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Original article

In vitro techniques of bovine oocyte maturation, fertilization and embryo culture resulting in the birth of a calf

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Summary — Oocyte cumulus complexes were aspirated from 3 to 5 mm follicles of cows prestimulated with 2.000 IU PMSG 24 h before slaughter. Oocytes matured in culture were fertilized *in vitro* by heparinized freshly ejaculated or epididymal spermatozoa. The cultivation procedure for fertilized eggs was the same as that used for cultivation of oocytes. From 163 matured oocytes, 109 cleaved to the 2-cell stage 24 h after fertilization and after 6 days of cultivation, 18 developed to the late morula and 18 to the blastocyst stages. Eleven blastocyts and 1 late morula were transferred surgically to the uteri of 7 recipient heifers. Two heifers became pregnant : one delivered a bull-calf at term, while the other pregnancy resulted in abortion at the 3rd month. The examination of some embryos by transmission electron microscopy showed an almost normal morphology for most cells. The degenerated cells contained mostly electron-dense residual bodies of unknown origin.

fertilization *in vitro* — development — bovine embryos

Résumé — Techniques de maturation ovocytaire, de fécondation et de culture embryonnaire *in vitro* permettant l'obtention d'un veau. *Des ovocytes entourés de leur* cumulus oophorus *sont aspirés de follicules de 3 à 5 mm de diamètre prélevés sur des vaches traitées avec 2 000 UI de PMSG 24 h avant l'abattage. Les ovocytes sont cultivés jusqu'à maturation, puis fécondés* in vitro *avec des spermatozoïdes héparinisés juste après récolte (sperme éjaculé ou épididymaire), d'après Pavlok* et al. *(1988). La technique de culture* in vitro *des œufs fécondés est la même que celle employée pour les ovocytes. Sur 163 ovocytes maturés, 109 se segmentent une fois en 24 h après fécondation et, au bout de 6 jours de culture, 18 atteignent le stade morula tardive et 18 le stade blastocyste. Après transfert chirurgical intra-utérin chez 7 génisses de 11 blatocystes et une morula, deux receveuses sont gestantes. Une met bas un veau mâle à terme, l'autre avorte au 3e mois. L'examen de quelques embryons au microscope électronique à transmission révèle une morphologie normale pour la plupart des cellules. Les cellules dégénérées renferment des corps résiduels opaques aux électrons.*

fécondation* in vitro — *développement* — *embryon bovin

INTRODUCTION

As described by Bavister (1988) the cleavage of cattle one-cell zygotes cultured *in vitro* is blocked at the 8-cell stage. However, follicular oocytes, matured and fertilized *in vitro*, can overcome this 8-cell block and develop to the morula or blastocyst stage, after deposition into oviducts of the same or foreign species (Boland, 1984; Sirard *et al.*, 1985; Lu *et al.*, 1987) or in coculture with oviductal or follicular cells (Eystone *et al.*, 1987; Fuduka *et al.*, 1988; Lu *et al.*, 1988; Thibault *et al.*, 1988).

This paper describes a simple and effective culture technique which supports the development of a high proportion of zygotes up to the morula and blastocyst stages. The transfer *in vivo* of some of them was used to give us information about the viability of such embryos. A short communication on the results obtained has recently been presented in Czech (Pavlok *et al.*, 1988a).

MATERIALS AND METHODS

Oocyte cumulus complexes were aspirated from 3—5 mm diameter follicles of slaughtered cows stimulated 24 h previously with 2 000 IU PMSG. Before treatment with PMSG, animals were preselected according to the presence of well-developed corpora lutea.

Modified Parker's medium (M 199; Sevac-Praha) supplemented with 2.92 mmol/l Ca-lactate, 2 mmol/l Na-pyruvate, 33.9 mmol/l Na-bicarbonate, 4.43 mmol/l Hepes buffer, 50 IU/ml penicillin, 50 µl/ml streptomycin sulphate and 20% of bovine inactivated serum (BOS; Sevac-Praha) designated as "MPM 20" was used for culture of oocyte cumulus complexes. This culture medium (150 µl) was placed in a watch-glass under paraffin oil and equilibrated at a temperature of 38.5 °C in 4% CO_2, 10% O_2 and 86% N_2 for at least 2 h. The culture of oocytes ≈ 20—30 per watch-glass was carried out for 24—26 h.

Four hundred µl of ejaculated semen of good sperm motility were diluted with MPM + 10% BOS ("MPM 10") 1—3 h after collection and centrifuged at ≈ 700 *g* in 4 ml tubes for 10 min. After discarding the supernatant, this procedure was repeated once with MPM 10 and once with 100 IU/ml of heparin in Krebs—Ringer phosphate. The appropriate portion of the centrifuged heparin-treated spermatozoa was resuspended in 1 ml of MPM 10 to a concentration of approximately 1.0—2.0 x 10^8/ml and incubated for 40—60 min at 38.5 °C. To obtain the epididymal spermatozoa, the ducti deferentes with adjacent distal caudae epididymis were dissected and spermatozoa flushed with 2 ml of MPM 10, stored at room temperature for ≈ 20 h. Of these spermatozoa 200—300 µl aliquots were treated as freshly collected ejaculates.

At the end of culture, the oocytes with expanded cumuli were transferred into 4—5 ml of MPM 10 and mixed with the upper layer of the nonsedimented part of preincubated spermatozoa to give a final concentration of 1.0—2.0 x 10^6 spermatozoa/ml. For fertilization and subsequent culture gametes and zygotes were maintained at the same temperature and atmosphere as the cultured oocytes. Twenty to 24-h-old zygotes together with the remains of adherent follicular cells were transferred into 150 µl of MPM 20 and cultured in the same manner as the oocytes. This medium was changed only once (after ≈ 90 h) during the whole culture period. At the same time, the evidently degenerated or retarded embryos were removed from the culture, together with the remains of follicular cells surrounding all embryos. The healthy-looking embryos without follicular cells were cultured 1—2 days more (see Table I).

Seven recipient heifers were synchronized with 500 µg of cloprostenol (Oestrophan— Spofa) injected during the luteal phase of their cycle to induce estrus at the day of the start of donor oocytes cultivation. Nine 7-day, two 6-day blastocysts and one 6-day morula from all 4 experiments were surgically transferred to the uteri of synchronized recipients (Table I).

Embryos not used for transfer were fixed in acetic acid—ethanol (1 : 3) for 24 h, stained

Table I. Development of bovins embryos cultured *in vitro*.

No. of			*Development in culture 7 days after fertilization (No. %)*					
Experiment	*animals*	*oocytes*	*non cleaved*	*2—7 cells°*	*8—31 cells°*	*> 32 cells*	*blastocysts (> 64 cells)*	*embryos for transfer*
1	5	54	16 / 29.6	10 / 18.5	7 / 13.0	7 / 13.0	10 / 18.5	4 / 7.4
2	4	30	13 / 43.4	4 / 13.3	1 / 3.3	3 / 10.0	6 / 20.0	3 / 10.0++
3	6	62	19 / 30.6	28 / 45.1	4 / 6.5	7 / 11.3	— / 0.0	4 / 6.5
4	2	17	6 / 35.3	4 / 23.5	3 / 17.6	1 / 5.9	2 / 11.8	1 / 5.9 +
Total	17	163	54 / 33.2	46 / 28.2	15 / 9.2	18 / 11.0	18 / 11.0	12 / 7.4

° Most cells showed degenerative changes.
+ 1 pregnancy originated from 6th-day early blastocyst resulted in abortion.
++ 1 pregnancy resulted in delivery of normal bull-calf after transfer of 2 6-day embryos (1 morula, 1 early blastocyst).

with 1% acetic orcein, differentiated in 30—50% acetic acid and evaluated by phase contrast microscopy. Attention was paid to the number of cells of each embryo.

In addition, 4 blastocysts were fixed, stained and sectioned for electron microscopy as described earlier (Fléchon and Pavlok, 1986). Ultrathin sections were examined with a Jeol 1200 EX electron microscope.

RESULTS

From 163 cultured and inseminated oocytes, 109 cleaved to the 2-cell stage ≈ 24 h after sperm—egg mixing. During the subsequent 6 days of *in vitro* cultivation, 18 embryos developed to the morula stage (> 32 cells) and 18 to the blastocyst stage (> 64 cells) (Table I, Fig. 1). Many blastocysts started to expand (Fig. 2).

The transfer of 11 blastocysts and 1 morula to the uteri of 7 recipients resulted in two pregnancies. One healthy bull-calf (from experiment 2, Table I) was born at term; however, the second pregnancy (from experiment 4, Table I) resulted in abortion at the 3rd month.

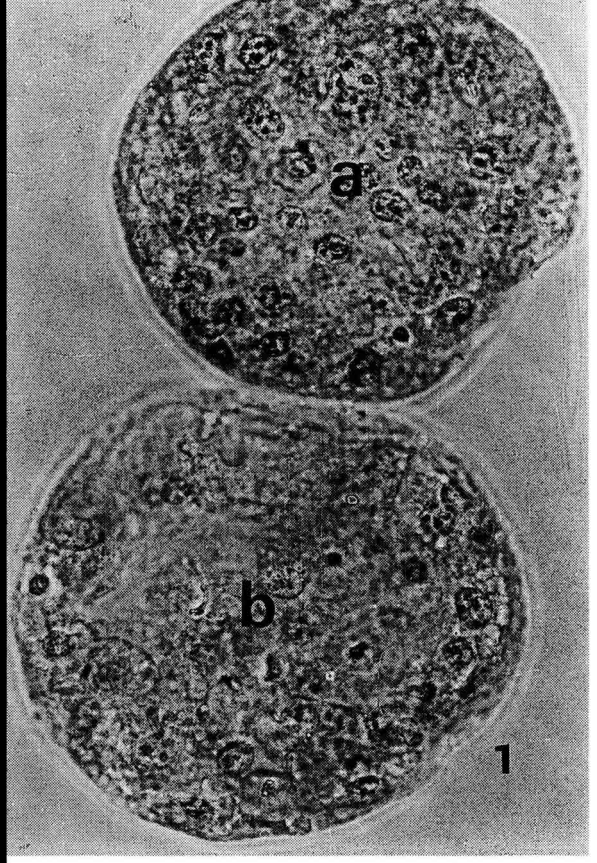
The observation of blastocysts in TEM showed that in healthy-looking blastocysts most of the cells were morphologically normal but contained a large amount of lipid material (Fig. 3). In well-developed blastocysts, only 1 or 2 cells per a middle embryoblast section contained electron-dense residual bodies as a sign of degenerative changes. In contrast, the electron-dense residual bodies were found in about a quarter or more of embryoblast cells of two retarded blastocysts (darker under the stereomicroscope and with a smaller irregular blastocell cavity).

DISCUSSION

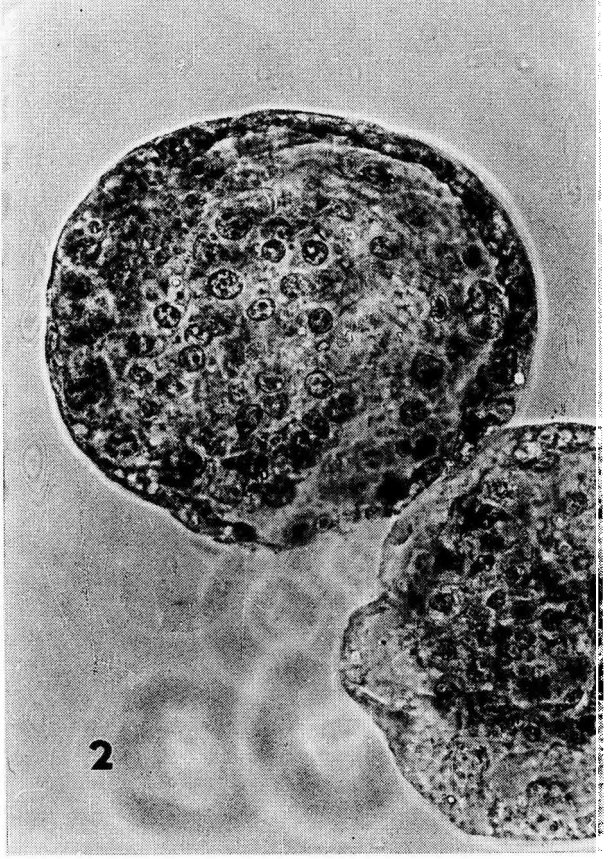
When bovine embryos were cocultured with a monolayer of oviductal cells (Eystone *et al.*, 1987; Lu *et al.*, 1988; Fukui and Ono, 1988; Thibault, 1988), or with granulosa cells (Fukuda *et al.*, 1988), the 8-cell cleavage block (Bavister, 1988) was somehow suppressed and some embryos were able to develop to the morula or blastocyst stage.

The authors cited above used mainly M 199 or Ham F 10 media, buffered either with Na-bicarbonate or Hepes and supplemented with fetal calf serum or œstrus cow serum. In our experiments the development of zygotes was also secured with M 199 and bovine serum, but in addition with increased amount of Na-pyruvate and with a combination of two buffer systems. In the absence of added cells, the development of embryos was probably supported by adherent corona radiata cells which surrounded practically all successfully developing embryos. As described earlier, these cells were easily removed mechanically 90 h later.

Figs. 1—3. Day 7 blastocysts after in vitro fertilization and cultivation observed by phase-contrast light microscopy (Figs. 1, 2) and transmission electron microscopy (Fig. 3). **1,** Early (**a**) and well-developed (**b**) blastocysts mounted *in toto,* fixed in acetic alcohol and stained with aceto-orcein. (≈ x 340). **2,** Expanded blastocyst (slightly collapsed during mounting on the slide) fixed and stained as those in Fig. 1. (≈ x 340). **3,** Section through the trophoblast (**T**) and the embryoblast (**E**) of day 7 blastocyst. Most of the cells appear normal, although containing large amount of lipids (**L**) and sometimes dense bodies (D).



1



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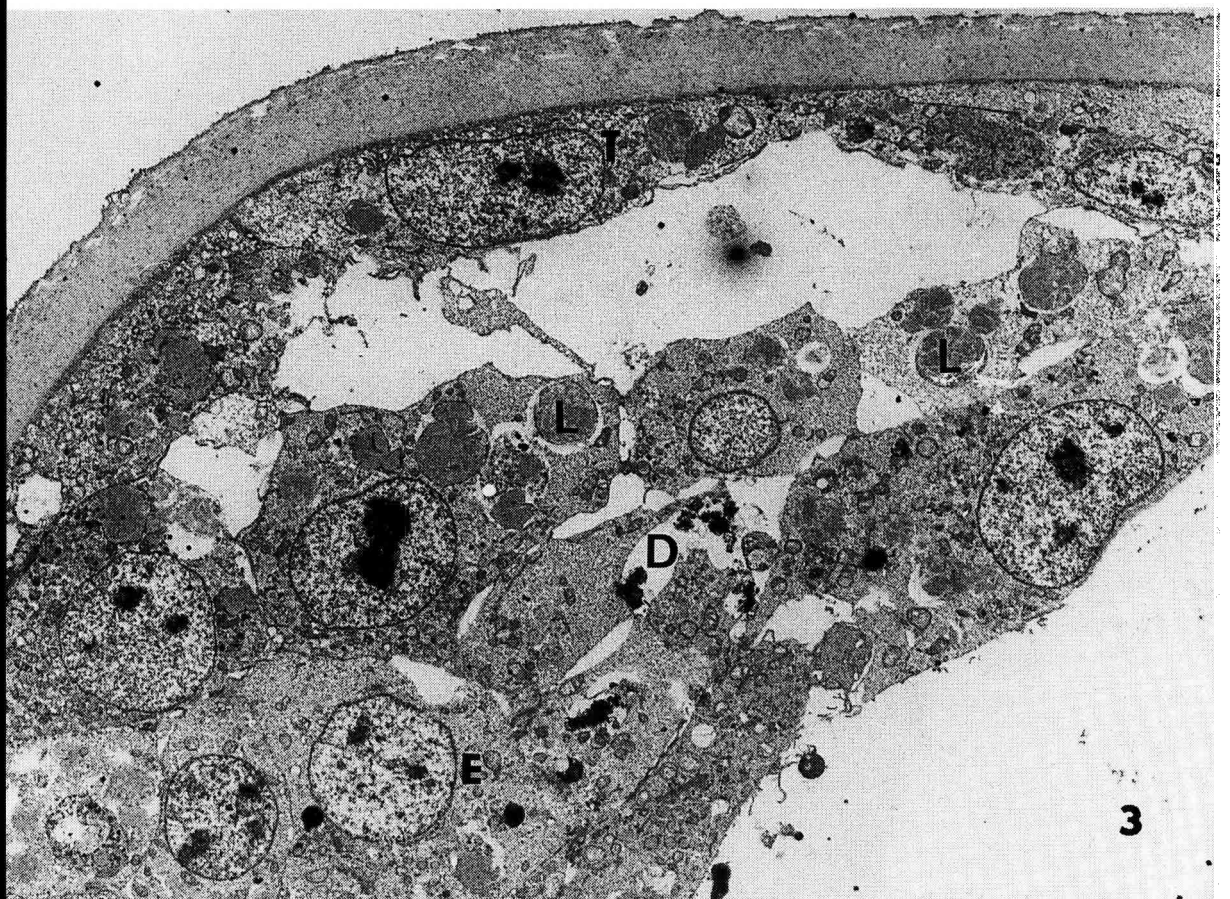

3

The ultrastructural study of *in vitro* developed blastocysts revealed healthy or degenerated cells as in blastocysts developed *in vivo* (Pivko *et al.*, 1986). However, we noted a very high density of lipid inclusions in the cells of blastocysts developed *in vitro*. This observation could correlate with a frequent lower transparency of practically all *in vitro* cultivated embryos under the stereomicroscope. The study of ultrastructure in connection with synthetic activities of single cells is in progress.

Even if the present technique for culture of bovine embryos seems to be superior to culture methods described by the authors cited above and also apparently to the culture in sheep oviduct (Sirard *et al.*, 1988), more effort is still necessary to obtain normal blastocysts under well-defined *in vitro* conditions and further research is required to understand the regulatory mechanisms of early embryonic development.

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