1-1-1993

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Recommended Citation

Moore, K., & Bondioli, K. (1993). Glycine and alanine supplementation of culture medium enhances development of in vitro matured and fertilized cattle embryos. *Biology of Reproduction, 48* (4), 833-840. https://doi.org/10.1095/biolreprod48.4.833

BIOLOGY OF REPRODUCTION 48, 833–840 (1993)

Glycine and Alanine Supplementation of Culture Medium Enhances Development of In Vitro Matured and Fertilized Cattle Embryos

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ABSTRACT

One-cell cattle embryos were prepared by in vitro oocyte maturation and fertilization (IVM/IVF) and cultured with or without oviductal cells. Embryos were evaluated after 7 days in culture to determine the percentage developing from the 1-cell stage to the morula or blastocyst stage. The combination of glycine (2 mM) and alanine (1 mM) with oviductal cells (experiment 1) improved embryo development over that in control culture (29 vs. 13%; $p < 0.05$). An optimum response was obtained with 10 mM glycine and 1 mM alanine in coculture (experiments 2 and 3). In experiment 4, the effects of glycine (0 or 10 mM), alanine (0 or 1 mM), and the presence or absence of oviductal cells were tested. In the absence of oviductal cells, the addition of glycine, alanine, or glycine and alanine combined improved embryonic development over that in control medium (45, 33, 42 vs. 24%, $p < 0.001$, $p < 0.05$, $p < 0.001$, respectively). However, the effect of the combination of glycine and alanine was not different from that of glycine alone when oviductal cells were absent (42 vs. 45%, $p > 0.10$). In the presence of oviductal cells, glycine or the combination of glycine and alanine improved embryonic development over that in the control medium with cells (47, 55 vs. 37%, $p < 0.01$, respectively). However, supplementation with alanine alone gave no improvement over controls when oviductal cells were present (40 vs. 37%, $p > 0.10$). These results indicate that glycine and alanine, when used independently, directly affect cattle embryo development, but in combination affect embryo development indirectly, possibly by altering oviductal cell function. Finally, oviductal cell-conditioned (48 h or 6 days) medium excluding glycine and alanine supplements was analyzed for free amino acids. Glycine and alanine were secreted by oviductal cells, suggesting that enhanced embryonic development achieved through coculture may be due, in part, to these amino acids.

INTRODUCTION

In vitro culture of the early cattle embryo has met with limited success due to an 8- to 16-cell block to development [1–3]. This block can be overcome through the use of coculture. Uterine fibroblasts [4, 5], trophoblastic vesicles [6, 7], cumulus cell monolayers [8], and oviductal cell monolayers as well as free-floating oviductal cell vesicles [9–13] have all been used to supplement culture systems for development of the early cattle embryo, with varied success. These studies all suggest that the somatic cells alter the culture medium in such a manner as to improve the environment for the developing embryo.

Attempts to isolate embryotrophic factors produced by the cells have been made, with little success. Difficulty in isolating such components arises from the complexity of the culture medium, serum supplements, and/or other metabolites produced by the somatic cells. Hence, a strong interest has been generated in formulating a simple, defined culture medium that is capable of supporting normal development of the early cattle embryo. Tervit et al. [14] formulated a medium similar in composition to oviductal fluid and obtained viable sheep and cattle embryos after culturing 8-cell embryos obtained from in vivo collections for a period of 3 days. Since this earlier experiment, attention has focused on defining the requirements of the preimplantation cattle embryo, as well as in-depth analysis of the reproductive tract environment.

Energy substrates and embryo metabolism have attracted the attention of recent investigators. Glucose was determined to be detrimental to development of preimplantation hamster [15, 16], mouse [17], and cattle [10, 11] embryos. Ellington et al. [11] suggest that culture of cattle embryos in the presence of glucose during the first 36 h results in a decrease in development of blastocysts. Conversely, an increase in the ratio of lactate to pyruvate appears to stimulate development of the mouse and cattle embryo [10, 17]. During early development, it appears that glycolysis results in the inefficient production of ATP, while energy generated through the Kreb's cycle is a preferred, more efficient route. Seshagiri and Bavister [18] suggest that glycolysis inhibits respiratory activity and oxidative metabolism, thus resulting in decreased development. Nieder and Corder [19] have found that the pregnant mouse oviduct preferentially secretes first pyruvate and then lactate at high levels during the first 5 days after ovulation, a trend not followed by the pseudopregnant animal. This suggests that lactate and pyruvate are normally preferred as metabolic substrates for the early embryo.

The effects of amino acids on embryonic development have also been studied. The addition of glutamine to culture medium has proven beneficial to developing hamster [20], mouse [17], and cattle [10] embryos. The results of Rieger et al. [21] suggest that glutamine is an energy substrate preferred over glucose for the early cattle embryo. Lawitts and Biggers [22] indicate, however, that glutamine may be

Accepted November 30, 1992.
Received March 4, 1992.
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TABLE 1. Composition of culture media used to culture 1-cell cattle IVM/IVF embryos.

Component[a]	Concentration (mM) BMOC	MBMOC[b]
NaCl	94.900	81.620
KCl	4.780	4.830
$CaCl_2 \cdot 2H_2O$	1.700	1.700
$MgSO_4$ (anhydrous)	1.190	1.190
$NaHCO_3$	25.100	25.120
KH_2PO_4	1.190	1.190
EDTA	0.116	0.110
Sodium Acetate	0.610	0.610
Glutamine	—	1.000
Glucose	5.550	—
Sodium Lactate	25.400	31.300
Sodium Pyruvate	0.509	0.270
Phenol Red	5.0 mg/L	5.0 mg/L

[a]Adapted from the formulations of the medium CZB as described by Chatot et al. [3].
[b]Components were obtained from Sigma Chemical Co., St. Louis, MO.

acting as an organic osmolyte to help maintain cell turgor in the developing mouse embryo.

Earlier successes in culture of the 2- and 8-cell hamster embryo utilized a defined medium supplemented with twenty amino acids [15, 16]. The concentrations of amino acids utilized in those experiments were based upon measurements of amino acid concentrations found in reproductive tract fluids and embryos of the mouse [23] and the rabbit [24]. Amino acid pools were highest in taurine, glycine, glutamate, and alanine for both species. In more recent experiments [20, 25], amino acids have been tested and classified as stimulatory (GLY, CYS, LYS), inhibitory (PHE, VAL, ILE, TYR, TRP, ARG), or neutral (PRO, SER, THR, HIS, ALA, LEU, ASP, MET) to the development of the early hamster embryo. It has been determined that the earlier 2- and 4-cell embryos require more extensive amino acid supplements [26, 27] while fewer amino acids are necessary, some even inhibitory, for the development of the later embryo (8-cell to blastocyst [26]). Xu et al. [12] obtained 31% morulae/blastocysts from 1-2-cell cattle IVM/IVF embryos cultured with oviductal cells in Ménézo's B2 medium [28]. Although the composition of B2 medium was not addressed by Xu et al., it includes high concentrations of glycine (5.33 mM) and alanine (0.76 mM), suggesting that these amino acids may be beneficial to early embryonic development in cattle. Amino acid requirements for the early cattle embryo have not been established.

Development of IVM/IVF 1-cell cattle embryos to blastocysts in a defined culture system has been reported. Pinyopummintr and Bavister [29] reported 37 to 50% viability of IVM/IVF embryos cultured for 8 days in tissue culture medium 199 (TCM199). However, the rate of development to blastocysts by 8 days appeared to be low (19 to 26%). This suggests that development was delayed and that other factors affecting normal developmental rates are still to be determined.

In a previous study [10], cattle embryo coculture with oviductal cells was supplemented with bovine oviductal fluid (BOF), resulting in enhanced embryonic development over that in coculture alone. This indicated that factors supplied by the oviduct are yet to be elucidated and are not provided through the use of coculture. The current set of experiments has examined this issue further by analyzing BOF for free amino acid composition and applying these findings to the modified Brinster's ovum culture medium (MBMOC) culture system to test the effects of amino acid supplementation on in vitro development of 1-cell cattle IVM/IVF embryos. Some of the results have appeared in abstract form [30].

MATERIALS AND METHODS

Oviductal Fluid and Conditioned Medium Preparation

Cattle oviducts, either determined to be obstructed at the time of collection or surgically ligated for not less than one estrous cycle, were surgically excised and fluid was recovered aseptically. Fluid was aliquoted and frozen at −20°C. Before freezing, several lots of fluid were tested for embryotrophic ability (data not shown) and only lots that improved embryo development were used for analysis.

Conditioned medium was prepared by culturing oviductal epithelial cells in drops of modified Brinster's ovum culture medium (MBMOC), excluding Nonessential Amino Acid solution (NEAA; Gibco, Grand Island, NY), as well as glycine and alanine supplements. Drops were cultured in environmental chambers with 5% CO_2: 5% O_2: 90% N_2 at 38°C for 48 h or 6 days. Treatment drops were pooled, and centrifuged to remove cellular debris and supernatant was harvested for analysis.

Free Amino Acid Determination

Samples of cattle oviductal fluid as well as oviductal cell-conditioned medium were submitted to the TAES Biotechnology Support Laboratory (Texas A & M University, College Station, TX) for analysis of free amino acid concentration. Samples were filtered through an M_r = 10 000 cutoff filter (Millipore Ultrafree; Bedford, MA) to remove proteins. Free amino acids were determined from 10-μl aliquots of each filtrate using a Model ALC 204 Liquid Chromatograph (Waters, Millford, MA) as described by Bidlingmeyer et al. [31].

Culture Medium

Embryos were cultured in Brinster's ovum culture medium (BMOC [32])(experiment 1) or MBMOC (experiments 2–4). The formulations of both media are given in Table 1. Media were prepared with high quality water (Continental Type I Reagent Grade Water, Continental Water Systems, San Antonio, TX), filter-sterilized with Nalgene 0.2-μm bottle filters (Nalgene, Rochester, NY), and stored at

TABLE 2. Free amino acid concentrations found in cattle oviductal fluid (mM).

Amino acid	Lot number				
	2	29	35	37	38
GLU	0.03	0.03	0.04	0.03	0.02
SER	0.05	0.03	—	0.02	0.02
GLY	2.35	1.42	1.01	1.11	1.13
ARG	0.03	0.04	0.03	0.02	—
THR	0.03	0.02	0.04	0.02	0.02
ALA	0.51	0.42	0.31	0.43	0.24
PRO	0.03	0.03	—	0.03	0.03
VAL	0.02	0.02	0.02	0.02	0.01
LYS	0.03	0.07	0.04	0.02	0.04

4°C for not more than 4 wk. Medium was supplemented on a daily basis, for treatment use, with 6 mg/ml BSA (Fraction V, Sigma Chemical Co., St. Louis, MO), 0.1 mM MEM NEAA, and 1% penicillin/streptomycin (10 000 U/ml Pen G sodium/10 000 μg/ml streptomycin stock solution; Gibco). Addition of NEAA to BMOC proved beneficial to cattle embryonic development in coculture with oviductal epithelia in earlier experiments (data not presented). While NEAA does contain glycine and alanine, their concentrations are far below those used as supplements in the following experiments. Glycine and alanine supplements were prepared as 100-strength stock solutions, aliquoted, and stored at −20°C until added to culture medium.

Oviductal Cell and Culture Drop Preparation

Oviductal epithelial tissue was collected from slaughterhouse oviducts via a procedure modified from that described by Eyestone and First [9]. Oviducts from cows early in gestation were transported in ice-cold PBS supplemented with 4 mg/ml BSA and 1% penicillin/streptomycin. Harvested cells were washed three times in HEPES-buffered tissue culture medium 199 (TCM199; Gibco), resuspended 1:50 in TCM199 with 10% fetal calf serum (FCS; HyClone, Logan, UT) and 1% penicillin/streptomycin, and cultured in 50-ml culture flasks (Nalgene, Rochester, NY) for 24 h at 38°C in 5% CO_2 in air prior to use.

Twenty-microliter culture drops were prepared in 60-mm culture plates (Costar, Cambridge, MA) 24 h prior to the addition of embryos. Drops were overlaid with Dow Corning 200 Fluid (Specialized Products, Houston, TX) and incubated at 38°C in a humidified environment of 5% CO_2 in air.

For treatments including coculture with oviductal cells, 12 to 20 free-floating oviductal cell aggregates exhibiting ciliary movement and vesicle formation were selected, washed in the appropriate treatment medium, and transferred in 1 μl of medium to culture drops during culture-drop preparation.

Embryo Preparation and Culture

Cattle embryos were derived from oocytes aspirated from slaughterhouse ovaries and matured and fertilized in vitro according to procedures outlined by Parrish et al. [33] and Critser et al. [34].

Eighteen to 24 h after fertilization (Day 0), cumulus cells were removed by vortexing. A sample of fertilized eggs was fixed and stained at this time and examined for pronuclei as an estimate of fertilization rate. Embryos without cumulus cells were selected and washed through 2 drops of the appropriate treatment medium prior to transfer to culture drops to ensure that cultures were not contaminated with cumulus cells. One-cell embryos were randomly assigned to appropriate treatments with not more than 15 embryos per culture drop. On occasion, repetitions contained 2 drops per treatment. Culture dishes were then transferred to environmental chambers (Billups-Rothenberg, Del Mar, CA) and sealed in a humidified environment of 5% CO_2: 5% O_2: 90% N_2 at 38°C for 7 days. Embryos were evaluated for developmental stage and quality on Day 7 of culture according to procedures described by Lindner and Wright [35]. The percentage viable was calculated from the total number of 1-cell embryos developing to the compact morula or blastocyst stage. Cultures without oviductal cells were observed after 7 days in culture to verify the absence of cumulus cells.

Experimental Design and Analysis

In experiment 1, the effects of glycine and/or alanine on embryonic development were tested. Amino acids were supplemented at 2 mM and 1 mM, respectively, in both the presence and absence of oviductal cells.

Experiments 2 and 3 tested concentration effects of glycine and alanine, respectively. Both experiments were conducted in two parts to determine optimal concentrations in coculture. Glycine was supplemented at 2, 10, 20, and 50 mM in experiment 2, part 1, and at 2, 5, 10, and 20 mM in part 2. During both parts of both experiments, alanine was held constant (1 mM). For experiment 3, glycine was held constant (10 mM) while alanine was tested at 1, 5, 10, 20, and 50 mM in part 1 and 1, 3, 5, 8, and 10 mM in part 2. Culture medium osmolarity was not adjusted after the addition of amino acids.

The fourth experiment tested the effects of glycine (0 or 10 mM), alanine (0 or 1 mM), and presence or absence of oviductal cells on cattle embryonic development in vitro.

Data for each experiment were analyzed for treatment differences by chi-square using two-way contingency analysis.

RESULTS

Analysis of Cattle Oviductal Fluid

Free amino acid concentrations determined for five lots of cattle oviductal fluid are given in Table 2. The amino acids highest in concentration were glycine and alanine with mean concentrations of 1.40 mM (range = 1.01 to 2.35) and

TABLE 3. Free amino acid concentrations (mM) in MBMOC[a] conditioned with oviductal cells 48 h or 6 days.

Amino acid	48-h Conditioned medium		6-Day Conditioned medium	
	Lot #1	Lot #2	Lot #1	Lot #2
SER	—	0.006	0.008	0.007
GLY	0.093	0.092	0.133	0.173
ALA	0.147	0.117	0.047	0.015
PRO	0.247	0.326	0.295	0.435
TYR	—	—	0.006	—
LEU	0.009	0.007	0.009	0.009
PHE	0.006	—	0.011	0.010
LYS	—	0.004	0.005	—

[a]Medium did not contain glycine or alanine supplements.

0.38 mM (range = 0.24 to 0.51), respectively. A number of amino acids were at concentrations too low to be detected, including ASP, ASN, GLN, HIS, TYR, CYS, ILE, LEU, PHE, and TRP.

Analysis of Conditioned Medium

Determinations of free amino acid concentrations for medium conditioned with oviductal cells for either 48 h or 6 days are given in Table 3. Glycine, alanine, and proline were the amino acids highest in concentration at both time points for all lots. Glycine tended to increase in concentration over time (48 h mean = 0.093 mM vs. 6-day mean = 0.153 mM), whereas alanine appeared to decrease over time (48 h mean = 0.132 mM vs. 6-day mean = 0.031 mM). Amino acids undetectable across all samples were ASP, ASN, GLU, and GLN.

Experiment 1

The results of four repetitions showed that the combination of glycine (2 mM) and alanine (1 mM) with oviductal cells (+ glycine + alanine with cells) increased embryonic development over that in control medium with cells (29 vs. 13%, $p < 0.05$). However, the combination did not improve development over that in control medium in the absence of oviductal cells (13 vs. 8%, $p > 0.10$) (Table 4). The combination in the presence of oviductal cells was not significantly different from +glycine with cells or +alanine with cells for improving embryonic development (29 vs. 23, 20%, respectively, $p > 0.10$). When either amino acid alone was used as a supplement, no benefit was seen over control medium with cells (23, 20 vs. 13%, $p > 0.10$) or without cells (18, 7 vs. 8%, $p > 0.10$). The average fertilization rate during the experiment was 60% (range = 43 to 76%). Cultures without oviductal cells did not contain cumulus cell contaminants following the 7-day culture period.

TABLE 4. Effect of amino acid supplementation on embryo development in vitro with or without oviductal cells (experiment 1).

Treatment[a]	No. of embryos	No. of replicates	Percentage morula/blastocyst
With oviductal cells			
+ Glycine + alanine	69	4	29
+ Glycine	69	4	23
+ Alanine	69	4	20
Control medium	69	4	13
Without oviductal cells			
+ Glycine + alanine	68	4	13
+ Glycine	68	4	18
+ Alanine	68	4	7
Control medium	68	4	8

[a]Glycine (2 mM) and alanine (1 mM) were used to supplement treatments designated "+".

TABLE 5. Effect of glycine concentration on cattle embryonic development in coculture (experiment 2).

Concentration (mM)	No. of embryos	No. of replicates	Percentage morula/blastocyst
Part I			
2	120	9	28
10	120	9	47
20	120	9	32
50	120	9	29
Part II			
2	115	9	47
5	115	9	38
10	115	9	50
15	115	9	52
20	115	9	43

Experiment 2

In part 1, a total of nine repetitions were conducted to test concentration effects of glycine. Glycine at 10 mM increased embryonic development as compared to of all other concentrations ($p < 0.05$), producing 47% compact morulae or blastocysts. All other treatments were not different ($p > 0.10$).

The concentration of glycine was optimized in part 2 of this experiment. Results from 9 repetitions showed that 10- and 15-mM concentrations were not different ($p > 0.10$), both giving the highest developmental rates of approximately 51%. Glycine at 10 or 15 mM improved development over the 5 mM treatment (50, 52 vs. 38%, $p < 0.05$), but were not different from the other treatments ($p > 0.10$). Results for parts 1 and 2 are given in Table 5.

TABLE 6. Effect of alanine concentration on cattle embryo development in coculture (experiment 3).

Concentration (mM)	No. of embryos	No. of replicates	Percentage morula/blastocyst
Part I			
1	77	6	56
5	77	6	57
10	77	6	49
20	77	6	43
50	77	6	39
Part II			
1	144	12	53
3	144	12	53
5	144	12	54
8	144	12	48
10	144	12	51

TABLE 7. Treatment effects on the development of IVM/IVF cattle embryos after 7 days in culture.

Treatment[a]	No. of embryos[b]	Stage (%)[c]				Total (%) morula/blastocyst
		CM	B	ExB	HB	
With oviductal cells						
+ Glycine + alanine	297	1	22	31	1	162 (55)
+ Glycine	297	—	24	22	1	141 (47)
+ Alanine	297	2	20	18	—	119 (40)
Control medium	297	—	26	11	—	110 (37)
Without oviductal cells						
+ Glycine + alanine	297	—	20	23	—	126 (42)
+ Glycine	297	—	24	20	1	134 (45)
+ Alanine	297	—	19	14	—	98 (33)
Control medium	297	—	16	8	—	73 (24)

[a]Glycine (10 mM) and alanine (1 mM) were supplemented to treatments designated "+".
[b]Number of total 1-cell embryos in 19 replicates per treatment.
[c]Developmental stage: CM = compact morula, B = blastocyst, ExB = expanded blastocyst, HB = hatching/hatched blastocyst; developmental stage was calculated from the total number of 1-cell embryos put into culture.

Experiment 3

In preliminary trials with alanine, results from 6 repetitions showed that the optimal concentration lay between 1 and 10 mM (Table 6). Alanine at 5 mM improved development significantly over the 20 and 50 mM concentrations (57 vs. 43, 39%, $p < 0.05$). The 1 mM alanine treatment was better than the 50 mM treatment ($p < 0.05$). Other treatments were not different ($p > 0.10$). When concentrations were refined to 1, 3, 5, 8, and 10 mM to test for optimal response, no differences were found after 12 repetitions between any of the treatments ($p > 0.10$; Table 6). The average development rate across the 5 treatments was 52%. During experiments 2 and 3, the average fertilization rate was 62% (range = 37 to 83%).

Experiment 4

A total of 19 repetitions were run to determine the effects of glycine, alanine, or the combination of the two on embryo development in the presence or absence of oviductal cells. Percentage fertilization averaged 62% during the experiment (range = 44 to 82%). Glycine supplementation alone, both with and without oviductal cells, greatly improved embryonic development over treatments without supplementation (for treatments with oviductal cells: 47 vs. 40%, $p < 0.10$; 47 vs. 37%, $p < 0.01$; for treatments without oviductal cells: 45 vs. 33%, $p < 0.005$; 45 vs. 24%, $p < 0.001$; Table 7).

Culture in the presence of alanine alone, without oviductal cells, enhanced embryo development over controls without cells (33 vs. 24%, $p < 0.05$). However, it did not improve development over the controls when oviductal cells were present (40 vs. 37%, $p > 0.10$; Table 7).

When both glycine and alanine were combined in the presence of oviductal cells, embryonic development improved over that in medium with single amino acid supplements or in control medium in the presence of oviductal cells (55 vs. 47%, $p < 0.10$; 55 vs. 40%, $p < 0.05$; and 55 vs. 37%, $p < 0.01$). There was no benefit for the combination as compared to glycine alone when oviductal cells were absent (42 vs. 45%, $p > 0.10$). However, the combination did improve development as compared to alanine alone or control medium when oviductal cells were absent (42 vs. 33%, $p < 0.10$; 42 vs. 24%, $p < 0.001$; Table 7).

The presence of oviductal cells improved development over that in medium alone when both amino acids were omitted (37 vs. 24%, $p < 0.05$). However, the addition of glycine to the medium alone made up for the absence of cells when compared to culture with oviductal cells without amino acid supplementation (45 vs. 37%, $p < 0.05$; Table 7). Cultures without oviductal cells did not contain cumulus cell contaminants after 7 days in culture.

Effects of treatment on stage of embryo development after 7 days in culture are given in Table 7. Greater than 95% of all viable embryos were blastocysts, expanded blastocysts, or hatching blastocysts, across all treatments, by 7 days.

DISCUSSION

In a previous study [10], it was determined that development of 1-cell IVM/IVF cattle embryos improved when bovine oviductal fluid (BOF) was used to supplement the MBMOC coculture system. This implies that factors exist that improve embryo development but cannot be provided through coculture alone. To improve our understanding of the oviductal environment, BOF was analyzed for free amino acid content. Glycine and alanine were the two most predominant amino acids, with mean concentrations of 1.40 and 0.38 mM, respectively. Concentrations of glycine ranging from 4.50 to 19.33 mM and alanine from 1.09 to 2.18 mM have been measured in rabbit oviduct fluid [24]. Ménézo et al. [36] tested mouse oviductal flushings for free amino acids and determined that both glycine and alanine were in high concentration. Similar trends have also been demonstrated for uterine fluids of the rabbit [24] and the mare [37]. It can be inferred from these results that glycine and alanine have a role in early embryonic development.

Experiment 1 was conducted to test this hypothesis by supplementing BMOC with glycine and alanine at concentrations similar to those found in BOF. Glycine and alanine supplementation when combined with oviductal tissue exerted a beneficial effect on development over coculture alone ($p < 0.05$). Previously it was reported [20, 25] that glycine (0.1 mM) as well as taurine was beneficial to the developing hamster embryo, while alanine was considered neutral, not proving beneficial or harmful at the 0.1 mM concentration. Ménézo's B2, a medium high in glycine and alanine (5.33 and 0.76 mM, respectively), was shown to improve development of cattle 1- and 2-cell IVM/IVF embryos in the presence of oviductal cells [12]. The results of the present study suggest that the two amino acids promote early development of the cattle embryo.

To optimize development in coculture, titration studies were performed for both glycine and alanine. The highest development rate was observed when glycine was used at 10 or 15 mM. Alanine concentrations from 1 to 10 mM produced the highest developmental rates. Since increased concentrations gave no further benefits, 10 mM glycine and 1 mM alanine were the concentrations selected as optimum and were used in all further studies. Culture medium osmolarity was not adjusted after addition of amino acids. This may have affected embryo development at the highest concentrations tested; however, development rates had plateaued at much lower concentrations. This suggests that higher concentrations, even if adjusted for osmolarity, would not have resulted in further improvement in development. The early mouse embryo can develop in medium osmolarities ranging from 0.2002 to 0.3542 osM [38]. Studies with other species indicate similar results [2], suggesting that osmolarity has minimal effects on embryonic development.

The fourth experiment was conducted to determine whether improved embryonic development was due to a direct effect of the amino acids or an indirect effect through alteration of oviductal cell function. Results from this experiment indicate that glycine and alanine, when used independently, directly interact with the cattle embryo to enhance development. When glycine and alanine were combined in the presence of oviductal cells, development was enhanced further. This action may occur indirectly by altering oviductal cell function, since this additional benefit was not seen in the absence of cells. These results may partially explain the benefits that were produced when BOF was used to supplement the MBMOC coculture system in a previous study [10].

While the present studies have shown glycine and alanine to be beneficial to the developing cattle embryo, it is not well understood how these amino acids are utilized. Hobbs and Kaye [39] have shown that glycine is taken up by the mouse blastocyst and that it can be converted to serine (5%) and alanine (17%) or incorporated into macromolecules, such as proteins and nucleic acids, and thus serve as energy substrates and precursors. Bavister and McKiernan [40] hypothesized that for the hamster embryo, glycine and alanine may be important for intracellular pH regulation by acting as proton shuttles. Others [22, 41] have suggested that these amino acids act as intracellular osmolytes and that they are important for protecting the mouse embryo from the osmotic stresses of the oviductal environment.

In experiment 4, the beneficial effects of the medium MBMOC could also be seen (Table 7). The control treatments with or without oviductal cells, but without amino acid supplements, resulted in 37 and 24% development, respectively. These rates are much improved over those of experiment 1 controls with which BMOC was utilized (13 and 8% development, respectively; Table 4). These results support the previous observation [10] that modification of metabolic substrates improves development.

When the two controls in experiment 4 are compared (Table 7), the treatment with oviductal cells significantly improved embryo development over that without cells ($p < 0.05$). Glycine-supplemented MBMOC, in the absence of cells, produced development rates greater than ($p < 0.05$) those obtained for control medium when oviductal cells were present but amino acid supplementation was absent. This implies that the oviductal cells may secrete glycine and/or alanine at low concentrations into the culture drop, while increased supplementation further benefits embryonic development.

Oviductal cell-conditioned MBMOC, without amino acid supplementation, was analyzed for free amino acids to test this hypothesis. For all lots at both time periods, glycine, alanine, and proline were higher in concentration in relation to other amino acids and the concentration of glycine appeared to increase between 48 h and 6 days of conditioning (Table 3). Ouhibi et al. [42] tested several cell types for coculture of mouse embryos and found that those cell types that were embryotrophic produced high concentrations of glycine. The current results indicate that the oviductal epithelia secrete glycine and alanine in vitro. The fact that development in coculture supplemented with glycine and alanine is higher than in medium supplemented with glycine and alanine without cells suggests that oviductal cells provide some factor(s) in addition to glycine or alanine that is embryotrophic.

In experiment 4, the majority of the viable embryos were blastocysts by Day 6 (data not shown), and greater than 95% of those considered viable were blastocysts, expanded blastocysts, and hatching blastocysts by 7 days in culture, across all treatments (Table 7). Pinyopummintr and Bavister [29] reported developmental rates for IVM/IVF cattle embryos for several treatments and obtained an average of 10% compact morulas and 23.3% blastocysts (best 4 treatments) after 8 days in culture. Eyestone et al. [43] reported 53% development to the compact morula or blastocyst stage when cattle embryos, retrieved 36 to 48 h postestrus, were cultured in the sheep oviduct for 5 days. The culture system

described here allows the cattle IVM/IVF embryo to develop at rates comparable to those observed with in vivo culture systems. Samples of 1-cell IVM/IVF embryos indicated that only 61% (mean fertilization rate) of the embryos put into culture were competent to develop.

Although the embryos in the current experiments were not transferred to recipient animals, this culture system has been used successfully for culturing pronuclear injected 1-cell IVM/IVF cattle embryos [44]. Those investigators report a 31% pregnancy rate at 60 days, from 494 transfers, resulting in 94 live calves. Their results are similar to those in a study by Biery et al. [45], in which 79 pregnancies were obtained at 60 days from 175 pronuclear injected embryos developing in the ligated sheep oviduct.

These studies have shown that cattle IVM/IVF embryos can develop in vitro from the 1-cell to the blastocyst stage in a defined, serum-free culture system. This culture system has been successfully used to culture pronuclear injected 1-cell embryos prior to transfer on Day 6, resulting in viable offspring (data not shown). Glycine and alanine supplementation of MBMOC, either with or without the use of oviductal cells, improved development and yielded 45 to 55% viability. Since these amino acids further improve development, possibly by altering oviductal cell function, it is likely that there are other factors produced by the oviduct yet to be discovered that affect embryonic development. The ability to achieve significant development with a defined medium without somatic cell support will be useful for the design of experiments testing the effects of suspected embryotrophic factors.

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