1988) and frozen-thawed semen for 15 min (Parrish *et al,* 1986) prior to mixing sperm with oocytes. The concentration of heparin varies according to the type of semen and between bulls (Leibfried-Rutledge *et al,* 1989). The mechanisms behind the induction of capacitation have been thoroughly described by First and Parrish (1987). In general, changes occur in the spermatozoa plasma membranes which allow uptake of Ca^{2+} activation of a c-AMP dependent protein kinase. In this context it is worthwhile emphasizing that glucose will inhibit the effect of heparin-induced capacitation (Parrish *et al,* 1985, 1989). Combined with a swim up technique which separates motile from immotile spermatozoa, treatment with 10 µg/ml heparin gives rise to repeatable and predictable fertilization rates of bovine oocytes matured *in vivo*, as well as *in vitro* (Parrish *et al,* 1986). Presently, this system seems to be the predominant treatment used to prepare sperm for fertilization *in vitro.* However, Fukui *et al* (1990) recently found that the optimal heparin dosage ranges from 25 to 100 µg/ml.

The effect of heparin may be enhanced by caffeine (Niwa and Ohgoda, 1988). It is also noteworthy that the spermatozoa from different bulls give different frequencies for both fertilization and embryonic development *in vitro* (Brackett *et al,* 1982; Sirard and Lambert, 1985; Leibfried-Rutledge *et al,* 1987; Miller and Hunter, 1987; Ohgoda *et al,* 1988; and Eyestone and First, 1989b). The research of Ohgoda *et al* (1988) indicates that this effect is unrelated to the bull's *in vivo* fertilizing capability. However, heparin at a low concentration (0.05 µg/ml) seems to be optimal for evaluating *in vivo* fertility of different bulls (Marquant-Le Guienne *et al,* 1989).

It is therefore necessary to test each bull to ascertain that optimal heparin concentration and/or number of sperm are used for IVF (Leibfried-Rutledge *et al,* 1989). It is also important to take into consideration that each bull has its own contribution to *in vitro* fertilization and embryonic development (Hillary *et al,* 1990; Shi *et al,* 1990).

Coculture of sperm and oocytes

The fertilization medium used for coculture of sperm and oocytes consists of TALP placed in 50-µl droplets, and overlaid with sterile silicone or paraffin oil (Ball *et al,* 1983; Leibfried-Rutledge *et al,* 1987). In general, 10 oocytes are added to each 50 microliter droplet, which also contains 1 x 10^6 live spermatozoa per ml. Coculture is conducted approximately 20–22 h at 39 °C in an atmosphere of 5% CO_2 in air.

Assessment of fertilization in vitro

Assessment of fertilization is important since it has been established that oocytes

matured both *in vivo* and *in vitro* may be pathenogenetically activated, therefore leading to invalid conclusions concerning actual fertilization rates (Xu *et al*, 1986b; King *et al*, 1988). This activation is apparently an age-dependent process (Ware *et al*, 1989). It is very important that one can distinguish between an activated oocyte and a fertilized egg; presence of part of the sperm tail (midpiece) and a maternal and paternal pronucleus indicate that fertilization has taken place. Abnormalities (polyspermy and others) are likely to occur (Xu *et al*, 1988b) due to deviating oocyte maturation and/or fertilization (Hyttel *et al*, 1989a and 1989b). In this study, fertilization and parthenogenetic activation were approximately 60% and 12%, respectively.

CULTURE OF FERTILIZED EGGS

Until recently, successful culture of fertilized bovine oocytes was limited to the use of sheep oviducts (Lu *et al*, 1987; Fukui *et al*, 1989; Leibfried-Rutledge *et al*, 1989; Lu *et al*, 1989) or rabbit oviducts (Fukui and Ono, 1988; Fukui *et al*, 1989). However, methods of *in vitro* culture that utilize coculture with bovine oviduct epithelial cells (BOEC) (Fukui and Ono, 1988; Fukui *et al*, 1989; Eyestone and First, 1989a; Gordon and Lu, 1990; Madison *et al*, 1991), granulosa cells (Goto *et al*, 1988; Berg and Brem, 1989), trophoblastic vesicles (Heyman and Ménézo, 1987; Aoyagi *et al*, 1989) or culture in conditioned medium (Eyestone and First, 1989a) are very effective and have become the preferred method for the culture of embryos produced *in vitro*. The rate of development may be improved by adding estrous cow serum, rather than FCS, to the culture medium and omitting hormones (Fukui, 1989). Culture in domestic chicken eggs has also been carried out successfully, although the method is not commonly used (Blakewood *et al*, 1989). *In vitro* results, which are comparable with those *in vivo*, have been reported by Marquant-Le Guienne *et al* (1989) who added TGFβ at the beginning of blastocyst formation.

Studies by Gandolfi and Moor (1988) and Gandolfi *et al* (1989) indicate that at least two proteins from oviductal fluid (92 and 46 kDa) are involved in supporting embryonic development. Future studies will indicate whether these two proteins or other substances, such as growth factors, are secreted by the BOEC and are thus responsible for the positive effect of using coculture with oviduct cells. In our laboratory using BOEC the average rates of cleavage (6- to 16-cells on day 3) and development to the morula/blastocyst stage for the total oocytes exposed to sperm were 40% and 34%, respectively.

The advantages of using the *in vitro* system are as follows: there is no need to maintain live animals in the laboratory vicinity; rates of recovery are higher than after agar embedding, transfer to the oviduct, and flushing 5-6 days later; there are more possibilities for detailed studies of embryonic development; and fertilized oocytes or embryos may be used at various stages for other experimental procedures, such as cloning and gene transfer. The disadvantages are a possible decrease in embryonic development rate and probably reduced viability after transfer to the final recipient, particularly if the embryos are previously frozen or split by micromanipulation.

FINAL COMMENTS

The techniques of *in vitro* maturation and *in vitro* fertilization of cattle oocytes and culture of fertilized eggs and embryos have

made tremendous progress during the last 5–10 years. It is now realistic to predict that readily available and inexpensive embryos can be produced from slaughterhouse ovaries and will be on the market for experimental and commercial purposes. The problems are, however, still many and must be solved before the efficiency of producing embryos or off-spring with *in vitro* techniques is greater than achieved with natural procedures.

It may be possible to improve the proportion of transferable embryos by ultrasonically guided transvaginal oocyte aspiration (Kruip *et al,* 1990). This technique may be performed two to three times during an estrous cycle in a cow for as long as 6 months (Pieterse *et al,* 1990; Greve, unpublished data, 1990). If this procedure yielded 5–10 oocytes per cycle, it would be possible with the current rates of fertilization and blastocyst formation to achieve 1–2 calves from the aspirations during a 3-week period.

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