# Influence of Oviductal Fluid on Ovine Embryo Viability

(embryo mortality / *in vitro* maturation / *in vitro* fertilization / embryo transfer / oviductal fluid )

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**Abstract. The severe loss of developmental competence affecting fertilized ova when removed from the oviductal environment suggests that this organ plays a functional role in early embryonic development. The purpose of this study was to determine the effect of sheep heat-inactivated OF on the mortality rate of ovine embryos produced *in vitro* and transferred into recipients. As control groups we used embryos fertilized and cultured in media supplemented with different kinds of proteins (FCS, BSA). Transfer of embryos in the two pronuclei stage to the oviducts of synchronized recipients resulted in 60% of successfully termed pregnancies after incubation of embryos in OF, 40% in BSA and only 10% after FCS. All ewes were further assessed for pregnancy by ultrasonography 33, 53 and 80 days after embryo transfer. The highest embryo mortality appeared between day 33 and 52. We concluded that incubation of ovine oocytes in OF during the final period of the maturation process may play a functional role at the time of fertilization and early embryonic development.**

There have been several attempts to create an optimal procedure for ovine embryo culture with the aim to avoid the high incidence of embryo mortality and appearance of some foetal abnormalities. Enhanced embryonic development has been noted when embryos were co-cultured with the intact oviductins (Minami et al., 1988), oviductal epithelium (Gandolfi and Moor, 1987) and oviduct-conditioned medium (Eyestone and First, 1989). While successful *in vitro* fertilization and embryonic development can also be achieved in the absence of these components, it was suggested that oviductal fluid (OF) and oviductal cells play a facilitatory role in fertilization and embryo development. Although the precise function of these proteins has not been determined, many studies have demonstrated that oviductal fluid proteins associate with the zona pellucida both *in vivo* and *in vitro*, suggesting a possible role in fertilization or embryonic development. In 1995, Nancarrow and Hill conducted studies on the effect of oviductal proteins (oEGP) on embryo development from fertilization or the first cleavage stage through to a hatched blastocyst and showed that these glycoproteins appear to regulate cell division and blastocyst formation rate. Studies by Broermann et al. (1989) indicated that pig follicular oocytes or embryos exposed to the oviduct microenvironment were more resistant to proteolysis than oocytes without oviductal exposure or embryos collected from the uterine environment, suggesting the uptake of protective agents from the oviduct. The presence of two identified major protease inhibitors in OF may account for this increased resistance to proteases by oocytes and embryos (Buhi et al., 1997; Kouba et al., 1997). The rate of blastocyst formation from ovine oocytes widely fluctuates, and survival to term after transfer of ovine-derived embryos is generally low (O'Brien et al., 1996; Ptak et al., 1999). The reasons for this high incidence of foetal loss are largely unknown.

We tested the hypothesis whether addition of OF to culture medium improves the developmental competence of embryos *in vitro* and after transplantation the embryo mortality *in vivo*.

## Material and Methods

### *Collection of OF*

As described previously, OF has been collected with permanent canullae inserted to the oviduct of adult sheep during the breeding season as we described previously (Slavík et al., 2000). Only samples taken at oestrus were used for the experiments. The OF was centrifuged. Supernatants after centrifugation at 1000 *g* for 20 min were heat-inactivated.

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Abbreviations: BSA – bovine serum albumin, FCS – foetal calf serum, IVF – *in vitro* fertilization, IVM – *in vitro* maturation, MM – maturation medium, OF – oviductal fluid.

### *In vitro maturation*

Composition of media used has been identical as described by Pavlok et al. (1988). If not noted else, all chemicals were purchased from Sigma (St. Louis, MO). Briefly, manipulating medium (MM) contained: 9.4 ml 10 x TCM 199 (Sevac Praha, Czech Republic); 2.1 ml 7.5% $NaHCO_3$ (Sevac); 9.5 mM HEPES, 1.82 mM Na-pyruvate, 3 $mg.ml^{-1}$ polyvinyl alcohol (PVA); 50 $IU.ml^{-1}$ penicillin K-salt, 50 $IU.ml^{-1}$ streptomycin sulphate; 125 $ng.ml^{-1}$ amphotericin B; deionized nanopure filtered water ad 100 ml. For the second step of culture, medium stimulating meiosis (MSM) was used, containing 8.4 ml 10 x TCM 199; 3.8 ml 7.5% $NaHCO_3$; 9.5 mM HEPES, 1.82 mM sodium pyruvate, 2.27 mM calcium lactate; 50 IU/ml penicillin K-salt, 50 $IU.ml^{-1}$ streptomycin sulphate, 125 $ng.ml^{-1}$ amphotericin B, and deionized nanopure filtered water ad 100 ml. Before use, this medium was supplemented with Pergonal (Serono, Roma, Italy) 1 $IU.ml^{-1}$ and one of the following proteins: 3 $mg.ml^{-1}$ crystalline bovine serum albumin (BSA), foetal calf serum (10% FCS) or heat-inactivated sheep OF (20% OF). Cultures were carried out at 38.5°C under humidified atmosphere of 5% $CO_2$ in air, and 4-well Nunclon dishes were used for all procedures, including *in vitro* fertilization.

### *In vitro fertilization*

Only ejaculates showing more than 80% of progressive motility after collection have been used. The same ejaculates were used in parallel experiments.

Two hundred microliters of freshly ejaculated ram semen were diluted with 2 ml of medium described previously by Oliphant and Brackett (1975) composed of 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 $IU.ml^{-1}$ penicillin K-salt, 50 $IU.ml^{-1}$ streptomycin sulphate, 125 $ng.ml^{-1}$ amphotericin B supplemented before use with BSA (3 $\mu g.ml^{-1}$). Sperm suspension was centrifuged at 500 *g* for 10 min. Supernatant was removed and washing was repeated three times. The concentration of the last sediment was set to $10^6.ml^{-1}$, heparin was added at a final concentration 5 $IU.ml^{-1}$ and 1 ml aliquots of suspension were divided into a fertilization dish (4-well Nunclon). Cumuli of the oocytes were removed by fine pipeting, oocytes were washed in drops of fertilization medium and immediately added to sperm suspension.

### *Evaluation of oocytes*

After 24 h under culture conditions described above, part of oocytes were fixed in acetyl alcohol (1 : 3), stained with 2% aceto-orcein and evaluated under a Nomarski interference contrast microscope. As fertilized were assigned oocytes with two pronuclei, two polar bodies and a sperm tail in the ooplasm.

### *Recipients and embryo transfer*

The oestrous cycles of recipients were synchronized in 30 matured Polish Longwool sheep ewes by placing Chronogest (Intervet) sponges 40 mg into vagina for 14 days. On the day of sponge removal, the ewes were administered 500 IU of pregnant mare serum gonadotropin (PMSG) (Bioveta, Ivanovice na Hané, Czech Republic). This treatment is commonly used under field conditions. The onset of oestrus was designated as day 0. Embryos in the two pronuclei stage were transferred via ampulla to the oviducts of recipients using a thin glass pipette. Into each oviduct, 6 embryos were introduced.

### *Pregnancy diagnosis*

The numbers of foetuses were recorded using ultrasonography at 33, 53 and 80 days after embryo transfer. Pregnant ewes were then allowed to proceed to term.

## Results

### *In vitro maturation and fertilization*

The proportions of oocytes undergoing maturation and fertilization are presented in Table 1. The cytological evaluation of embryos before cleavage revealed a high penetration rate of fertilization and it did not differ significantly between groups of oocytes matured in media supplemented with different proteins. The highest penetration rates were noted for oocytes matured in media supplemented with FCS (89.7%), but concomitantly this group was characterized with significantly increased incidence of polyspermic fertilization. The monospermic fertilization rate fluctuated notably between groups. While in the group where BSA was used we found 63.9 % monospermically fertilized oocytes, in the group with FCS it was less than 30%.

### *Pregnancy rate and embryo survival*

Data for the number of recipient ewes pregnant, as determined by ultrasound, are presented in Table 2. To test the developmental ability, embryos in the two pronuclei stage were transferred into recipients. Embryos from group 3 were characterized as the highest rate of embryo viability *in vivo* after transfer, embryo survival recorded at ultrasound sonography exceeded 60%, which, in comparison to group 1, was a statistically significant difference. In group 1, a very high rate of embryo mortality was noted, only one pregnancy developed to 80 days.

The highest loss of embryos in all experimental groups appeared between days 33 and 53. One abortion on day 65 of gestation was noted in group 1 (lamb from twin pregnancy).

*Table 1. Effect of different protein media supplement on the penetration rate of ram sperm into sheep oocytes matured in vitro*

| Groups | Protein in culture medium (%) | Number of oocytes | Penetration rate (%) | Monospermically fertilized oocytes (%) |
|---|---|---|---|---|
| 1. | (FCS) | 78 | 89.7 | 26.9 |
| 2 | (BSA) | 72 | 84.7 | 63.9 |
| 3 | (OF) | 156 | 77.6 | 55.8 |

*Table 2. In vivo survival of sheep embryos produced in vitro and transferred to recipients*

| Groups | No. of recipients | No. of recipients pregnant | | |
|---|---|---|---|---|
| | | at 33 days (%) | at 53 days (%) | at 80 days (%) |
| 1 (FCS) | 10 | 5 (50) | 2 (20) | 1 (10) |
| 2 (BSA) | 10 | 8 (80) | 4 (40) | 4 (40) |
| 3 (OF) | 10 | 7 (70) | 6 (60) | 6 (60) |

## Discussion

The production of a viable oocyte is the first step towards a successful *in vitro* embryo achievement. Because it is known that embryonic development can be readily compromised by deficiencies introduced during the process of oocyte maturation (Moor et al., 1998), in our experiment we tried to improve this process by introducing OF to the culture medium.

OF proteins have been found to associate with the zona pellucida in many species including sheep (Gandolfi et al., 1989). The oviduct provides a passive source of protein, derived from serum and other fluids as a transudate. The major components are serum proteins, but several biologically active substances are also synthesized in the oviduct. Protein composition of OF has been described repeatedly, but the latest studies have shown that the secreted proteins include an oestrogen-dependent oviduct-specific glycoprotein, which is significantly conserved across many species (Buhi et al., 2000). Total protein has been measured and in general appears to be lower in comparison with serum (Hunter, 1988), and that is why in our experiment we used 20% OF to increase the protein level to become comparable with media containing 10% of serum.

Embryo mortality is a very common and difficult problem of ovine embryo production *in vitro*. Acquisition of the developmental competence involves synthesis and storage of a wide range of molecules during oocyte maturation, fertilization and early embryogenesis. After finished maturation, oocytes are exposed to a very high concentration of spermatozoa during IVF, which may ultimately have a negative effect on embryonic development and implantation, since dying spermatozoa produce oxygen radicals (Aitken, 1995; Aitken et al., 1996). Based on the fact that oviduct most probably provides the optimal conditions for early reproductive events, embryonic development and implantation rate (Hunter, 1988), we paid great attention to oviductal secretion as the protein supplement in the conducted experiments.

The incidence of early embryonic loss in adult sheep after natural mating is generally 20-30% and occurs predominantly during the first 5 weeks of pregnancy (Edey, 1976). Death of cultured embryos following transfer occurs largely between approximately day 15 and day 60 of pregnancy, most likely around the time of implantation (Bolet, 1986) and for all postcompaction-stage embryos; regardless of the source. The ability to elongate and to begin the initial stages of organogenesis in conjunction with attachment to the endometrium is the most critical time for the developing of ovine conceptus (Thompson et al., 1994). Thompson (1997) has demonstrated that *in vitro* produced embryos have a low survival rate (40%), and embryo/foetal loss occurs at 30–35 days after transfer.

In addition to implantation and organogenesis, the embryo mortality depends on several factors such as *in vitro* culture system, stage and number of transferred embryos. Slavík and Fulka (1992) received a high embryo mortality rate following transfer of a large number of embryos produced *in vitro* from randomly chosen oocytes. This factor also affects the embryonic mortality in prolific breeds (Hanrahan, 1980). Furthermore, Kleeman et al. (1990) have demonstrated that the rate of embryonic loss increases with an increasing ovulation rate. In our study we used one defined sheep breed as donors and recipients. Survival of embryos following incubation of oocytes in OF exceeds 60% after the transfer. Supplementation of culture medium with FCS did not appear to be very successful. Only 1 out of 10 ewes was pregnant at 80$^{th}$ day of gestation. This high loss rate was probably due to an increased incidence of polyspermic fertilization followed by a reduced developmental capacity of embryos. There were no statistical differences between groups; that is why we claim that OF can be an alternative source of protein supplement to the culture media. Due to the presence of biologically active substances it may represent conditions more close to those *in vivo*. After transfer of embryos incubated in oviductal pro-

teins, the large offspring syndrome was not recorded, while in the group of embryos using a routine protocol, large offsprings were observed (our preliminary experiments). More extended experiments focused on the effect of oviductal proteins on the incidence of the large offspring syndrome are in run.

Perhaps, culture of embryos *in vitro* to the morula or blastocyst stage could increase the pregnancy rate and allow selection based on the embryo viability before transfer. Using this scheme, O'Brien et al. (1996) received 65.7% pregnant recipients diagnosed by ultrasound following transfer of embryos in the blastocyst stage. In our study we transplanted embryos in the two pronuclei stage, avoiding long-term *in vitro* culture of embryos. Our method did not allow selection based on the advanced cell stage; on the other hand, this system maximizes exposure of embryos to the oviductal environment. In summary, OF added to the culture medium increased the survival rate after embryo transfer into recipients.

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