Effectiveness of Menuzo's B2 Medium with Buffalo Rat Liver Cells for Development of In Vitro Matured/In Vitro Fertilized Bovine Oocytes

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Purpose: The effectiveness of two culture media on the development of bovine embryos in a buffalo rat liver (BRL) coculture system was investigated.

Methods: Bovine oocytes were matured and fertilized in vitro, then cocultured, 25 per well, for 7 days in 500 μ l of modified M199 or modified Menuzo's B2 medium over a BRL cell monolayer at 39°C in an atmosphere of 5% CO₂ in air. Medium 199 was modified by the addition of 10% of (v/v) fetal bovine serum (FBS), 9 mg/ml bovine serum albumin, 2 mM glycine, 1 mM alanine, and 0.1 mM nonessential amino acids (NEAA). Menuzo's B2 medium was modified by the addition of 10% (v/v) FBS, 1 mM alanine, and 0.1 mM NEAA.

Results: Modified Menuzo's B2 medium improved embryo development to the morula or blastocyst stage compared to modified M199 (121/353, 34.3%, versus 99/362, 27.3%, $P < 0.05$).

Conclusions: These results suggest that Menuzo's B2 medium with modifications in a BRL coculture system can provide a significant benefit for culture of early bovine embryos over the traditional use of medium 199.

KEY WORDS: bovine; buffalo rat liver cells; embryo culture.

INTRODUCTION

In the past, in vitro culture of preimplantation bovine embryos has been complicated by the 8- to 16-cell block to development (1,2). Initially, this block was overcome using in vivo culture in the ovine oviduct (3) or in vitro culture in explanted mouse oviducts (4). Successful development in vitro has been achieved

using coculture with various types of somatic cells (5), including cumulus cells (6,7), trophoblastic vesicles (8,9), oviductal cell monolayers (10,11), free-floating aggregates (12), and buffalo rat liver (BRL) cells (13). In addition, medium conditioned by oviductal cells (14) and BRL cells (15) is effective. It has been hypothesized that the beneficial effect of somatic cells on bovine embryos is due to the removal of inhibitory substances from the culture medium (16).

The complexity of these culture systems makes it difficult to examine the energy requirements of the embryo. Ellington *et al.* (10), working with a simple, defined medium, suggested that glucose is inhibitory to bovine embryo development if present during the early days of culture. This result was later confirmed by other investigators (17–19). It appears that glucose utilization by the bovine embryo is low until the morula stage (20). Lactate or pyruvate seem to be necessary for successful development (21–23). Amino acids have been examined for their effect on embryo development. Supplementation with a mixture of essential and nonessential amino acids in the absence of coculture is beneficial to embryo development (24) and increases the cell number of in vitro cultured blastocysts (23). Specifically, glycine and alanine were found in media conditioned with oviductal cells, suggesting a possible mechanism for improved development in coculture systems (12). Even with these advances, chemically defined, protein-free medium can support development of bovine embryos only to a limited extent (25). For the production of large numbers of morulae and blastocysts, most investigators continue to use coculture systems. The present experiment compared the effectiveness of Menuzo's B2 medium, which contains glucose, lactate, and pyruvate, to that of M199, which contains only glucose, used in conjunction with BRL

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cell coculture, on the development of in vitro matured/ in vitro fertilized (IVM/IVF) bovine embryos.

MATERIALS AND METHODS

Bovine oocytes were purchased (EmTran, Inc., Elizabethtown, Pennsylvania, and Utah State University, Logan, Utah) and shipped overnight in 1-ml maturation medium at 39°C in a temperature-controlled portable incubator (Minitube of America, Madison, Wisconsin). Maturation medium consisted of M199 (GIBCO, Long Island, New York) supplemented with 10% (v/v) fetal bovine serum (FBS HyClone, Logan, Utah), follicle stimulating hormone (bFSH) and luteinizing hormone (bLH) (0.01 U/ml of each gonadotropin; Nobl Labs, Sioux Center, Iowa), and 1% penicillin-streptomycin (GIBCO). Oocytes were matured for 24 hr, then washed three times in TL Hepes medium and placed into fertilization medium, 50 oocytes in 500 µl.

A Percoll separation procedure was used to prepare frozen semen for in vitro fertilization. Frozen 0.5-ml straws of semen were thawed at 35°C for 1 min. Semen was layered on top of a Percoll (Sigma Chemical, St. Louis, MO) density gradient (90:45%) in a 15-ml centrifuge tube and centrifuged for 30 min at 700 g. After centrifugation, the live sperm concentration in the soft pellet was determined. Sperm were added to fertilization wells to give a final concentration of 1.0×10^6 sperm/ml. Heparin (5.0 mg/ml) and PHE (20 mM penicillamine, 10 mM hypotaurine, 1 mM epinephrine) were included. After 18 hr of incubation with spermatozoa, the oocytes were vortexed in 1 ml TL Hepes for 2 min 15 sec to remove cumulus cells.

Embryos were cocultured in wells prepared with BRL cells as described by Voelkel and Hu (13). Coculture wells (Nunc, Roskilde, Denmark) were plated with 200,000 BRL cells in 500 µl medium 24 hr prior to use. Embryos were placed, 25 per well, into the culture plates and maintained in a modular incubator chamber (Billupsa-Rothenberg, Del Mar, California) in 5% CO₂:95% air at 39°C. On day 4 of culture (fertilization = day 0) embryos were moved to fresh culture wells and cultured to day 7.

Two culture media were used to prepare coculture wells. One medium used for coculture consisted of tissue culture medium (M199; GIBCO) supplemented with 10% (v/v) fetal bovine serum (FBS; HyClone), 1 mg/ml bovine serum albumin (BSA; Sigma Chemical), 0.1 mM nonessential amino acids (NEAA; GIBCO), 2 mM glycine, 1 mM alanine (Sigma Chemical), and 1% (v/v) penicillin-streptomycin (GIBCO). The sec-

Table I. Select Components of Media Used to Culture IVM/IVF Cattle Embryos

Component	Concentration (mg/L)	
	Modified Menuso's B2	Modified M199
Alanine	157	139
Glycine	400	800
Glucose	1200	1000
Lactate	50	0
Pyruvate	250	0

ond medium used for coculture consisted of Menuso's B2 (IMI, Minneapolis, MN) (26) supplemented with 10% (v/v) FBS (HyClone), 0.1 mM NEAA (GIBCO), 1 mM alanine (Sigma Chemical), and 1% (v/v) penicillin-streptomycin (GIBCO). The composition of both culture media used is presented in Table I. After fertilization, embryos were randomly allocated to one of these two coculture media.

After 7 days of coculture all embryos were assessed for stage of development attained and given a morphological grade from 1 (best) to 4 (worst). Embryos reaching the compact morula or blastocyst stage and having a morphological grade of 1, 2, or 3 were judged to be viable.

The proportion of embryos judged viable at day 7 of culture in each medium treatment was compared by chi-square analysis.

RESULTS

The results of coculture of IVM/IVF bovine embryos for 7 days on BRL cells with two types of media are presented in Table II. When modified M199 was used, 99 viable embryos were produced from 362 fertilized ova (27.3%). Culture with modified Menuso's B2 produced 121 viable embryos from 353 fertilized ova (34.3%). The difference in developmental results between these two medium was significant ($P < 0.05$). There was no difference in the morphological grade of viable embryos produced between the two medium treatments.

DISCUSSION

BRL cells have become widely used for culturing one-cell bovine embryos since their introduction by Voelkel and Hu (13). There are several advantages of the BRL coculture system. One is that there is no need

Table II. Development of IVM/IVF Bovine Embryos in BRL Cell Coculture with Two Types of Media

Medium ^a	Viable embryos (%)	Development stage ^b				
		Compact morula	Early blast	Blast	Expanded blast	Hatching blast
modB2	121/353 (34.3)*	7	23	34	48	9
mod199	99/362 (27.3)*	7	24	31	35	2

^a modB2, modified Menuzo's B2 medium; mod199, modified M199 medium.

^b Blast, blastocyst.

* Treatments differ ($P < 0.05$).

for slaughterhouse-derived material, as is the case if bovine oviductal epithelial cells are used for coculture. BRL cells are also easily managed, culture conditions are highly reproducible, and the cells can be stored frozen indefinitely. Finally, embryos cultured on BRL cells have a higher survival rate after cryopreservation than embryos cultured with bovine oviductal epithelial cells (27), an important consideration for commercial application of bovine IVM/IVF technologies.

As more information concerning the early metabolic requirements of bovine embryos becomes available, medium can be formulated to produce higher rates of viable morula and blastocysts from IVM/IVF oocytes. The use of chemically defined, protein-free culture medium is extremely valuable in studies to determine the regulatory mechanisms and nutritional requirements of bovine embryos. However, coculture systems still provide the optimum method to produce high-quality viable morulae and blastocysts from IVM/IVF bovine oocytes. It is unclear whether the coculture cells exert their positive benefit to the embryos by removing inhibitory substances from the culture medium (16) or by producing embryotropic factors. BRL cells are known to secrete polypeptides in culture that are similar to insulin-like growth factors (28). These cells also secrete differentiation inhibiting activity, which is homologous to leukemia inhibiting factor, both acting to inhibit differentiation and stimulate growth (29).

Menuzo's B2 medium has been used successfully to culture human embryos to early cleavage stages and to produce pregnancies after transfer (26). Used in combination with bovine oviductal epithelial cells, Menuzo's B2 medium has been shown effective in producing high percentages of bovine morulae and blastocysts, with cell numbers comparable to in vivo produced embryos (29/94; 30.9%) (30). Substituting Menuzo's B2 medium, with the addition of FBS,

nonessential amino acids, and alanine, for the commonly used M199 in a BRL coculture system resulted in greater embryonic development in this study. Because Menuzo's B2 medium contains glucose, embryos are subjected to this energy substrate throughout the culture period. Glucose was shown to be inhibitory to early bovine embryo development (23). Coculture using BRL cells with Menuzo's B2 medium without glucose for the early culture period may provide a more beneficial environment for preimplantation bovine embryo development.

In summary, BRL cell coculture with modified Menuzo's B2 medium can be used to produce a large number of morulae and blastocysts from IVM/IVF bovine oocytes. This system offers a convenient way to culture bovine embryos successfully without the use of abattoir-derived oviductal epithelial cells.

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