BIOLOGY OF REPRODUCTION 55, 703–708 (1996)

Development of Ovine Embryos in Synthetic Oviductal Fluid Containing Amino Acids at Oviductal Fluid Concentrations

S.K. Walker,[1,3] J.L. Hill,[2,4] D.O. Kleemann,[3] and C.D. Nancarrow[4]

South Australian Research and Development Institute,[3] Turretfield Research Centre, Rosedale, SA 5350, Australia
CSIRO,[4] Locked Bag 1, Delivery Centre, Blacktown, NSW 2148, Australia

Downloaded from https://academic.oup.com/biolreprod/article/55/3/703/2760562 by U.S. Department of Justice user on 16 August 2022

ABSTRACT

The effects of supplementing synthetic oviductal fluid (SOF) with amino acids, at oviductal fluid concentrations, on the development of ovine in vitro-matured/in vitro-fertilized embryos was examined in three experiments. In the first, embryo development in SOF, SOF + 2% human serum (HS), SOF + 20% HS, and SOF + BSA, with and without amino acid supplementation, was examined. Development of zygotes to the blastocyst and hatching blastocyst stages was highest in medium containing 20% HS (64.8% and 54.4%, respectively) irrespective of amino acid supplementation. However, supplementation was significantly beneficial in all other media, with up to 42.1% of zygotes developing into hatching blastocysts. In these media, supplementation also significantly increased the mean number of nuclei per newly formed blastocyst (up to a mean of 70.8) and reduced the time during which blastocysts formed. Experiment 2 was an examination of the effect on embryo development of three amino acid preparations (oviduct amino acid concentrations vs. Eagle's Basal Medium (BME) essential + Minimum Essential Medium (MEM) nonessential vs. MEM essential + MEM nonessential concentrations) and the presence or absence of BSA. Both the amino acid and BSA treatments significantly influenced the percentage of zygotes that developed to the hatching blastocyst stage but not to the blastocyst stage. The preferred medium contained amino acids at oviductal fluid concentrations and BSA (54.5% hatching rate). The amino acid treatments did not significantly influence the mean number of nuclei per newly formed blastocyst, but the addition of BSA had a significant effect (70.7 ± 1.14 vs. 75.7 ± 1.13). In experiment 3, embryo development to Day 13 was examined after culture in SOF containing amino acids at oviductal fluid concentrations. Embryos were cultured in the presence of either BSA, polyvinyl alcohol (PVA), or no additional supplement and were transferred to recipient ewes on either Day 0 (after in vitro fertilization), 3, or 5. The addition of BSA or PVA had no significant effect, but significantly more embryos developed to Day 13 following transfer on Day 0 (60.0%) than on either Day 3 or 5 (overall 45.4%). It is concluded that SOF containing oviductal fluid concentrations of amino acids 1) facilitates the development of a high percentage (57.5%) of blastocysts, 2) improves embryo morphology compared with that observed in medium containing HS, 3) significantly improves hatching rates compared with those obtained in SOF containing commercially available preparations of amino acids, and 4) produces embryos with relatively high levels of viability to Day 13 of pregnancy.

INTRODUCTION

Oviductal fluid is a complex medium formed by selective transudation from the blood and by active secretion from cells of the epithelium. The ovine embryo is bathed in this fluid for 3 days after fertilization [1], during which time it develops to the 8–16-cell stage and initial transcription of the embryonic genome occurs [2]. Development of the embryo during this period is likely to be regulated by subtle changes in the composition of oviductal fluid induced by endocrine stimuli and influenced by both paracrine and autocrine effects. The significance of the oviductal environment to early embryo development is evident by the way in which disturbances of this environment can affect fetal development and the well-being of subsequent offspring [3–6].

Development of embryos in vitro depends, in part, on the ability to mimic the physical and chemical properties of oviductal fluid. Components of oviductal fluid have been studied in several species including the mouse [7, 8], rabbit [9], hamster [7], cow [10–12], and sheep [13]. All amino acids in blood are present in oviductal fluid [14], but there are variations in their concentrations during the estrous cycle [15]. Nancarrow et al. [16] examined the concentrations of 20 amino acids in oviductal fluid of sheep throughout the cycle and found that concentrations of most (e.g., methionine, leucine, phenylalanine, lysine, aspartic acid, glycine, alanine, taurine, tyrosine) were higher than in plasma while a small number (e.g., threonine, serine, ornithine) were at lower concentrations. These authors concluded that because amino acids have a pivotal role in metabolism, their inclusion in culture media at appropriate concentrations might be beneficial to early embryo development.

Studies on the effect of amino acid supplementation on the in vitro development of embryos of livestock species have concentrated on the evaluation of media containing amino acids at concentrations used by Eagle [17]. Generally, supplementation has improved embryo development in both the sheep [18] and cow [19–21]. In the sheep, improvements in embryo morphology and rates of blastocyst development have been observed. However, Eagle's concentrations of amino acids are based on the requirements for the growth of fibroblasts rather than embryos. Consequently, the current study was conducted to examine the effect on embryo development of supplementing synthetic oviductal fluid (SOF) with amino acids at oviductal fluid concentrations. Comparisons were made with development in SOF containing human serum (HS) and, in a separate experiment, in SOF containing Eagle's concentrations of amino acids. In a third experiment, the viability of embryos cultured in SOF containing oviductal fluid concentrations of amino acids was estimated.

MATERIALS AND METHODS

In Vitro Maturation (IVM) and In Vitro Fertilization (IVF) of Oocyte-Cumulus Complexes (OCCs)

Ovaries were collected from a local slaughterhouse and transported to the laboratory in PBS warmed to 30°C. Follicles (> 2 mm in diameter) were aspirated using an

Accepted May 8, 1996.
Received January 31, 1996.
[1]Correspondence. FAX: 6185249088;
e-mail: walker.simon@pi.sa.gov.au
[2]Current address: Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, MA 01003.

18-gauge needle and a vacuum pressure of approximately 25 mm Hg. The OCCs were collected in Hepes-buffered tissue culture medium 199 (TCM-199; Sigma Chemical Co., St Louis, MO) containing 2% (v:v) heat-inactivated sheep serum (SS) and 100 IU/ml heparin (Commonwealth Serum Laboratories, Parkville, Victoria, Australia). Unselected OCCs were subsequently rinsed five times and matured for 24 h in TCM-199 containing 20% (v:v) SS, 5 μg/ml FSH (Folltropin; Vetrapharm, London, ON, Canada), 5 μg/ml LH (Lutropin; Vetrapharm), and 1 μg/ml estradiol-17β (Sigma). Approximately 40 OCCs were placed in culture wells (Nunc Inc., Naperville, IL) each containing 0.8 ml medium covered with paraffin oil (BDH Ltd., Poole, Dorset, UK). Maturation occurred at 38.5°C in an humidified atmosphere of 5% CO_2 in air.

After maturation, the OCCs were rinsed briefly in SOF + 2% SS (IVF medium) containing 500 IU/ml hyaluronidase (Sigma). After three subsequent washes in IVF medium, OCCs were placed in groups of 30–35 per well. Each well contained 0.45 ml of IVF medium covered with paraffin oil. Motile sperm were obtained using "swim-up" procedures in which 0.3 ml of frozen-thawed semen (pellets from 3 rams combined) was layered below 1 ml IVF medium in a 10-ml tube (Falcon; Becton Dickinson, Melbourne, Victoria, Australia). The concentration of sperm in the upper fraction of the medium was determined 2 h later, and approximately 0.5×10^6 sperm were placed into each well. The OCCs and sperm were subsequently incubated at 38.5°C for 24 h in a humidified atmosphere of 5% CO_2 in air.

Embryo Culture

Remnant cumulus cells were removed from putative zygotes (i.e., pronuclear embryos) by gentle pipetting. Zygotes were subsequently rinsed twice in the IVF medium and then twice in the appropriate culture medium before being randomly allocated (in groups of 20–25) to wells each containing 0.8 ml of culture medium covered with paraffin oil. Culture occurred in a humidified atmosphere of 5% CO_2:5% O_2: 90% N_2 at 38.5°C. Development was assessed approximately 48 h after the commencement of culture, and putative zygotes that had failed to cleave were removed. The morphology of embryos was observed on Days 2, 4, 5, and 6 (Day 0 = day of commencement of culture), and final hatching rates were determined on Day 9. Morphological characteristics observed included the incidence of lipid inclusions in the cytoplasm, the incidence of cytoplasmic fragments, compaction of blastomeres in Day 4 and older embryos, and blastocoele formation in Day 5 and older embryos.

Preparation of Amino Acid Stocks

The concentrations of amino acids used in this study were those determined in sheep oviductal fluid by Nancarrow et al. [16]. Solutions of individual amino acids, with the exception of arginine, cystine, lysine, and tyrosine, were prepared in SOF. Arginine and lysine were mixed in 0.1 N HCl, and cystine and tyrosine were mixed in 0.1 N NaOH. The concentration of cystine in oviductal fluid was not available, but cystine was included in media at a concentration of 50 μM. Stock solutions were prepared every two weeks. All amino acids were of the L-form and all were obtained from Sigma.

Preparation of Culture Media

Synthetic oviductal fluid was prepared according to the formula of Tervit et al. [22] minus glucose. All chemicals were analytical grade and obtained from Mallinckrodt Specialty Chemical Co. (Paris, KY) with the exception of pyruvic acid, lactic acid, and penicillin (Sigma; all cell culture grade). Media were prepared weekly and equilibrated in the appropriate atmosphere for 2 days before use. Protein supplements were either BSA (fraction V; Boehringer Mannheim, Mannheim, Germany) or HS; BSA was used at a concentration of 4 mg/ml and HS at either 2% or 20% (v:v). The HS was prepared weekly by collection of 50 ml of whole blood from a 24-h-fasted subject. The sample was immediately centrifuged (500 × *g* for 20 min) and, after clotting, the serum was harvested by compression of the clot. Serum was then heat inactivated at 56.0°C for 30 min and stored at 4°C before use.

Between 6.7 and 47.8 μl of each amino acid stock was added to 20 ml of SOF to give the required final concentrations. The volume of each stock was proportionately reduced when serum was added to the medium; this reduction was based on the concentrations of amino acids previously determined in SOF + 20% HS. The concentration of each amino acid was determined in media used in experiment 1 to validate the formulation and mixing procedures. These concentrations were determined using the Waters amino acid analyzer (Waters, Millford, MA). Amino acids were identified on the basis of their elution position and reaction with ninhydrin.

Experimental Comparisons

In experiment 1 the effect of amino acid supplementation at oviductal fluid concentrations (OVaa) was examined in SOF containing various protein supplements. Media compared were SOF, SOF + 2% HS, SOF + 20% HS, and SOF + BSA. Hence the study was of a 2 × 4 factorial design (± amino acid supplementation, 4 media). Embryo development was assessed in terms of the percentage of zygotes that progressed to the blastocyst and hatching blastocyst stages (7 replicates) and the mean number of nuclei per newly formed blastocyst (10 replicates).

In experiment 2, embryo development in SOF + OVaa was compared with that obtained in media containing amino acids at Eagle's concentrations . The latter media were 1) SOF + Eagle's Basal Medium (BME) essential + Minimum Essential Medium (MEM) nonessential amino acids and 2) SOF + MEM essential + MEM nonessential amino acids; glutamine was added at a concentration of 205 μM. Embryo development in each medium was compared in the presence and absence of BSA. Hence, the study was of a 3 × 2 factorial design (3 amino acid treatments, 2 concentrations of BSA). The percentages of zygotes that developed to the blastocyst and hatching blastocyst stages were assessed in 4 replicates, while 7 replicates were used to determine the mean number of nuclei per newly formed blastocyst.

In experiment 3, the viability of embryos cultured in either SOF + OVaa, SOF + OVaa + PVA (ICN Biochemicals Inc., Cleveland, OH), or SOF + OVaa + BSA was assessed. Embryos were cultured for either 0, 3, or 5 days after IVF and then transferred to either oviducts (Day 0) or the uterine lumen (Days 3 and 5) of synchronized recipient ewes. Estrous cycles were synchronized using intravaginal pessaries containing 45 mg flugestone acetate (Chronogest; Laboratoire Pharmaceutique Porges, Paris, France) fol-

Downloaded from https://academic.oup.com/biolreprod/article/55/3/703/2760562 by U.S. Department of Justice user on 16 August 2022

TABLE 1. Amino acid concentrations (μM) in oviductal fluid (OV) and in SOF with various supplements (experiment 1).[a]

	OV*	20% HS	OVaa	2% HS + OVaa	20% HS + OVaa	BSA + OVaa
Arginine	100	24 ± 0.4	90 ± 0.1	92 ± 0.7	91 ± 2.5	81 ± 5.5
Asp. acid	20	4 ± 0.2	22 ± 0.4	21 ± 0.5	10 ± 0.6	27 ± 2.3
Asparagine	20	14 ± 1.0	24 ± 2.0	20 ± 1.9	30 ± 2.6	26 ± 1.6
Alanine	500	86 ± 0.1	431 ± 6.0	440 ± 9.6	484 ± 10.8	440 ± 2.1
Cystine	50**	NA	40 ± 0.4	26 ± 0.3	11 ± 1.6	22 ± 0.9
Glu. acid	50	20 ± 0.8	54 ± 0.8	50 ± 0.8	42 ± 3.0	53 ± 2.1
Glutamine	210	49 ± 8.3	101 ± 18.1	100 ± 17.2	125 ± 23.1	97 ± 19.5
Glycine	1500	60 ± 1.1	1390 ± 24.9	1381 ± 26.9	1419 ± 22.8	1371 ± 27.7
Histidine	50	16 ± 0.1	42 ± 0.2	42 ± 0.1	44 ± 0.1	41 ± 0.7
Isoleucine	100	19 ± 0.2	97 ± 2.5	100 ± 1.5	105 ± 0.7	97 ± 1.4
Leucine	200	36 ± 0.5	194 ± 0.5	195 ± 2.3	203 ± 1.1	194 ± 0.1
Lysine	220	40 ± 0.1	194 ± 3.2	196 ± 0.1	182 ± 3.1	191 ± 2.1
Methionine	50	NA	55 ± 0.9	49 ± 4.0	43 ± 0.2	49 ± 0.3
Ornithine	20	16 ± 2.1	19 ± 0.3	21 ± 0.7	18 ± 0.4	21 ± 0.2
Phenylal.	100	19 ± 0.3	95 ± 2.3	97 ± 1.1	94 ± 0.5	93 ± 1.2
Proline	50	NA	NA	NA	NA	NA
Serine	10	31 ± 2.2	9 ± 0.1	9 ± 0.7	31 ± 0.6	8 ± 1.0
Taurine	50	9 ± 0.4	49 ± 0.4	48 ± 0.8	46 ± 0.2	49 ± 0.7
Threonine	10	30 ± 0.6	9 ± 0.1	10 ± 2.1	30 ± 0.6	9 ± 0.3
Tyrosine	110	18 ± 0.6	89 ± 0.8	83 ± 1.5	91 ± 0.9	90 ± 0.7
Valine	270	62 ± 0.8	264 ± 0.4	262 ± 1.2	285 ± 0.5	261 ± 1.3

[a] NA, not available.
* Values of Nancarrow et al. [16].
** Nominal concentration.

lowed by the administration (i.m.) of eCG (400 IU per ewe; Heriot Agvet Pty Ltd, Melbourne, Victoria, Australia) at the time of pessary removal. Embryos were transferred during midventral laparotomy in groups of 20–30 per ewe. All cultured embryos were transferred irrespective of their stage of development. Day 13 conceptuses were subsequently recovered by flushing each uterine horn with 20 ml saline. Three replicates were conducted.

Number of Nuclei Per Blastocyst

In experiments 1 and 2, newly formed blastocysts were removed from media every 12 h and fixed for 5 days in a solution of ethanol and glacial acetic acid (3:1 proportion). Embryos were subsequently stained with orcein (BDH Ltd.), and nuclei were counted using phase-contrast optics (×125 magnification).

Statistical Analysis

Chi-square analysis (CATMOD procedure in Statistical Analysis Systems [23]) was used to test the effect of treatment on the proportions of blastocysts, hatching blastocysts, and Day 13 conceptuses. Analysis of variance procedures (General Linear Models in Statistical Analysis Systems [24]) were used to interpret treatment effects on mean number of nuclei. All first-order interactions were tested initially, and only those that were significant ($p < 0.05$) or were of specific interest were included in the final model.

RESULTS

Amino Acid Concentrations in Media

The concentrations of amino acids in media used in experiment 1 approximated those reported in oviductal fluid (Table 1) with the exception of glutamine and cystine, which were lower than expected (Table 1). The storage of media for 1–2 wk before assay was presumably responsible for the reduced glutamine concentration, whereas the reduced concentration of cystine may have resulted from oxidation during the same period. The concentrations of proline were not determined because of difficulty with resolution during the analysis. The concentration of serine was greater in SOF + 20% HS than in oviductal fluid. Amino acids were not detected in either SOF or SOF + BSA, while concentrations in SOF + 2% HS were proportional to those in SOF + 20% HS.

Experiment 1

The effect of amino acid supplementation on embryo development varied with the medium as indicated by a significant ($p < 0.01$) interaction between main effects. Supplementation of SOF + 2% HS significantly ($p < 0.001$) increased the percentage of embryos that developed to the hatching blastocyst stage (but not the blastocyst stage) and also increased the mean number of nuclei per newly formed blastocyst compared with that obtained in the unsupplemented medium (Table 2). On the other hand, the devel-

TABLE 2. The effect of amino acid supplementation at oviduct fluid concentrations on the development of IVM/IVF zygotes in SOF (experiment 1).

Medium*	Blastocysts (%)	Hatching blastocysts (%)	Blastocyst nuclei no.**
SOF + 2% HS	46.2^{a}	16.1^{ae}	45.4 ± 1.8^{a}
SOF + 2% HS + OVaa	52.1^{ad}	42.1^{b}	57.9 ± 1.6^{bd}
SOF + 20% HS	64.8^{b}	53.5^{c}	53.5 ± 1.6^{c}
SOF + 20% HS + OVaa	60.6^{b}	54.4^{c}	54.4 ± 1.5^{bc}
SOF + BSA	34.4^{c}	0.0^{d}	61.1 ± 2.2^{df}
SOF + BSA + OVaa	57.9^{bd}	21.3^{e}	70.8 ± 1.7^{e}
SOF	22.2^{c}	0.0^{d}	60.3 ± 3.4^{bcdf}
SOF + OVaa	57.5^{bd}	13.4^{a}	65.7 ± 1.8^{f}

* BSA concentration = 4 mg/ml.
** Nuclei number is least-squares mean ± SEM.
[a,b,c,d,e,f] Values for subclass means with at least one common superscript do not differ significantly ($p > 0.05$). Results are from 2 studies in which embryos were able to develop to the hatching blastocyst stage (n = 126–160 per treatment) or were stained for the determination of nuclei number in newly formed blastocysts (n = 40–137 per treatment).

Downloaded from https://academic.oup.com/biolreprod/article/55/3/703/2760562 by U.S. Department of Justice user on 16 August 2022

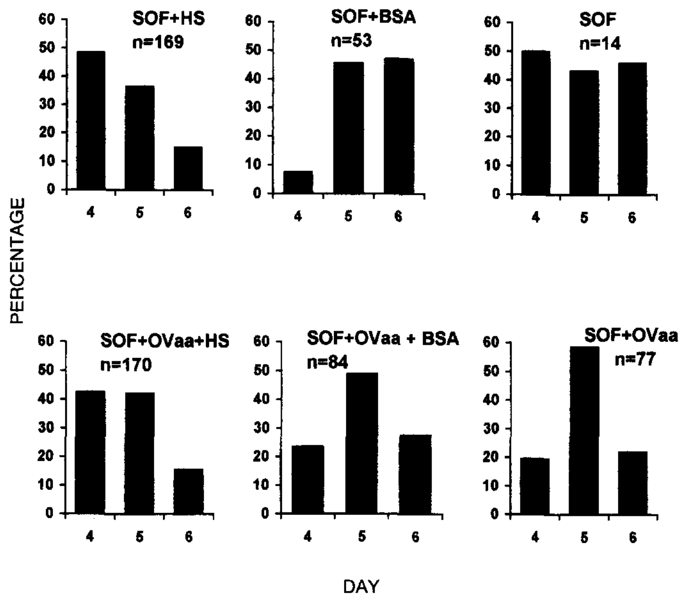

FIG. 1. The effect of supplementing SOF with amino acids at oviductal fluid concentrations (OVaa) on the day of blastocyst formation. SOF contained either HS (results combined for media containing 2% and 20% HS), BSA (4 mg/ml), or no protein supplement (Day 0 = day of insemination) (experiment 1).

opment of embryos to the blastocyst and hatching blastocyst stages in SOF + 20% HS was not significantly affected by supplementation; the highest rate of blastocyst formation occurred in this medium. Amino acid supplementation did not significantly influence the day of blastocyst formation in medium containing either 2% or 20% HS (Fig. 1).

The percentage of zygotes that developed to the blastocyst or hatching blastocyst stages in SOF or SOF + BSA was significantly ($p < 0.05$) improved following amino acid supplementation (Table 2). Supplementation also significantly ($p < 0.05$) increased the mean number of nuclei per newly formed blastocyst in SOF + BSA but not in SOF alone. The addition of amino acids to either SOF or SOF + BSA resulted in a time of blastocyst formation that was significantly ($p < 0.001$) more synchronized than in media without amino acids (Fig. 1).

The morphology of embryos that developed in SOF + OVaa was improved compared with that of embryos cultured in media containing HS. Improvements included a reduced incidence of lipid inclusions in the cytoplasm, fewer cytoplasmic fragments, and the ability of morulae to compact (Fig. 2). Furthermore, there was a more synchronous time of blastocyst development (Fig. 1) when embryos were cultured in SOF + OVaa.

Experiment 2

The percentage of embryos that developed to the blastocyst stage was not significantly influenced by either the amino acid treatment or the presence or absence of BSA (Table 3). However, amino acid treatment significantly ($p < 0.001$) influenced hatching rates, with SOF + OVaa (54.5%) being the preferred treatment. The addition of BSA also significantly ($p < 0.001$) improved hatching rates. Treatments did not significantly influence the time of blastocyst formation, and there were no significant interactions.

Amino acid treatment had no significant effect on the mean number of nuclei per newly formed blastocyst (Table 3). However, the addition of BSA significantly ($p < 0.01$) improved nuclei number (70.7 ± 1.14 vs. 75.7 ± 1.13). Nuclei number was also significantly ($p < 0.05$) influenced by the day of blastocyst formation and by replicate. It was significantly ($p < 0.001$) higher in blastocysts that formed on Day 5 compared with Day 6 (80.0 ± 1.11 vs. 66.4 ±

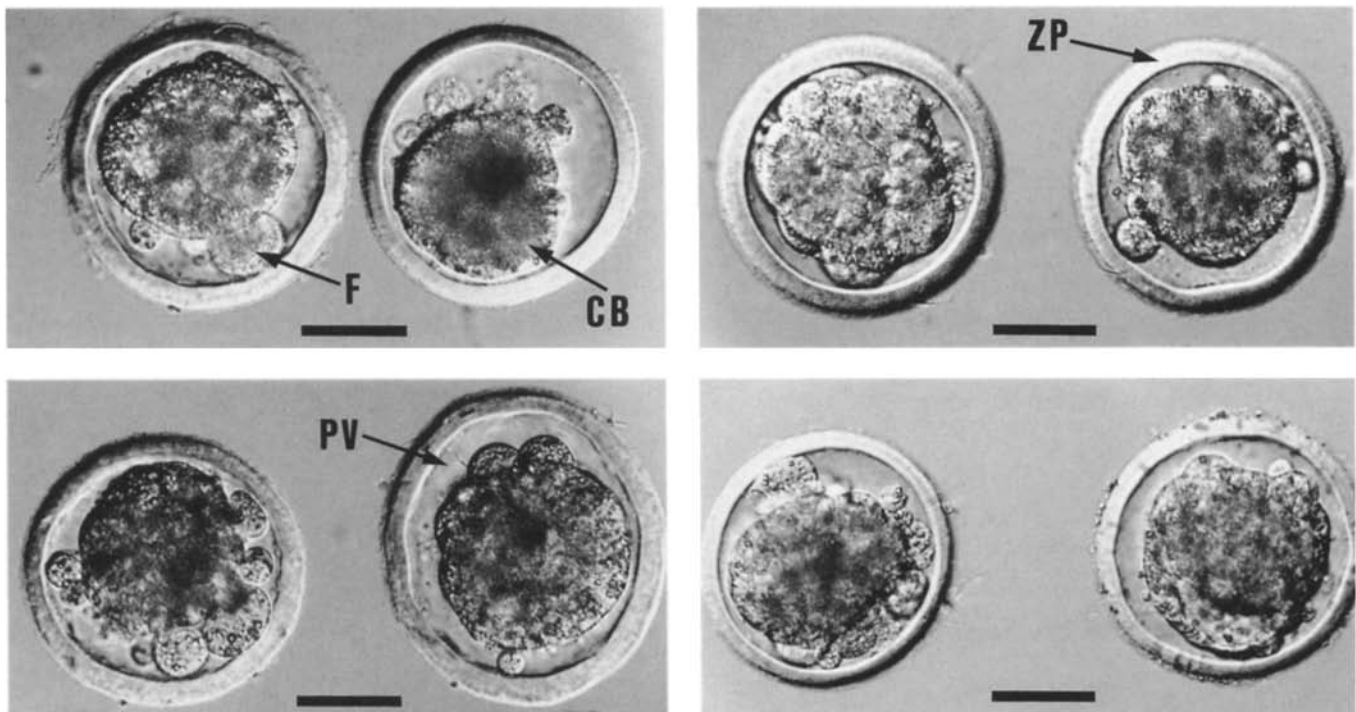

FIG. 2. Selected embryos cultured in SOF + OVaa showing varying degrees of compaction 5 days after the time of insemination. Micrographs were taken using Nomarski differential interference contrast optics (×250). PV, perivitelline space: CB, compact blastomeres; F, cytoplasmic fragment or blastomere excluded from the compaction process; ZP, zona pellucida. Scale bars = 60 μm.

Downloaded from https://academic.oup.com/biolreprod/article/55/3/703/2760562 by U.S. Department of Justice user on 16 August 2022

TABLE 3. The effect of various preparations of amino acids on the development of IVM/IVF zygotes in SOF with and without BSA (4 mg/ml) (experiment 2).

Amino acid preparation	BSA	Blastocysts (%)	Hatching blastocysts (%)	Blastocyst nuclei no.*
OVaa	−	68.6	39.1[a]	72.1 ± 1.9[a]
OVaa	+	74.0	54.5[b]	73.6 ± 1.9[abc]
BME essential + MEM nonessential	−	70.1	23.1[c]	69.1 ± 1.9[a]
BME essential + MEM nonessential	+	72.3	41.2[a]	75.5 ± 1.9[bc]
MEM essential + MEM nonessential	−	65.8	17.5[c]	70.9 ± 2.0[ab]
MEM essential + MEM nonessential	+	72.6	41.6[a]	77.9 ± 2.0[c]

* Nuclei number is least-squares mean ± SEM.
[a,b,c] Values for subclass means with at least one common superscript do not differ significantly ($p > 0.05$). Results are from 2 studies in which embryos were able to develop to the hatching blastocyst stage (n = 113–123 embryos per treatment) or were stained for the determination of nuclei number in newly formed blastocysts (n = 89–104 per treatment).

1.19); insufficient blastocysts developed on Day 4 to give a valid comparison. There were no significant interactions of relevance.

Experiment 3

The morphology of cultured embryos on the day of transfer (Day 3 or 5) was similar between treatments. The proportion of embryos that developed to Day 13 was significantly ($p < 0.05$) higher when embryos were transferred on Day 0 compared with either Day 3 or 5. There was no significant difference between the latter two days, and the effect of medium (i.e., SOF + OVaa vs. SOF + OVaa + PVA vs. SOF + OVaa + BSA) was also not significant. Overall, 60.0% (60 of 100) of embryos transferred on Day 0 developed to Day 13 compared with 45.4% (197 of 434) for all other treatments.

DISCUSSION

The results of this study demonstrate that when ovine zygotes produced by IVM/IVF are cultured in a chemically defined medium containing amino acids at oviductal fluid concentrations, high percentages develop into blastocysts. Furthermore, a relatively high percentage of unselected embryos develop to Day 13 of pregnancy when transferred to recipient ewes after either a 3- or 5-day culture period. Synthetic oviductal fluid is similar to several "simple" media and is based on the concentration of salts and energy sources in the oviductal fluid of sheep [13]. Serum is usually added to the medium to facilitate embryo development; and while a high proportion (> 90%) of in vivo-derived sheep zygotes develop into blastocysts in this medium, abnormalities occur in both embryos and resultant offspring [4, 5]. In experiment 1, the highest rates of blastocyst and hatching blastocyst formation were obtained in media containing 20% HS. The replacement of serum with amino acids at oviductal fluid concentrations did not significantly affect the rate of blastocyst formation but did significantly improve the mean number of nuclei per newly formed blastocyst. These results were obtained in association with substantial improvements in embryo morphology. The incidence of lipid inclusions in the cytoplasm was reduced, and most morulae also underwent compaction (Fig. 2), a feature not observed in embryos cultured in SOF containing serum. Furthermore, the time of blastocyst formation was similar to that observed in embryos developed in vivo [5].

This study also demonstrates that oviductal fluid concentrations of amino acids in SOF are more advantageous to embryo development than are Eagle's concentrations. Hatching rates of embryos cultured in SOF + OVaa were significantly higher than those of embryos cultured in the presence of Eagle's amino acids. However, general morphology, rates of blastocyst formation, and nuclei number per newly formed blastocyst were similar. The increased hatching rates observed in SOF + OVaa may have resulted from differences in the concentrations of amino acids involved in the hatching process or, alternatively, from a more permissive influence of the culture medium on embryo development. The addition of Eagle's amino acids to culture media has generally been associated with improved embryo development in both the sheep [18] and cow [19–21]. However, Eagle's concentrations are based on those required for the growth of fibroblasts [17] and consequently may not be able to meet the precise requirements of the embryo. These concentrations are generally higher than those in oviductal fluid. The clear exceptions are 1) glycine and alanine, which are at higher concentrations in oviductal fluid and 2) ornithine and taurine, which are not present in Eagle's amino acid preparations but are present in oviductal fluid. Glycine and alanine are the predominant amino acids in the oviductal fluid of several species (cow [12], rabbit [9], horse [25]), indicating an active role for each in early embryo development. Glycine, alanine, and taurine are implicated in the regulation of intracellular pH and in protecting the embryo from osmotic stress [26, 27]. Both glycine and alanine are known to have a stimulatory effect on the development of the bovine embryo [12], and glycine is also known to have a beneficial effect on the hamster zygote [28]. Similarly, taurine is reported to improve embryo development in both the mouse [29] and pig [30]. It is possible that glycine and alanine are present in Eagle's amino acid preparations at concentrations less than optimal for embryo development and that this deficiency is exacerbated by the absence of taurine.

Although 45.4% of unselected embryos developed into Day 13 conceptuses after culture for either 3 or 5 days (experiment 3), this was nonetheless a figure significantly below that obtained (60.0%) when embryos were transferred immediately after IVF. In our experience, the latter figure is also below that normally obtained following the comparable transfer of zygotes produced in vivo (70.0% [4, 31]). The observation that the addition of BSA to SOF + OVaa had no significant effect on embryo viability to Day 13 was surprising given that BSA significantly improved hatching rates in both experiments 1 and 2. The addition of PVA to the medium was also without significant effect. Albumin is thought to function by stabilizing cell membranes [32] and so to prevent the leakage of endogenous amino acids. It is possible that membranes of embryos

Downloaded from https://academic.oup.com/biolreprod/article/55/3/703/2760562 by U.S. Department of Justice user on 16 August 2022

cultured in serum-free media containing amino acids are sufficiently stable to negate any long-term benefit of including BSA in the medium. That membrane stability is improved in the presence of amino acid supplements is supported by the observation that morulae are able to compact, a procedure requiring the formation of tight gap junctions.

Previous studies have shown that some sheep embryos cultured in the presence of serum develop into unusually large lambs after transfer [4, 5]. The physiological basis for this phenomenon is not understood, but the incidence of large lambs may be associated with the morphological abnormalities that occur during culture [5, 33]. Given the improved morphology of embryos cultured in SOF + OVaa, a pertinent question is whether such embryos would develop normally after transfer. Logistical restraints prevented the production of lambs in this study. However, in the study of Thompson et al. [33], normal lambs were born after the transfer of embryos cultured in serum-free SOF containing Eagle's concentrations of amino acids. Given that embryo morphology in that study was, in many respects, comparable to that observed in SOF + Ovaa, it is highly likely that embryos cultured in the latter medium would develop normally. It is also noteworthy that supplementing SOF + 20% HS (or 2% HS) with amino acids so that their concentrations approximated those in oviductal fluid did not reduce the incidence of developmental abnormalities during culture. The adverse effects of serum on embryo morphology in vitro is therefore unlikely to result from inappropriate concentrations of amino acids.

In conclusion, this study has demonstrated that IVM/IVF sheep zygotes cultured in the defined medium SOF + OVaa are able to develop into conceptuses at relatively high rates. The efficacy of this medium does not require the presence of macromolecules, and only further studies will determine the extent to which embryo development is normalized. Subject to these findings, the availability of such a medium is likely to play an important role in establishing the determinants of normal embryo and fetal development.

ACKNOWLEDGMENTS

We thank Ms. C. Brand and Mr. J. Evans for assistance with these experiments.

REFERENCES

1. Holst PJ. The time of entry of ova into the uterus of the ewe. J Reprod Fertil 1974; 36:427–428.
2. Crosby IM, Gandolfi F, Moor RM. Control of protein synthesis during early cleavage of sheep embryos. J Reprod Fertil 1988; 82:769–775.
3. Wilmut I, Sales DI. Effect of an asynchronous environment on embryo development in the sheep. J Reprod Fertil 1981; 61:179–184.
4. Walker SK, Heard TM, Seamark RF. In vitro culture of sheep embryos without co-culture: successes and perspectives. Theriogenology 1992; 37:111–126.
5. Walker SK, Heard TM, Bee CA, Frensham AB, Warnes DM, Seamark RF. Culture of embryos of farm animals. In: Lauria A, Gandolfi F (eds.), Embryonic Development and Manipulation in Animal Production. London: Portland Press Ltd; 1992: 352–358.
6. Kleemann DO, Walker SK, Seamark RF. Enhanced fetal growth in sheep administered progesterone during the first three days of pregnancy. J Reprod Fertil 1994; 102:411–417.
7. Kaye PL. Metabolic aspects of the physiology of the preimplantation embryo. In: Rossant J, Pedersen RA (eds.), Experimental Approaches to Mammalian Embryonic Development. Cambridge: Cambridge University Press; 1986: 411–417.
8. Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism *in vitro*. J Reprod Fertil 1990; 88:361–368.
9. Miller JGO, Schultz GA. Amino acid content of preimplantation rabbit embryos and fluids of the reproductive tract. Biol Reprod 1987; 36:125–129.
10. Stanke DF, Sikes JD, De Young DW, Tumbleson ME. Proteins and amino acids in bovine oviduct fluid. J Reprod Fertil 1974; 38:493–496.
11. Roberts GP, Parker JM, Symonds HW. Proteins in the luminal fluid from the bovine oviduct. J Reprod Fertil 1975; 45:301–313.
12. Moore K, Bondioli KR. Glycine and alanine supplementation of culture medium enhances development of in vitro matured and fertilized cattle embryos. Biol Reprod 1993; 48:833–840.
13. Restall BJ, Wales RG. The fallopian tube of the sheep. 3. The chemical composition of fluid from the Fallopian tube. Austr J Biol Sci 1966; 19:687–698.
14. Thibault C. Physiology and physiopathology of the Fallopian tube. Int J Fertil 1972; 17:1–13.
15. Menezo YJR, Laviolette P. Les constituants aminés des sécrétions tubaires chez la lapine. Ann Biol Anim Biochim Biophys 1972; 12: 383–396.
16. Nancarrow CD, Hill JL, Connell PJ. Amino acid secretion by the ovine oviduct. In: Proc Aust Soc Reprod Biol; 1992; Adelaide, Australia. Abstract 71.
17. Eagle H. Amino acid metabolism in mammalian cell cultures. Science 1959; 130:432–437.
18. Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins and culturing embryos in groups stimulate development. Biol Reprod 1994; 50:390–400.
19. Takahashi Y, First NL. In vitro development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology 1992; 37:963–978.
20. Kim J-H, Niwa K, Lim J-M, Okuda K. Effects of phosphate, energy substrates and amino acids on development of in-vitro matured, in vitro-fertilized bovine oocytes in a chemically defined, protein-free culture medium. Biol Reprod 1993; 48:1320–1325.
21. Rosenkrans CF, First NL. Effects of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. J Anim Sci 1994; 72:434–437.
22. Tervit HR, Whittingham DG, Rowson LEA. Successful culture in vitro of sheep and cattle ova. J Reprod Fertil 1972; 30:493–487.
23. Stanish WM, Sall JP. The CATMOD procedure. In: SAS User's Guide, Statistics. Cary, NC: Statistical Analysis System Institute, Inc.; 1985: 171–253.
24. Spector PC, Goodnight JH, Sall JP, Sarle WS. The GLM procedure. In: SAS User's Guide, Statistics. Cary, NC: Statistical Analysis System Institute, Inc.; 1985: 433–506.
25. Engle CC, Foley CW, Plotka ED, Witherspoon DM. Free amino acids and protein concentrations in reproductive tract fluid of the mare. Theriogenology 1984; 21:919–930.
26. Van Winkle LJ, Haghighat N, Campione AL. Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions in oviduct fluid. J Exp Zool 1990; 253: 215–219.
27. Bavister BD, McKiernan SH. Regulation of hamster embryo development in vitro by amino acids. In: Bavister BD (ed.), Preimplantation Embryo Development. New York: Springer-Verlag; 1993: 57–72.
28. Bavister BD, Arlotto T. Influence of single amino acids on the development of hamster one-cell embryos in vitro. Mol Reprod Dev 1990; 25:45–51.
29. Dumoulin JCM, Evers JLH, Bras M, Pieters MHEC, Geraedts JPM. Positive effect of taurine on preimplantation development of mouse embryos in vitro. J Reprod Fertil 1992; 94:373–380.
30. Reid ML, Illera MJ, Petters RM. In vitro culture of pig embryos. Theriogenology 1992; 37:95–109.
31. Walker SK, Smith DH, Frensham A, Ashman RJ, Seamark RF. The use of synthetic gonadotropin releasing hormone treatment in the collection of sheep embryos. Theriogenology 1989; 31:741–752.
32. Cholewa JA, Whitten WK. Development of two-cell mouse embryos in the absence of a fixed-nitrogen source J Reprod Fertil 1970; 22: 553–555.
33. Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. Biol Reprod 1995; 53:1385–1391.

Downloaded from https://academic.oup.com/biolreprod/article/55/3/703/2760562 by U.S. Department of Justice user on 16 August 2022