An Attempt to Reduce Polyspermic Penetration in Lamb Oocytes

(prepubertal oocyte / fertilization *in vitro* / polyspermy)

T. SLAVÍK[1], M. LIBIK[2], E. WIERZCHOS[2], J. FULKA[1]

[1]Institute of Animal Physiology and Genetics, Liběchov, Czech Republic
[2]University of Agriculture, Department of Sheep and Goat Breeding, Krakow, Poland

Abstract. The incidence of polyspermy in lamb oocytes matured and fertilized *in vitro* is very high and this results in a reduced developmental potential of embryos arising from them. We have attempted to produce oocytes more resistant to this fertilization anomaly. The oocytes from prepubertal lambs 7–12 weeks old were matured in a medium supplemented with various blood sera and oviductal fluid and fertilized *in vitro*. Significantly higher monospermic penetration was found in a medium supplemented with BSA – 3 mg/ml (63.9%) and OF – 20% concentration (55.8%). Lower monospermy was recorded in the presence of 10% LS (44.6%) or 10% SS (40.8%), and particularly in a medium with 10% FCS (26.9%). In contrast, high monospermy (78.7%) was observed in oocytes from adult donors matured and fertilized in an identical system. In another set of experiments we estimated whether polyspermy can be reduced by improvement of the cytoplasmic maturation of prepubertal oocytes using a two-step maturation protocol. After artificial arrest of the maturation for 24 h with a specific cdk inhibitor – BL-I, 50 μM – more than 80% oocytes from prepubertal and adult donors did not resume meiosis. When incubated thereafter in a drug-free medium for another 24 h, the oocytes of both categories progressed to MII in the rate comparable with control (80% to 90% MII). However, after fertilization no significant differences in the level of monospermic penetration was recorded between the arrested group (59.8%) and control (58.8%), both matured in the presence BSA, and 46.6% and 52.3% after treatment with OF. Also, no significant difference was observed between the arrested and control oocytes from adult donors (72.6% and 84.8%, respectively). These results suggest that high polyspermy in prepubertal oocytes is caused by developmental imperfection and can't be fully eliminated either by modifying the composition of culture media or by prolongation of the culture interval.

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Corresponding author: Tomáš Slavík, Institute of Animal Physiology and Genetics, 277 21 Liběchov, Czech Republic. E-mail: slavik@iapg.cas.cz

Abbreviations: BL-I – butyrolactone I, cdk – cyclin-dependent kinases, CG – cortical granule, FCS – foetal calf serum, GV – germinal vesicle, MII – metaphase II, LS – lamb serum, OF – oviductal fluid, SS – sheep serum, ZP – zona pellucida.

The oocytes obtained from pre-pubertal females represent a potential source of gametes for the production of embryos that could be then used in different breeding programmes, but attempts to exploit them gave almost exclusively disappointing results, because embryos derived from them are evidently developmentally less competent than those produced from oocytes obtained from cycling animals (Revel et al., 1995; O'Brien et al., 1996; Ledda et al.,1997; Ptak et al., 1999). Although the oocytes from juvenile donors are smaller in size, they resume and complete maturation when cultured *in vitro* similarly as oocytes collected from adult females (Ledda et al., 1997). The reason for their low developmental ability is not known in detail. It is generally accepted that structural and biochemical characteristics of embryos (lamb, calf) derived from oocytes obtained from juvenile donors do not secure their further regular cleavage (Ledda et al., 2000; Salamone et al., 2001). The fate of embryos originating from pre-pubertal donors is also negatively influenced by the high incidence of polyspermy. It was found that in oocytes from most adult mammals fertilized *in vivo*, zona pellucida (ZP) forms the main barrier to polyspermy. In oocytes matured *in vitro* and particularly in oocytes from pre-pubertal animals, ZP represents a less effective barrier against the polyspermy (Yanagimachi, 1988). The exposure of ovarian oocytes to the secretion of oviductal cells alters the surface properties of an extracellular investment, leading to the reduction of polyspermy in several species (Kano et al., 1994; Kim et al., 1996; Duby et al., 1997; Wang et al., 1998; Vatzias et al., 1999). In addition, Slavík and Fulka (1999) reported that oviductal secretion also contributes to the establishment of a ZP species-specific barrier leading to the protection of the oocyte against penetration by alien spermatozoa (Slavík et al., 1990; Slavík and Fulka, 1992).

In contrast to oocytes from adult donors, pre-pubertal oocytes also differ in certain parameters of cytoplasmic maturation, including the uneven distribution of cytoplasmic organelles, namely, cortical granules (CGs) (Gandolfi et al., 2000). The exocytosis of their content leads to a cortical reaction, which is followed by the zona reaction, and thus in the establishment of

the block against polyspermy. Under current culture conditions, the complete dispersion and localization of CGs underneath the plasma membrane, which is common for the final phases of physiological maturation, does not occur (Damiani et al., 1996). New two-step maturation protocols, which are based on a reversible arrest of oocytes at the germinal vesicle (GV) stage, provides the opportunity for prolongation of the culture interval and thus for eventual redistribution of cytoplasmic organelles resulting in the production of more competent oocytes. For the maturation arrest at GV stage, the oocytes are at present incubated with specific inhibitors of cyclin-dependent kinases (cdk) (Kubelka et al., 2000; Motlík et al., 2000). These treatments have no obvious detrimental effect on subsequent embryonic development after fertilization (Mermillod et al., 2000).

The aim of our experiments was to check whether various supplements to the maturation medium such as oviductal fluid (OF) or various molecules from blood sera can alter the properties of ZP and thus influence the frequency of polyspermic penetration in oocytes from pre-pubertal donors. Further, we investigated whether the incidence of polyspermy can be decreased when oocytes are matured in a two-step system.

Material and Methods

Donors of oocytes

As donors of oocytes we used 60 lambs at the age 7–12 weeks and 53 adult ewes, all of Olkulka-Polish prolific breed x Merino. Some animals in both categories were hormonally stimulated with FSH (Folicotropin, Spofa, Prague, Czech Republic) with 120 IU for lambs and 240 IU for ewes, applied in six consecutive equal doses in 12-h intervals. The animals were slaughtered 12 h after the last treatment.

Culture and fertilization media

The media used in these experiments were essentially identical to those described earlier by Pavlok et al. (1988). Unless mentioned otherwise, all chemicals were purchased from Sigma (St. Louis, MO).

The basic culture medium contained 8.4 ml TCM 199 (x10 conc., Sevac, Prague, Czech Republic), 3.8 ml $NaHCO_3$ (7.5% solution, Sevac), 9.5 mM HEPES, 1.82 mM sodium pyruvate, 2.27 mM calcium lactate; 50 IU/ml penicillin, K-salt; 50 IU/ml streptomycin sulphate, 125 ng/ml amphotericin B, and deionized nanopure filtered water added to 100 ml. Before use, this medium was supplemented with Pergonal (Serono, Rome, Italy) 1 IU/ml. To examine the effect of various macromolecules, the basic medium was supplemented with i/ either crystalline bovine serum albumin (BSA, 3 mg/ml), ii/ foetal calf serum (FCS, 10%), iii/ sheep serum (SS, 10%), iv/ lamb serum (LS, 10%) or v/ heat-inactivated sheep oviductal fluid (OF, 20%). To assess the inhibitory effect of butyrolactone I (BL-I, Funakoshi, Tokyo, Japan), the culture medium was supplemented with 50 μM of this drug.

OF was collected with permanent canullae inserted into oviducts of adult sheep during the breeding season, as described previously by Slavík et al., (2000). Supernatant, after centrifugation at 1000 *g* for 20 min, was heat-inactivated and stored at –20° C until use. Only samples taken at the oestrus were used for the experiments.

For sperm preparation and fertilization, the medium described by Brackett and Oliphant (1975) with slight modification was used. Its composition was as follows: 112 mM NaCl, 4.02 mM KCl, 2.25 mM $CaCl_2.2H_2O$, 0.83 mM $NaH_2PO_4.H_2O$, 0.52 mM $MgCl_2.6H_2O$, 37.0 mM $NaHCO_3$, 1.25 mM sodium pyruvate, 2.27 mM calcium lactate, 50 IU/ml penicillin K-salt, 50 IU/ml streptomycin sulphate, 125 ng/ml amphotericin, supplemented before use with BSA (3 mg/ml).

All experiments were carried out in Nunc 4-well dishes (Nunc, Roskilde, Denmark) at 38.5°C under a humidified atmosphere of 5% CO_2 in air.

In vitro maturation

Ovaries of slaughtered animals were transported at room temperature to the laboratory, rinsed briefly in ethyl alcohol, three times in physiological saline and sectioned serially in a manipulation medium supplemented with heparin (1000 IU/ml). After isolation of oocytes from ovaries and washing them in a culture medium without heparin, oocytes were cultured in 500 μl of media in an atmosphere of 5% CO_2 in air and temperature of 37°C for 24 h. The oocytes cultured initially with BL-I were washed after 24 h of culture four times in a drug-free medium and allowed to mature for another 24 h.

In vitro fertilization

Two hundred μl of freshly ejaculated ram semen were diluted with 2 ml of Brackett and Oliphant medium. Sperm suspension was centrifuged at 500 *g* for 10 min. The supernatant was discarded and washing was repeated three times. The concentration of the last sediment was adjusted to 10^6 sperm/ml and thereafter heparin was added (final concentration 5 IU/ml). The quality of spermatozoa was controlled and the suspension (1 ml) pipetted into fertilization dishes. Cumulus cells were removed by fine pipetting and oocytes were washed in several drops of the fertilization medium immediately before their transfer into the sperm suspension and incubated in it for 10 h.

Evaluation of oocytes

At the beginning and after each step of culture, some oocytes were fixed in acetic acid-ethanol (1 : 3) overnight, stained with 2% aceto-orcein and evaluated under a Nomarski interference contrast microscope.

The presence of metaphase II (MII) chromosomes and the first polar body served as a criterion for the completion of maturation. The oocytes with two pronuclei, two polar bodies and sperm tail in the ooplasm were judged as regularly fertilized. The oocytes with more than one male pronucleus were evaluated as polyspermic. The parthenogenetically activated oocytes containing two or more pronuclei without sperm tail, fragmented oocytes and oocytes with vacuolated ooplasm or pycnotic chromatin were not included in the evaluation.

Statistical analysis

χ^2-test has been used for data analysis (SigmaStat 3.0).

Results

All oocytes collected from prepubertal and adult donors were immature and contained, irrespective of hormonal stimulation, an intact GV. In controls that were evaluated 24 h after culture, nuclear maturation of more than 80% oocytes progressed, in all modified media with different supplements, up to MII.

The comparison of the effect of various supplements on the frequency of polyspermy is shown in Table 1. From the results is evident the high penetration rate in all experimental groups, irrespective of the previous treatment. However, significant differences were recorded in monospermic fertilization between groups of pre-pubertal oocytes. While oocytes matured in the presence of BSA were fertilized by one sperm in 63.9% instances, monospermy in the oocytes matured with FCS was observed only in 26.9%. Relatively positive results (55.8%) were found in the oocytes pretreated with OF. From Table 1 it is clear that monospermy in pre-pubertal oocytes remained far below the level of counterparts from adult females that were matured and fertilized in an identical system and achieved 78.7%. The results suggest that some, at present not yet defined molecules from blood sera and OF, are involved in sperm egg interaction and modify the properties of ZP, but they are in no case able to substantially reduce polyspermy in pre-pubertal oocytes.

Table 1. Penetration and monospermic fertilization of lamb oocytes matured in media supplemented with BSA, LS, SS, FCS or OF and oocytes from adult donors matured in the presence of BSA (4 replicates)

Supplement of maturation medium	No. of oocytes	Penetration rate (%)	Monospermic fertilization (%)
LS	74	82.4	44.6
SS	71	74.6	40.8[d]
FCS	78	89.7	26.9[a,b,c]
BSA	72	84.7	63.9[a]
OF	156	77.6	55.8[c]
A/BSA	128	84.5	78.7[b,d]

Superscripts of different letters are significantly different ($P < 0.05$). A – adult

The results in Table 2 show the ability of BL-I to arrest oocytes from pre-pubertal and adult donors at GV stage for 24 h. After this period 81.8% oocytes from adult and 83.6% from lambs did not resume meiosis. Control oocytes of both categories progressed in BL-I-free medium to MII in 83.7% and 86.5% instances. After further cultivation of inhibited oocytes for another 24 h in a BL-I-free medium, in 87.5% oocytes from adult donors and in 81.0% oocytes from lambs, MII and first polar bodies were visible. Fertilization and the incidence of monospermy are summarized in Table 3. As in previous investigation, a high penetration rate was typical for all experimental groups. However, the best results were again in the oocytes from adult donors, irrespective of previous treatment with BL-I. In prepubertal oocytes that were matured in the presence of BSA or OF and arrested transiently in GV, no significant improvement in monospermic penetration was recorded. The results with BSA-matured control oocytes (59.8%) were fully comparable with reversibly inhibited oocytes (58.8%). In the oocytes matured with OF the monospermic fertilization was a little lower, but no significant differences were found between the control and the experimental group (52.3% and 46.6%, respectively). The results suggest that oocytes from prepubertal and adult donors can be safely and reversibly arrested in GV stage for at least 24 h, but this treatment

Table 2. Maturation of lamb and adult sheep oocytes in control and BL-I-supplemented medium and reversibility of inhibition after 24 h (3 replicates)

Medium	No. of cultured oocytes	Stage of maturation (%) of oocytes cultured for 24 h		24 h in BL-I-free medium	
		GV	MII	No. of oocytes	No. (%) of MII oocytes
A (control)	43		36 (83.7)		
A+BL-I	33	27 (81.8)		40	35 (87.5)
L (control)	37		32 (86.5)		
L/BL-I	61	51 (83.6)		42	34 (81.0)

A – oocytes from adult donors; L – oocytes from lambs

Table 3. Penetration and monospermic fertilization of lamb and adult sheep oocytes arrested for 24 h in GV stage with BL-I. The oocytes were matured in the presence BSA or OS (4 replicates).

Oocytes / maturation medium	Total No. of oocytes	Penetrated oocytes (%)	Monospermic fertilization (%)
A/BL-I/BSA	62	79.0	72.6
A/BSA	66	88.9	84.8
L/BL-I/BSA	228	80.8	59.8
L/BSA	267	78.3	58.8
L/BL-I/OF	249	77.1	46.6
L/OF	193	72.5	52.3

A – adult; L – lamb

has little benefit on the intrinsic deficiency of pre-pubertal oocytes.

Discussion

The aim of our study was to analyse the possible contribution of various treatments on the incidence of polyspermic penetration of oocytes obtained from the lambs and to check whether this fertilization anomaly can be reduced. The present results show that the hormonal treatment of lambs aged 8–12 weeks stimulates their ovaries, which may then be used as a source of oocytes. The exploitation of these oocytes for the production of viable embryos remains restricted. As our experiments and previous studies indicate (Ledda et al., 1997; O'Brien et al., 1997), these oocytes mature well in culture, but the high polyspermy represents, among others, a serious problem, which is responsible for subsequent developmental failure of embryos produced from these oocytes. The high polyspermy is also a problem in *in vitro* matured oocytes in some other mammals, particularly in the pig (Wang et al., 1991; Wang et al., 1994; Kouba et al., 2000). Some experiments also showed certain differences in the level of polyspermic penetration between ovulated and *in vitro* matured oocytes (Kim et al., 1996; Wang et al., 1998). This allowed us to hypothesize that some molecules of oviductal origin associate with oocytes and regulate the sperm-egg interactions. Several reports support this hypothesis and demonstrate that the oviductal secretion (Kouba et al., 2000) and oviduct cell-conditioned media (Nagai and Moor, 1990; Kano et al., 1994; Vatzias et al., 1999) significantly reduce the polyspermic penetration *in vitro*. It was also observed that a species-specific barrier, which is absent in ovine and bovine oocytes matured in culture, can be almost fully established when these oocytes are incubated in the medium supplemented with oestrus oviductal secretion (Slavík et al., 1990; Slavík and Fulka, 1999). The mechanisms involved in the formation of a species-specific barrier and the functional block to polyspermy are still unknown. It is speculated that certain oviductal proteins in the incubation medium compete with sperm receptors for binding of ZP ligands and stimulate the rate of acrosome reaction and thus reduce the number of capacitated spermatozoa that can attach to the surface of the oocytes (Funahashi and Day, 1997).

The aim of the present experiments was to assess whether certain molecules from OF and blood sera can influence the polyspermy of pre-pubertal lamb oocytes. It appeared from previous observations that particularly the oviductal fluid might be a good candidate for this purpose. In fact, a certain variability in the frequency of polyspermy after supplementation of the maturation medium with various sera was recorded. The highest polyspermic penetration was observed in the oocytes matured in the presence of FCS. These results are in agreement with those published by Cran and Cheng (1986), who found incomplete CG exocytosis in the presence of FCS, resulting in delayed ZP block. On the other hand, lower polyspermy in the oocytes matured in the medium with BSA might be caused, among others, by the acceleration of cortical reaction and induction of more effective ZP block. We have, however, expected a much higher effect of OF after its addition to the culture media. In any case the monospermic penetration rate of lamb oocytes was comparable with that observed in oocytes from adult donors matured and fertilized in an identical system. In addition, the polyspermy in ovulated ewe oocytes after fertilization *in vitro* occurs very rarely (unpublished observations). It cannot be excluded that 20% of OF in a final volume of the maturation medium was not the optimal concentration. On the other hand, the same volume of OF was effective in establishing a species-specific block in *in vitro* matured ovine oocytes (Slavík and Fulka, 1999) and for improving the ZP reaction of porcine ovarian oocytes (Kim et al.,1996). The presented data show that biologically active molecules might be involved in the sperm-egg interaction, but in any case they could reduce the frequency of polyspermy substantially. Lower polyspermy in oocytes from adult donors treated similarly suggests that this fertilization anomaly was not caused by external factors only and thus it may be attributed to an intrinsic deficiency of lamb oocytes.

It has been shown that final stages of maturation are accompanied, among other events, by the redistribution of cytoplasmic organelles, including CGs (Hyttel et al., 1997), which are essential for the induction of cortical

and zona reactions. In oocytes from pre-pubertal donors these structural changes are delayed and incomplete and may contribute to failures of appropriate ZP alterations (Gandolfi et al., 2000). New maturation protocols based on the reversible arrest of nuclear maturation by specific inhibitors of cdk provide an opportunity to gain additional time for a more physiological localization of cytoplasmic organelles. BL-I and roscovitine, which are most commonly used for this purpose, block mammalian oocytes at GV stage for at least the first 24 h of the initial phase of cultivation, and this prolongation of maturation interval may hypothetically provide additional time-space for the realization of structural and biochemical cytoplasmic changes. After subsequent cultivation in a drug-free medium, bovine and porcine oocytes mature to MII at a high rate (Kubelka at al., 2000; Mermillod et al., 2000; Motlík et al., 2000), undergo normal fertilization and develop to healthy looking blastocysts. By using this approach, we wanted to generate more competent lamb oocytes that would also be better protected against polyspermic penetration.

At least the experiments with growing bovine oocytes that did not resume meiosis *in vitro* indicated a significant enhancement of meiotic competence after pre-incubation in the same concentration of BL-I. They matured afterwards at a high rate and synthesized, in collaboration with cumulus cells, vitally important molecules necessary for maturation and further development after fertilization (Pavlok et al., 2000). The results of our studies show that a low concentration of BL-I indeed blocks lamb and ewe oocytes at GV stage very effectively for at least 24 h. After subsequent incubation in a drug-free medium for another 24 h the oocytes of both categories completed maturation to MII at a rate comparable with controls. However, no beneficial effect of this transient maturation arrest on the frequency of polyspermy was observed. Whether the period of nuclear arrest used in our experiments was too short to induce the expected cytoplasmic changes or whether, due to their biochemical, structural and physiological properties, oocytes from pre-pubertal donors were not able to respond to such treatments remains unclear.

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