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Some factors affecting the efficacy of oviduct tissue-conditioned medium for the culture of early bovine embryos

W. H. Eyestone*, J. M. Jones and N. L. First

Department of Meat and Animal Science, University of Wisconsin, Madison, WI 53706, USA

Summary. Oviduct tissue-conditioned medium was evaluated for the culture of IVM-IVF bovine zygotes to the compact morula (cM) and blastocyst (BL) stages. Development was unaffected ($P > 0{\cdot}50$) by freezing and thawing of conditioned medium: no. cM + BL/no. cleaved ova obtained after culture in nonfrozen, frozen–thawed, and control treatments were 31/148 (21%), 26/124 (21%) and 5/86 (6%), respectively. The greatest proportion of normal development was obtained after a conditioning period of 48 h ($P < 0{\cdot}05$): no. of cM + BL/no. cleaved ova in media conditioned for 5, 24, 48 and 96 h were 23/114 (20%), 38/112 (34%), 43/115 (37%) and 36/125 (29%), respectively. Development declined with increasing dilutions of conditioned media ($P < 0{\cdot}005$): no. cM + BL/no. cleaved ova for 100, 75, 50, 25 and 0% conditioned medium were 32/94 (34%), 29/94 (31%), 17/82 (21%), 10/94 (11%) and 11/73 (15%), respectively. The oestrous cycle stage from which oviducal tissue was obtained did not affect development ($P > 0{\cdot}75$); no. cM + BL/no. cleaved ova was 21/63 (33%) at oestrus and 21/79 (27%) in the luteal phase.

Keywords: embryo; blastocyst; *in vitro*; cattle; oviduct

Introduction

Early bovine embryos cultured *in vitro* fail to develop past the 8–16-cell stage in a variety of media (Wright & Bondioli, 1981), including follicular fluid (Thibault, 1966), simple defined media (BMOC; McKenzie & Kenney, 1973) and 'complex' media (Camous *et al.*, 1984; Heyman *et al.*, 1987; Eyestone & First, 1989).

Recently, the co-culture of embryos with oviducal tissue (sheep: Rexroad & Powell, 1986, 1988a, b; Gandolfi & Moor, 1987; goat: Sakkas *et al.*, 1989; cattle: Eyestone & First, 1989) has been shown to permit cleavage past the 8–16-cell stage; indeed, compact morulae and blastocysts have been routinely obtained in these systems. In addition, medium conditioned by oviducal tissue was shown to be as effective as co-culture in supporting development from zygote to blastocyst, and to the hatched blastocyst stage (Eyestone & First, 1989). Moreover, the transfer of morulae and blastocysts from conditioned medium to recipient cattle resulted in the birth of calves. Thus, medium conditioned by oviducal tissue supported normal development of early bovine embryos *in vitro*.

Conditioned medium presents several advantages over co-culture, namely, in eliminating the confounding presence of additional tissue and facilitating biochemical procedures in the search for soluble embryotrophic factors. The objective of the present study was to evaluate factors

*Present address: Department of Biochemistry, Gorlaeus Laboratory, Leiden University, 2333 CA Leiden, Netherlands.

affecting the preparation, storage and use of oviduct-conditioned media, with the aim of optimizing conditions for embryo development.

A portion of this work has appeared in abstract form (Eyestone *et al.*, 1990).

Materials and Methods

All incubations were performed in a humidified atmosphere of 5% CO_2 in air at 39°C. Gametes and embryos were cultured in either 100 µl (oocyte maturation) or 50 µl (fertilization and embryo culture) droplets of medium under paraffin oil. Gentamycin sulphate (25 µg/ml) was included in all media.

Embryo source. Embryos were generated by in-vitro oocyte maturation and in-vitro fertilization according to methods described by Sirard *et al.* (1988). Intact cumulus–oocyte complexes were matured in TCM199 + 10% heat-treated foetal calf serum, supplemented with 5·0 µg ovine luteinizing hormone (NIAMDD-oLH-024)/ml, 0·5 µg ovine follicle-stimulating hormone (NIAMDD-oFSH-015)/ml and 1·0 µg oestradiol-17β/ml (20 complexes/droplet). After 24 h, cumulus–oocyte complexes with expanded cumulus masses were transferred to fertilization droplets, which consisted of Tyrode's medium, modified according to Bavister *et al.* ('TALP'; 1983) and supplemented with 2·0 µg heparin/ml, and to which $5{\cdot}0 \times 10^4$ swim-up separated spermatozoa were added. Cumulus–oocyte complexes were removed from fertilization droplets after 18 h, at which time cumulus cells were removed by gentle pipetting. At this point, 20–30 zygotes were fixed in ethanol:acetic acid (3:1, v/v), stained with 1% aceto-orcein (in 40% acetic acid), and examined by phase-contrast microscopy at ×400 to determine fertilization frequency (percentage of ova with 2 pronuclei and a sperm tail).

Preparation of oviduct tissue-conditioned medium. Conditioned medium was prepared according to Eyestone & First (1989). Oviducts were obtained at slaughter and transported to the laboratory on ice. Luminal tissue was harvested by gently scraping intact oviducts with a glass slide, washed 5 times in TALP (suplemented with 10mM Hepes), then suspended in TCM199 + 10% heat-treated foetal calf serum (M199 + FCS) to a tissue:medium ratio of 1:50. Media were conditioned in 50 ml 'T' flasks containing 5 ml oviduct tissue suspension. Conditioned medium was prepared from the supernatant after centrifuging tissue suspensions at 13 000 ***g*** for 10 min. Media droplets, covered with paraffin oil, were incubated for 2 h to permit pH to equilibrate before adding zygotes.

Embryo culture. Zygotes were placed in culture droplets approximately 20 h after insemination. Initial cleavage ($\geq$2 cells) was assessed at 42 h after insemination. Media were not changed during the course of incubations. Criteria for normal development consisted of attainment of the compact morula (cM) or blastocyst stage (BL), or of the expanded blastocyst stage (xBL), by Day 6 or 9 after insemination, respectively. Development results are expressed as a function of cleaved ova.

Experiment 1. The cryostability of the embryotrophic property in conditioned medium was tested. Conditioned medium was prepared after 24–48 h incubation with oviducal tissue. A portion of the medium was frozen at −20°C, while another was held at room temperature. After 1 h, the frozen portion was thawed and culture droplets prepared from both portions. Control droplets consisted of non-frozen M199 + FCS.

Experiment 2. The influence of length of conditioning period on embryo development was investigated. Media were conditioned for 5, 24, 48 or 96 h and stored at −70°C until use. All time treatments were represented in each replicate.

Experiment 3. The effect of dilution of conditioned medium on embryo development was examined. Conditioned medium, prepared as described in Exp. 1, was diluted with M199 + FCS to yield media containing 100, 75, 50, 25 and 0% conditioned medium.

Experiment 4. Embryo development in media conditioned by tissue from oviducts of cows at oestrus or in the luteal phase was compared. Oviducts were obtained from the abattoir and judged as 'oestrous' if they were accompanied by ovaries with regressing corpora lutea and follicles of at least 1·5 cm diameter, and cervices with copious amounts of clear mucus. Luteal-phase tracts were judged by the presence of large, healthy corpora lutea and the presence of scant quantities of opaque, viscous cervical mucus.

Statistics. Proportions were compared by χ^2 analysis (Steel & Torrie, 1960).

Results

Experiment 1

Development to the compact morula and blastocyst stages at Day 6 after insemination was similar between frozen–thawed and nonfrozen conditioned media ($P > 0{\cdot}75$) but higher in either type of conditioned medium than in M199 + FCS ($P < 0{\cdot}005$; Table 1).

Table 1. Effect of freezing and thawing of conditioned medium on development of cow zygotes to the compact morula (cM) and blastocyst (BL) stages (4 replicates)

Treatment	Initial cleavage (%)	cM + BL[2] (%)
Fresh	148/177 (84)	31/148 (21)[a]
Frozen–thawed*	124/159 (78)	26/124 (21)[a]
Control (M199 + foetal calf serum)	86/126 (69)	5/86 (6)[b]

* −20°C for 1 h.

Development to cM + BL did not differ between fresh and frozen–thawed treatments ($P > 0{\cdot}75$); $\chi^2 = 0{\cdot}021$, d.f. = 1.

[a,b]Proportions differed ($P < 0{\cdot}005$); fresh vs. control, $\chi^2 = 14{\cdot}71$; frozen–thawed vs. control, $\chi^2 = 8{\cdot}96$; d.f. = 1.

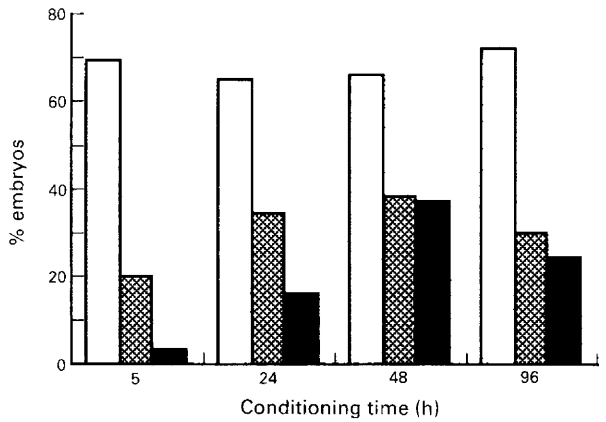

Fig. 1. Effect of conditioning time on cow embryo development in conditioned media. Zygotes were placed in conditioned media 20 h after insemination, examined for cleavage on Day 2 after insemination (open bars), for development to compact morula or blastocyst on Day 6 after insemination (cross-hatched bars) and expanded blastocyst on Day 9 after insemination (solid bars). Fertilization frequency: 77%; $n = 679$, 4 replicates.

Experiment 2

The yield of compact morulae and blastocysts on Day 6, and of expanded and hatched blastocysts on Day 9 after insemination, increased with increasing length of conditioning time up to 48 h ($P < 0{\cdot}05$, and $P < 0{\cdot}005$, respectively; Fig. 1). The yield of normally developed embryos was similar between 48 and 96 h of conditioning and was similar for embryos at Day 6 ($P > 0{\cdot}10$) and Day 9 ($P > 0{\cdot}25$). Initial cleavage was unaffected by conditioning time ($P > 0{\cdot}975$).

Experiment 3

The proportion of embryos that developed normally by Day 6 and Day 9 decreased with increasing dilutions of conditioned medium with M199 + FCS ($P < 0{\cdot}005$; Fig. 2), but initial cleavage was similar at all dilutions ($P > 0{\cdot}05$).

Experiment 4

Development to compact morula or blastocyst stage was similar in medium conditioned by oviducal tissue from the oestrus or luteal phase ($P > 0{\cdot}50$). Development was higher in conditioned medium from either phase than in M199 + FCS ($P < 0{\cdot}005$; Table 2).

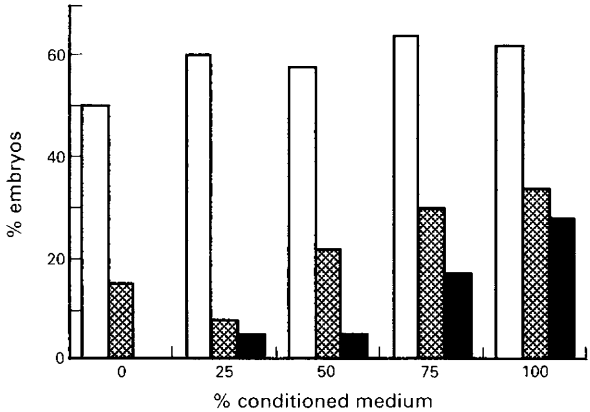

Fig. 2. Effect of dilution on cow embryo development in conditioned media. Zygotes were placed in conditioned media 20 h after insemination, examined for cleavage on Day 2 after insemination (open bars), for development to compact morula or blastocyst on Day 6 after insemination (cross-hatched bars) and expanded blastocyst on Day 9 after insemination (solid bars). Fertilization frequency: 69%; $n = 735$, 4 replicates.

Table 2. Cow embryo development in medium conditioned by oviduct tissue from the oestrous or luteal phases of the cycle (3 replicates)

Cycle stage	Cleavage (%)	cM + BL (%)
Oestrous	63/88 (72)	21/63 (33)[a]
Luteal	79/101 (78)	21/79 (27)[a]
Control (M199 + foetal calf serum)	68/88 (77)	5/68 (7)[b]

Development to compact morula (cM) + blastocyst (BL) did not differ between oestrous and luteal treatments ($P > 0{\cdot}50$); $\chi^2 = 0{\cdot}477$, d.f. = 1.

[a,b]Proportions differed ($P < 0{\cdot}005$); oestrous vs. control, $\chi^2 = 12{\cdot}29$; luteal vs. control, $\chi^2 = 8{\cdot}01$; d.f. = 1.

Discussion

The results of this study confirm our previous observation (Eyestone & First, 1989) that medium conditioned by oviducal tissue would support development from zygote to the expanded and hatched blastocyst stages. We have extended that work here to define several parameters that facilitate the preparation and optimize the performance of oviduct tissue-conditioned medium for embryo culture.

The ability of oviduct-conditioned medium to support embryo development remained stable through a single cycle of freezing and thawing (Table 1). This property alleviated the need to prepare new batches of conditioned medium for each experiment. It permitted single batches of conditioned medium to be stored frozen in small volumes so that the same batch could be used over several replicates and in a number of experiments. Although not specifically tested here, this approach should reduce potential variations in embryo development due to batch differences in conditioned medium.

Initial cleavage was unaffected by any of the treatments tested here, despite the fact that zygotes were transferred to culture treatments about 12 h before the first cleavage division (Eyestone &

First, 1988; Barnes & Eyestone, 1990). Indeed, the rate of cleavage from the 1- to 8-cell stage may be similar *in vitro* and *in vivo* (Barnes & Eyestone, 1990). Thus, the beneficial effect of oviducal tissue may not occur until around the 8-cell stage, at which point in-vitro cultured embryos encounter a block to development (Thibault, 1966; Camous *et al.*, 1984; Heyman *et al.*, 1987; Eyestone & First, 1989).

The mechanism by which oviducal tissue imparted its beneficial effect to the medium is unclear. Possible explanations include the addition of embryotrophic substances to the medium, or the removal of embryo-suppressive components from the unconditioned M199 + FCS. No embryotrophin (i.e. a substance that specifically regulates development) has been identified in mammals. A protein of M_r 92 000 secreted by cultured sheep oviduct tissue has been shown to cross the zona pellucida and bind to sheep embryos (Gandolfi *et al.*, 1989). Whether this protein, or any other protein, was responsible for the positive effect of oviduct co-culture on early embryo development *in vitro* was not determined in that study. Our data are consistent with the existence of a secretory factor, possibly a protein, which accumulates over time in culture (Fig. 1) and whose activity is reduced by dilution.

The beneficial effect of conditioned medium could also be mediated by the removal or modification of embryo-suppressive substances. For example, glucose and inorganic phosphate induced a 2-cell block in hamster embryos (Schini & Bavister, 1988), pyruvate induced a 4-cell block in pigs (Davis & Day, 1978) and hypoxanthine induced a 2-cell block in mice (Loutradis *et al.*, 1987). In each case, the substances mentioned inhibited development at the precise stages where each species normally exhibits a block to in-vitro development.

Embryo development in conditioned medium was not affected by the oestrous cycle stage from which the oviduct tissue was obtained (Table 2). This result is consistent with those obtained after in-vivo culture in either rabbit (reviewed by Boland, 1984) or sheep (Willadsen, 1982; Moore *et al.*, 1983; Eyestone *et al.*, 1987) oviducts, in which development was similar in the oestrous and luteal phases, or in anoestrous and ovariectomized animals. The ability of the oviduct to support embryo development does not seem to vary during the oestrous cycle, despite cyclic changes in secretory activity and oviducal fluid composition (reviewed by Leese, 1988).

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