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Function of the Cumulus Oophorus Before and During Mammalian Fertilization

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Contents

Fertilization encompasses a series of different steps which have to be performed in a well-orchestrated way to create a new individual. They include sperm capacitation, sperm binding and penetration of the zona pellucida, traversing the perivitelline space, binding and fusion with the oolemma, activation of the oocyte and decondensation of the sperm head to form the male pronucleus. In most mammalian species, cumulus cells surround the oocyte at the time of fertilization. Removal of the cumulus oophorus at this point of time often leads to a drop in fertilization rates. It is not yet known how cumulus cells interact with the oocyte or with spermatozoa to promote fertilization. There are different possibilities:

1 cumulus cells cause mechanical entrapment of spermatozoa and guide hyperactivated spermatozoa towards the oocyte, while preventing abnormal spermatozoa to enter the cumulus matrix;

2 cumulus cells create a micro-environment for the spermatozoa which favours their capacitation and penetration into the oocyte;

3 cumulus cells prevent changes in the oocyte which are unfavourable for normal fertilization; these changes can be located in the zona pellucida or in the cytoplasm.In this review, studies in several species are listed to prove the importance of these three cumulus cell functions and the current lines of research are highlighted. Moreover, different ways to improve *in vitro* fertilization of bovine cumulus-denuded oocytes are discussed.

Introduction

The cumulus oophorus is unique to the egg of eutherian mammals (Bedford and Kim 1993). It consists of a mass of granulosa cells surrounding the oocyte. The cumulus oophorus expands after ovulation due to the deposition of a proteoglycan matrix. The major carbohydrate in this muco-elastic matrix is hyaluronic acid (Ball et al. 1982; Salustri et al. 1999).

Cumulus cells are involved in oocyte growth and maturation. In primary follicles which contain growing oocytes, the surrounding granulosa cells start to proliferate and at the time of antrum formation, two specific populations of granulosa cells can be distinguished: (1) cumulus granulosa cells, which enclose the oocyte with the corona cells as the innermost layers; and (2) mural granulosa cells which are lining the follicular wall (Buccione et al. 1990; Cortvrindt and Smitz 2001). Cumulus and mural granulosa cells together with the oocyte form a gap-junction-mediated syncytium, which is essential for oocyte growth to proceed. The granulosa cells supply the oocytes with nutrients and connect them to the external world. The vascular supply of the follicle is situated in the theca interna (Redmer and Reynolds 1996). If one considers that in some species a mature follicle can easily reach a diameter of 2–4 cm, it is clear that pre-ovulatory oocytes are at a relatively long distance from the vascular supply of oxygen, nutrients and signals. Fortunately, the membrane connections provided by cumulus gap junctions allow a quick transfer of small metabolites and regulatory molecules into the oocyte. In fact, it is this property of cumulus cells, together with their specific metabolizing capacity, which is of major importance in the regulation of oocyte maturation (Tanghe et al. 2002). Cumulus cells keep the oocyte under meiotic arrest (Dekel 1988; Eppig 1989), participate in the induction of meiosis by conducting the LH signal to the oocyte (Mattioli and Barboni 2000) and they are responsible for proper cytoplasmic maturation of the oocyte (Staigmiller and Moor 1984; Mori et al. 2000). Adequate cytoplasmic maturation of the oocyte is crucial for the developmental potential of the embryos which result after fertilization. Since cumulus cells are partially responsible for oocyte maturation, gene expression patterns in biopsied cumulus cells might actually serve as non-invasive markers to evaluate oocyte cytoplasmic maturation (Smitz et al. 2001).

Despite all these data on the importance of cumulus–oocyte interaction during oocyte growth and maturation, there is no consensus as to the exact role of the cumulus oophorus during fertilization. At fertilization, the actual connections between the oocyte and cumulus cells are broken down, but the oocyte still remains embedded in the proteoglycan matrix and is surrounded by the corona cells and by dislodging cumulus cells. Spermatozoa approach the ovulated cumulus–oocyte complex with the ultimate goal to fertilize it. Fertilization encompasses a series of different steps which have to be performed in an orchestrated way to create a new individual. These steps are classically defined as sperm capacitation, sperm binding and penetration of the zona pellucida, binding and fusion with the oolemma, activation of the oocyte and decondensation of the sperm head to form the male pronucleus (Töpfer-Pedersen 1999). Rather surprisingly, sperm–cumulus interaction is almost never mentioned in this series of events although the first contact the capacitated sperm cell has after leaving the oviductal isthmus is with the cumulus of the ovulated oocyte. In most mammals the cumulus oophorus is present at the time of fertilization, both under *in vivo* and *in vitro* conditions. It is the purpose of this review to put the importance of the cumulus oophorus during fertilization into its context.

Is the cumulus oophorus necessary during fertilization in all mammals?

The first question to be considered is whether the cumulus oophorus is necessary for *in vivo* fertilization.

The answer to this question is that this is surely dependent upon the species. In many mammals, especially those with a spacious ampulla, the cumulus cells remain embedded in the expanded mucified matrix for a variable period of time and may actually be involved in fertilization (Bedford 1996). In mammals with a relatively narrow oviduct, such as shrews and canines, there is no expansion of the persistent cumulus cells before fertilization, and in marsupials where the upper oviduct diameter is comparable to the diameter of the egg, the cumulus cells are even shed before ovulation (Bedford 1999). In small insectivores such as the musk shrew, *Suncus murinus,* and the least shrew, *Cryptotis parva,* it has been shown that the cumulus oophorus is an essential mediator of fertilization, probably by inducing the acrosome reaction (Bedford et al. 1997a, b). Although in ruminants the cumulus cells are shed within a few hours (Lorton and First 1979) to 10 h after ovulation (Hyttel et al. 1988), it has been noticed that cumulus-free cattle oocytes, surgically collected from the oviduct a few hours after (super)ovulation are no longer fertilizable (Moyaert and Geldhof, personal communication). Moreover, in hamster, *in vivo* fertilization also decreased when naked oocytes as compared to cumulus-intact oocytes were transferred to oviducts of mated hamsters (Moore and Bedford 1978).

A second question is whether the cumulus oophorus is equally important during *in vitro* fertilization as it is during *in vivo* fertilization. It is known that perfectly viable embryos can be obtained by injecting spermatozoa immediately into the cytoplasm of the oocyte, with numerous successes described in humans (Bonduelle et al. 1999), and with less success also in other species (Catt and Rhodes 1995). However, this technique cannot be considered as a normal fertilization process, as it brings about plasmalemma disruption and elimination of the normal interaction between the surfaces of both gametes preceding gamete fusion (Tesarik 1996). During a 'natural fertilization' process, i.e. bringing oocytes and spermatozoa together as happens in a Petri dish, the cumulus cells definitely improve the fertilization rates in most mammalian species. Removal of cumulus cells before *in vitro* fertilization has decreased sperm penetration in cattle (Zhang et al. 1995; Tajik et al. 1993) and in pigs (Wang et al. 1995; Suzuki et al. 2000). However, here also, a species dependence is obvious since it was found that in different mouse strains cumulus removal did not affect fertilization rates (Vergara et al. 1997). Thus although cumulus cells are not critical for fertilization *in vitro*, they do in some species definitely improve *in vitro* fertilization rates.

Indications for cumulus removal

Why is it necessary to devote this much attention to the possible influence of cumulus cells on fertilization, if it is perfectly alright to leave the cumulus oophorus where it belongs during fertilization, i.e. around the oocyte? Indeed there are situations in which it would be convenient to study fertilization of cumulus-free oocytes, especially in cattle.

First, it could be used to assess the presence of polar bodies, since extrusion of the first polar body is an indication of oocyte meiotic maturation. This feature has been used in a elegant study, in which the timing of the appearance of the first polar body in combination with timing of insemination influenced the sex of the resulting embryos (Dominko and First 1997a) and the developmental competence of the zygotes (Dominko and First 1997b).

Another aspect indicative of oocyte maturation is the quality of the oocyte cytoplasm. Assessment of cytoplasm vacuolization and granulation is very difficult in cumulus-intact oocytes: after cumulus removal, the oocyte cytoplasm can be evaluated as even, dense and finely granulated or as coarse granular or with mixed light and dark areas. However, grading of cattle oocytes on the basis of ooplasm appearance had no significant influence on fertilization outcome (Hawk and Wall 1994).

An often neglected feature of the mature oocyte is the extent and the contents of the perivitelline space. It has been described that the perivitelline space is wider in *in vivo*-matured oocytes than in *in vitro*-matured bovine oocytes, and *in vivo*-derived zygotes develop more readily to advanced embryonic stages (Van Soom and de Kruif 1992). Moreover, the contents of the perivitelline space consist of granules and protein depositions, from the cumulus cell projections or, after ovulation, also from the oviduct (Gandolfi et al. 1991). It has even been proposed that the perivitelline fluid composition can be sampled by means of micropuncture to permit evaluation of metabolic activities of the oocyte or embryo (Hunter 1994). Evaluation of the size of the perivitelline space might be an interesting approach to evaluate oocyte developmental competence.

Sperm–zona interaction has by definition to be studied in cumulus-denuded oocytes because it is very difficult to assess any interaction with the zona in cumulus-intact oocytes. These oocytes are in general artificially denuded of cumulus cells using mechanical forces or enzymatic treatment. As long as the role of the cumulus cells during fertilization has not completely been clarified, it might be wise to interpret these studies with the necessary caution, since sperm–zona interaction might not have taken place in a natural way.

Last but not least, cumulus cells must be removed before intracytoplasmic sperm injection (ICSI) or cloning. This is necessary to ensure that one does not inject the sperm into the oocyte DNA (ICSI) or that the oocyte nucleus has been adequately removed (cloning). However, since natural fertilization is circumvented for these techniques, it is not necessary to investigate the role of the cumulus cells in this process. Their role must be very limited and can only be related to the oocyte itself and not to the spermatozoa.

Cumulus cells entrap and guide spermatozoa towards the oocyte

If one takes a first look at the oocytes in Figs 1 and 2, the most obvious difference between both of them is

their apparent difference in size. However, at closer observation, it soon becomes apparent that the size of the oocytes themselves is in fact the same: it is only the increased volume of the cumulus mass which makes the difference.

This increased volume of the cumulus oophorus is important, especially in species with a wide ampulla and a relatively low number of spermatozoa in the oviduct (Bedford and Kim 1993) since it makes it more likely that a sperm cell will encounter the oocyte. However, this also implies a kind of chemotaxis, because how can the spermatozoa otherwise be attracted to the cumulus cells? Evidence for chemotactic effects of the cumulus oophorus has been provided in a study in which the products of ovulation (oocytes, cumulus oophorus and follicular fluid) stimulated sperm transport in the hamster oviduct (Ito et al. 1991). Moreover it has been suggested that the radial orientation of the cumulus cells themselves could guide the spermatozoon to the oocyte (Bedford 1999) and that secretions of the cumulus cells closest to the oocyte create an attractant gradient for spermatozoa within the cumulus oophorus

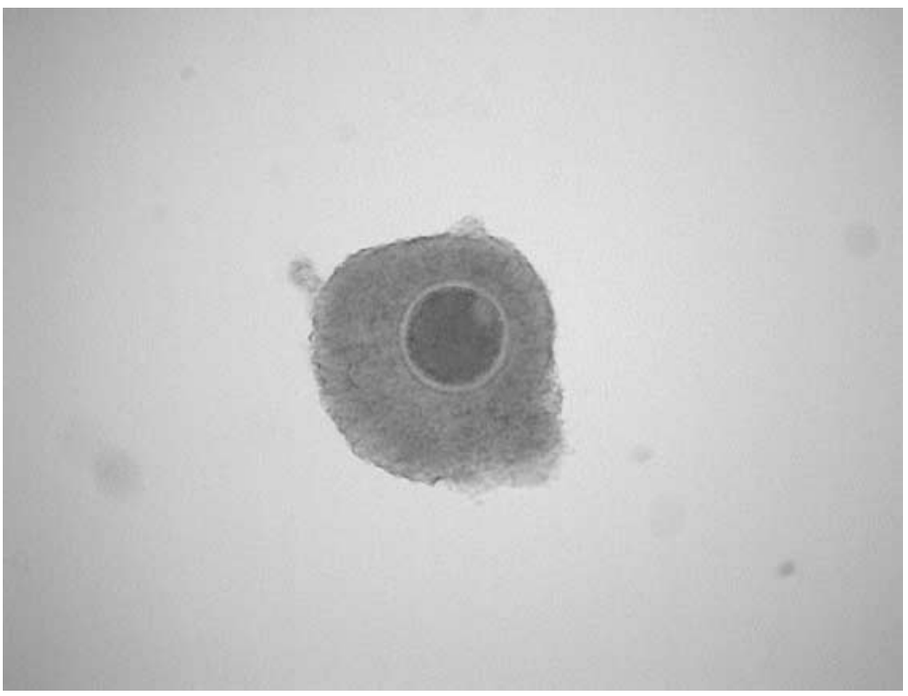

Fig. 1. Immature bovine cumulus–oocyte complex. Note the dense cumulus cells

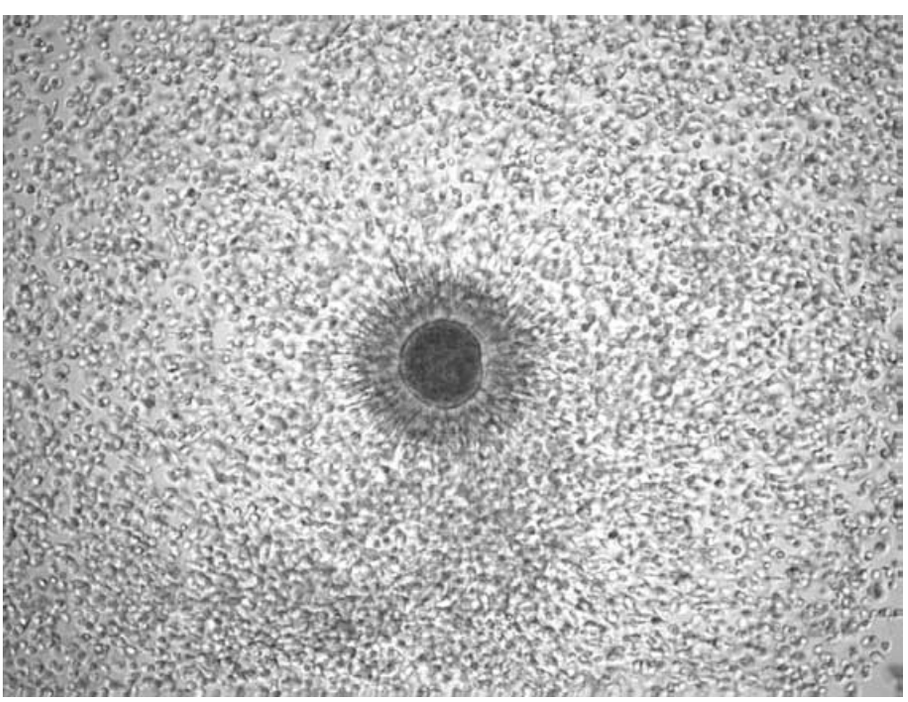

Fig. 2. The same cumulus–oocyte complex after 24 h of maturation. Note the expanded appearance caused by intercellular deposition of hyaluronic acid

(Eisenbach 1999). Studies in different mammalian species have provided sufficient evidence for this role of the cumulus oophorus during *in vivo* fertilization. It is a long-standing notion that the cumulus functions physically to entrap spermatozoa and guide them to the oocyte (Austin and Walton 1960). A more precise description of this function has been given by Hunter (1993), who has hypothesized that molecular factors released by the oocyte and/or its investments could act upon co-ordination of sperm penetration into oocytes, especially in polytocous animals. In polytocous animals such as the mouse, rat and pig, there is a strong argument for oocytes which are already activated after fertilization to release molecular signals to divert 'free spermatozoa' towards oocytes as yet unfertilized (Hunter 1997). Molecular signals which could be involved, as listed by Hunter (1993), are (1) matrix from cumulus cells; (2) glycoprotein material from zona pellucida; (3) molecules released from fluid within the perivitelline space; (4) oocyte plasmalemma material; (5) cortical granule contents; and (6) material from the fertilizing spermatozoon. If it were true that fertilized cumulus–oocyte complexes can secrete specific factors to divert spermatozoa away from them (in some cases towards other cumulus–oocyte complexes) there might be a role for the cumulus oophorus to prevent polyspermy, as suggested by Bedford and Kim (1993). More *in vivo* studies are needed to elucidate the nature of this signals.

But what about *in vitro* fertilization? During *in vitro* fertilization, an excess number of spermatozoa is added to the oocytes, which is in sharp contrast to the situation *in vivo* where sperm–egg ratios are close to one (Hunter 1993). Whether the cumulus oophorus is then actually capable of increasing the numbers of possibly fertilizing spermatozoa around the oocyte is not clear, because also when cumulus-free oocytes are used, spermatozoa swim near the oocyte in apparently great numbers. One *in vitro* study has been published which is indicative for a chemo-attractive role of the cumulus oophorus during bovine fertilization (Chian et al. 1996). In this choice assay, spermatozoa migrated preferably towards medium containing cumulus–oocyte complexes (COCs). However, since it is rather difficult to establish a functional migration assay without interference of physical forces, more research is needed to confirm these results. This has been tried in the authors' laboratory with a sperm migration assay as shown in Fig. 3. The migration medium used was IVF-TALP supplemented with heparin, to induce capacitation, because also in the oviduct and in IVF conditions, capacitated spermatozoa swim towards the oocyte. Frozen–thawed bull

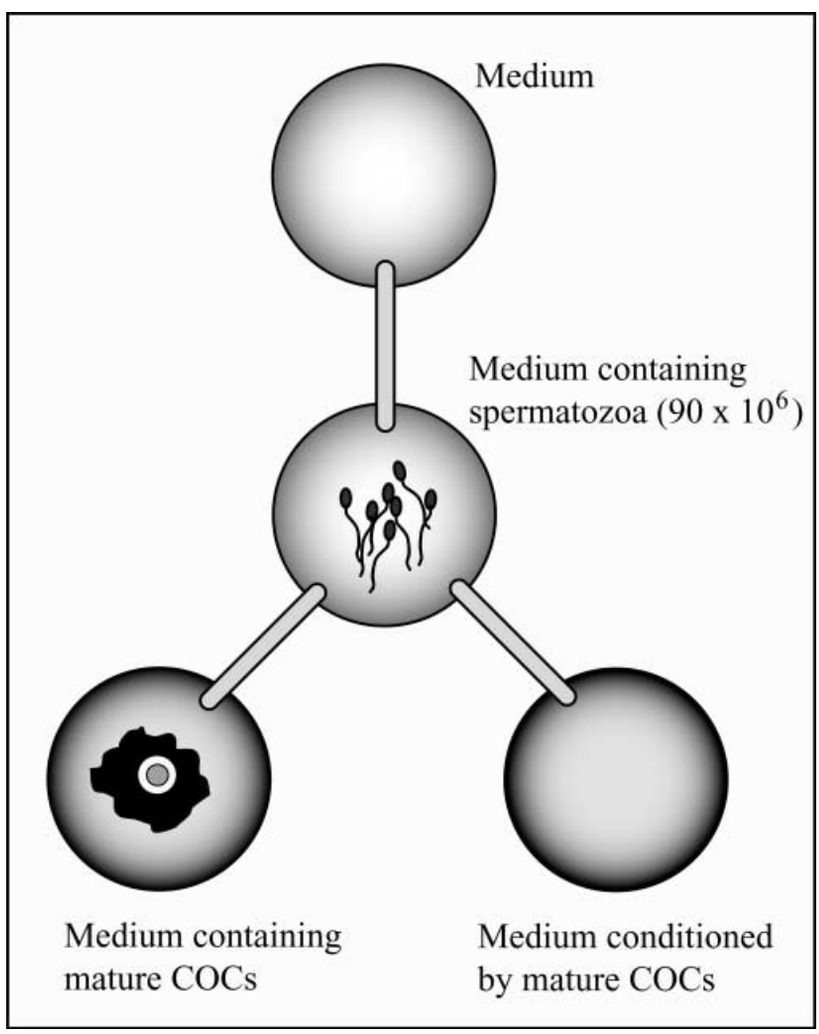

Fig. 3. Sperm migration assay

Table 1. Migration after 1 h incubation of bovine frozen–thawed spermatozoa towards IVF-TALP, IVF-TALP conditioned with mature cumulus–oocyte complexes (COCs) or IVF-TALP containing mature COCS (three replicates)

Medium	Mean no. (± SD) of spermatozoa per recipient	Percentage migrated spermatozoa
IVF-TALP	207 442 ± 193 825[a]	0.23
IVF-TALP after conditioning	364 167 ± 68 754[a]	0.40
IVF TALP with 50 COCs	421 333 ± 96 257[b]	0.47

Values with different superscripts within the same column display borderline significance ($p < 0.10$)- One way ANOVA –LSD test.

spermatozoa were added after Percoll separation at a concentration of 30×10^6 sp./ml into a 3 ml central Petri dish. This central Petri dish was linked to the peripheral Petri dishes by means of silicone tubing to generate a migration distance of 3 cm. The peripheral Petri dish contained control IVF-TALP, IVF-TALP with 50 matured COCs and IVF-TALP conditioned by 50 COCs. After 1 h incubation at 38.5°C, the contents of the three peripheral Petri dishes was collected and centrifuged (720 ***g*** for 10 min). The concentration of spermatozoa in each sperm pellet was determined by means of a fluorimeter. The experiment was repeated three times and the results are presented in Table 1.

Due to the large standard deviation, only borderline significance could be shown between the control and the Petri dish containing mature COCs ($p = 0.09$). However, these results indicate the need to refine comparative assays to test for chemotactic substances secreted by the cumulus cells. Maybe the use of larger groups of oocytes as a chemotactic source is necessary, although *in vivo*, chemotactic signals are often derived from a single ovulated oocyte.

Modulation of sperm penetration by the cumulus oophorus to decrease polyspermy could not be confirmed by *in vitro* studies. Cumulus denuded oocytes actually display lower levels of polyspermy than cumulus-enclosed oocytes (Chian et al. 1995; Tanghe et al. 2001a). Probably the oocyte itself does not have enough time to react upon the huge numbers of oocytes which are in its immediate environment during IVF, since the cortical reaction takes several minutes after the initiation of sperm–egg fusion (Yanagimachi 1988). This might enable a second or third spermatozoon to penetrate the zona pellucida and oolemma.

Cumulus cells create favourable circumstances for sperm capacitation and penetration into the oocyte

Carbohydrates are necessary for a range of complex interactions which are essential for successful fertilization (Ivell 1999). Several carbohydrate moieties have for some years been recognized as key elements in the interaction between spermatozoa and the zona pellucida, and more recently also in sperm–oviduct interactions (reviewed by Töpfer-Pedersen 1999). However, similar data on carbohydrate involvement in sperm–cumulus interaction are scarce. Early studies in hamsters have focused on the role of cumulus components in sperm capacitation and/or acrosome reaction. Both cellular and matrix-related cumulus components have been shown to be effective in inducing capacitation and/or acrosome reaction of golden hamster spermatozoa (Gwatkin et al. 1972; Bavister 1982). Interestingly, sialic acid was the key factor for sperm–cumulus interaction in those early studies in the golden hamster (Gwatkin et al. 1972), whereas the same sugar was also involved in sperm–oviduct interaction in this species (DeMott et al. 1995). The sharing of identical carbohydrate sequences by the oviductal epithelium and the zona pellucida has been mentioned before (Töpfer-Pedersen 1999) and maybe also the cumulus oophorus can be added to this group. Binding ability of spermatozoa to other cells develops rapidly both under non-capacitating or capacitating conditions, but it is only followed by sperm penetration under capacitating conditions.

More investigations are needed to verify which oligosaccharide structures are recognized by the spermatozoa on the cumulus cells or on the three-dimensional structure of the cumulus matrix, and whether this sperm–cumulus binding is promoting sperm capacitation. However, since spermatozoa are already capacitated when they are released from the epithelium of the oviductal isthmus (Suarez 1998), improvement of sperm capacitation and acrosome reaction are probably not the main functions of the cumulus oophorus. Capacitation under *in vivo* conditions has to be accomplished before the spermatozoa reach the oocyte. As far as induction of the acrosome reaction is concerned, it is now generally accepted that a physiological acrosome reaction is not co-ordinated by the cumulus, but by the zona pellucida (Töpfer-Pedersen 1999). In fact acrosome-reacted spermatozoa cannot penetrate the cumulus because they remain stuck to the surface (Cherr et al. 1986).

Despite these findings, the huge drop in fertilization rate of cumulus-denuded oocytes to about half that of cumulus-intact oocytes (Fukui 1990; Chian et al. 1995) urges further investigation of this presumed cumulus function.

How can cumulus cells create circumstances which are favourable for sperm penetration? Spermatozoa may undergo various stages of capacitation and the cumulus oophorus might play a role in this. This could either be due to something inherent to the cumulus cells themselves (e.g. specific binding of the sperm leading to improved capacitation/penetration or a change in the physicochemical environment of the spermatozoa) or to some specific secretion of the cumulus cells into the culture medium.

In the authors' laboratory, investigations have until now predominantly been focused on specific secretions of cumulus cells. For this purpose, media conditioned by cumulus cells were used to improve the fertilization rates of cumulus-denuded oocytes, but although fertilization rates were improving, conditioned media could not equal the fertilization of cumulus-intact oocytes, as has also been shown by others (Saeki et al. 1994; Tanghe et al. 2001b). This could mean that the cumulus-conditioned medium did not contain all of the necessary factors that could support sperm penetration or that the factor was diluted to an inefficient

level. It can be stated that the factor involved is not progesterone, since addition of progesterone to the fertilization medium of cumulus-denuded oocytes did not affect the formation of pronuclei in any way (Tanghe et al. 2001a). Other factors which are currently under investigation are hyaluronic acid, and sperm motility factors, such as caffein. Hyaluronic acid, a glycosaminoglycan, is an important part of the expanded cumulus matrix (Ball et al. 1982). Hyaluronic acid has been proposed to bind to sperm plasma membrane PH-20. As a consequence these spermatozoa have higher basal calcium levels and are more responsive to induction of acrosome reaction after binding to the zona pellucida, at least in primates (Sabeur et al. 1998; Cherr et al. 1999). Hyaluronic acid might therefore be a candidate molecule to improve fertilization of cumulus-free oocytes.

A sperm motility-maintaining factor, which is heat-labile and non-dialysable, is secreted by bovine granulosa cells and oviductal cells (Ijaz et al. 1994). Interestingly, it is possible to partially mimic the beneficial effect of cumulus cells by adding oviduct cells to cumulus-denuded oocytes, which means that the cumulus effect is not completely dependent upon cell origin (Tanghe et al. 2001c).

If the beneficial effect of cumulus on fertilization cannot completely be maintained in the absence of living cells, two further approaches can be followed. First it can mean that the positive action of cumulus cells is located in its binding properties to the spermatozoa. This could be investigated by binding-inhibition studies, or by determining lectin-binding patterns. Second the presence of the cumulus cells may evoke the scavenging of toxic products of sperm metabolism or may even simply be involved in lowering oxygen tension in the immediate vicinity of the oocyte. This last hypothesis is also under investigation at the authors' laboratory.

Cumulus cells prevent oocyte changes that are unfavourable for fertilization

Oocyte changes that are unfavourable for fertilization are, for example, premature cortical exocytosis, resulting in zona hardening and subsequent decreased fertilization rates.

In mice, the removal of the cumulus oophorus during oocyte maturation results in an increased resistance of the zona pellucida to proteolytic digestion and sperm penetration (Downs et al. 1986). Furthermore, in pigs, mechanical removal of the cumulus cells resulted in premature cortical reaction (Galeati et al. 1991). These results support the hypothesis that the cumulus oophorus participates in the prevention of spontaneous zona hardening. However, in cattle it has been speculated that spontaneous zona hardening probably does not occur or does not prevent sperm penetration after maturation of both cumulus-intact or cumulus-denuded oocytes (Chian et al. 1994). This speculation remains to be proven by a zona digestion test. Damage to the oocyte or to the zona pellucida by cumulus removal is less probable, since it has been shown that fertilization rates are almost normalized by fertilizing cumulus-denuded oocytes on a cumulus monolayer (Tanghe et al. 2001a).

Conclusions

In conclusion it can be stated that there is substantial evidence that the cumulus oophorus is indeed important during fertilization, both under *in vivo* and *in vitro* conditions. They might be involved in the trapping of spermatozoa or guiding them to the oocyte: a chemotactic effect cannot be excluded thus far but needs to be confirmed in further studies. Some cumulus-specific secretions, which are different from progesterone, are partially responsible for the improvement of sperm penetration, but cell contact and maybe also secretions that are not derived from the cumulus seem to be necessary. Protection of the oocyte by the cumulus cells against zona hardening or against more subtle damage to the cytoplasm or sperm binding sites could be an issue but seems unlikely. The question as to how cumulus cells improve fertilization rates has not been completely resolved yet, but the answer can hopefully be given in the near future.

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