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Lamb Birth Weight Is Affected by Culture System Utilized during In Vitro Pre-Elongation Development of Ovine Embryos

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ABSTRACT

It has previously been reported that ovine embryos cultured in Synthetic Oviduct Fluid medium supplemented with 20% human serum (SOF + HS) develop into lambs with a high birth weight. We have investigated this phenomenon by culturing ovine zygotes in SOF + HS or a serum-free version of Synthetic Oviduct Fluid with BSA and amino acids (SOFaaBSA) in place of serum. Zygotes were either obtained from superovulated and naturally mated ewes or produced in vitro. Embryos were subsequently transferred to synchronized recipient ewes (n = 63). An additional group of ewes (n = 16) served as flock fertility and lambing controls. Development of zygotes to stages suitable for transfer (i.e., good to excellent compact morulae or blastocysts) was not affected by medium (SOFaaBSA = 53 ± 5% vs. SOF + HS = 59 ± 5%) but was affected by source (in vivo-derived = 74 ± 5% vs. in vitro-derived = 35 ± 5%, $p < 0.001$). Embryos incubated in SOF + HS were morphologically different from those incubated in SOFaaBSA, having abundant lipid droplets. Pregnancy rate (65%) and embryo survival (48%) of recipients determined by ultrasonography on approximately Day 60 of pregnancy did not differ between medium treatments or source of embryo. Mean weight of lambs from embryos cultured in SOF + HS (4.2 ± 0.2 kg) was significantly heavier than that of controls (3.4 ± 0.2 kg, $p < 0.01$) or of lambs from embryos cultured in SOFaaBSA (3.5 ± 0.2 kg, $p < 0.05$). Furthermore, mean gestation length was longer in recipients receiving embryos incubated in SOF + HS (147 ± 1 days) than in SOFaaBSA (145 ± 1 day, $p < 0.05$). Reasons for this birth weight and gestation length difference are unclear, but our data suggest that different culture conditions can produce embryos with differing morphology, apparent chemical composition, and rate of development, resulting in lambs with differing gestation length and birth weight.

INTRODUCTION

There are several reports about the development of ovine zygotes to the blastocyst stage during in vitro culture (for example, [1–4]). In particular, both embryo development and post-transfer viability have been examined in some detail for one culture system, Synthetic Oviduct Fluid medium [5] supplemented with human serum and incubation under a low-oxygen atmosphere (SOF + HS) [2, 6, 7]. Although a high proportion (generally over 70%) of zygotes and early embryos will develop when cultured in this system, several abnormalities are known to occur, including premature blastulation [6], accumulation of cytoplasmic "lipid-like" inclusions [4], and an increase in both gestation length and birth weight of lambs following transfer of cultured embryos [6]. The reasons for these abnormalities have yet to be identified.

The addition of human serum has been shown to stimulate ovine embryo development during in vitro culture [2, 3]. However, recent reports demonstrate that serum can be replaced by BSA and amino acids [4, 8, 9]. In particular, when zygotes were cultured in Synthetic Oviduct Fluid medium supplemented with both Eagle's MEM essential and nonessential amino acids and 8 mg/ml BSA (SOFaaBSA) and transferred into fresh medium every second day, over 90% developed to the blastocyst stage. Furthermore, blastocysts had cell numbers comparable to those of in vivo-derived blastocysts of equivalent stage of development [4] and had fewer "lipid-like" inclusions than those from SOF + HS [4]. We report here the in vitro development and post-transfer viability of both in vivo- and in vitro-derived zygotes cultured in SOF + HS or SOFaaBSA and demonstrate that the culture system used affects embryo morphology, gestation length, and birth weight. A preliminary account of this work has been presented elsewhere [10].

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MATERIALS AND METHODS

Collection of Human Serum

Blood was obtained from 3 volunteers (2 female, 1 male) at the Blood Donor Centre, Waikato Hospital, Hamilton, New Zealand. Serum was recovered after the blood was allowed to clot overnight at 4°C. The serum was pooled, heat-treated at 56°C for 30 m, sterilized by use of a 0.22-μm filter (Gelman, Ann Arbor, MI), aliquoted, and stored frozen at −20°C before use.

In Vivo Embryo Production

Day 1.5 sheep embryos (day of estrus = Day 0) were recovered from superovulated and mated mature age Romney × Dorset (n = 23) and Coopworth × Dorset (n = 7) ewes according to procedures described elsewhere [11]. In brief, the estrous cycles of ewes were synchronized with two sequentially administered, controlled intravaginal progesterone-releasing devices (CIDR, Type G; InterAg, Hamilton, New

Zealand); the first device was inserted for 9 days, and the second was inserted immediately after removal of the first and left in place for another 3 days [11]. During these three days, horse anterior pituitary extract (total dosage 40 mg/ewe), prepared from a single ethanol extraction, was administered twice daily, and the second CIDR was withdrawn at the sixth and final injection. In addition, 200 IU eCG (Folligon; Intervet, Amsterdam, The Netherlands) was administered the morning after the second CIDR insertion concurrent with the first dose of horse anterior pituitary extract. Fertile rams were introduced at CIDR withdrawal, and a GnRH analogue (100 μg/ewe, Fertagyl; Intervet) was administered at noon the following day (at approximately the time of estrus onset). Rams were introduced to ewes in rotation over the estrous period to maximize fertilization of oocytes. Putative zygotes were recovered by flushing the oviduct of each ewe, under surgical conditions, with a HEPES-buffered version of Synthetic Oviduct Fluid supplemented with 0.3% w/v BSA (fatty acid-free prepared from Fr.V BSA; Sigma, St. Louis, MO) (HSOF) [12]. Ewes were randomly allocated to one of two days of embryo collection (and therefore initiation of superovulation treatment).

In Vitro Embryo Production

Ovaries were collected from mature ewes (from the same flock as the superovulated embryo donors) and placed in saline (35°C) at an abattoir on two separate occasions and transported within 2 h back to the laboratory. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 1–5-mm follicles by an 18-gauge needle and negative pressure delivered through a vacuum pump (50 mm Hg). COCs were collected into HEPES-buffered TCM 199 medium supplemented with 10 μg/ml heparin (from porcine intestinal mucosa, Sigma) and 0.4% w/v BSA (affinity column-purified, ABRD; Immuno-Chemical Products (ICP), Auckland, New Zealand). Prior to in vitro maturation, COCs were assessed morphologically, and only those that had a compact, nonatretic cumulus oophorus-corona radiata and a homogeneous ooplasm were selected. All selected COCs were washed thoroughly in HEPES-buffered TCM 199 medium supplemented with 10% fetal calf serum (FCS; Life Technologies, Auckland, New Zealand) before being washed once in maturation medium and placed in 50 μl maturation medium in wells of a 96-well, flat-bottom microtiter plate (Nunc, Roskilde, The Netherlands). The medium used for maturation was TCM 199 supplemented with 10% FCS, 10 μg/ml ovine FSH (Ovagen; ICP), 10 μg/ml ovine LH (ICP), and 1 μg/ml estradiol (Sigma). Microtiter plates were placed in culture chambers held at 39°C and gassed continually with humidified 5% CO_2:7% O_2: 88% N_2 [4] for 24 h. After maturation, the cumulus-corona was mostly removed by rapidly pipetting COCs with a fine-bore Pasteur pipette in a 0.01% solution of hyaluronidase (from bovine testes, Sigma) in HSOF.

Spermatozoa were prepared from frozen-thawed semen samples from three rams that were used to mate the embryo donor ewes. The contents of three 0.25-ml straws (each containing approximately 1×10^8 sperm/ml in TRIS/egg yolk/glycerol diluent) were layered upon a Percoll gradient (45%/90%), and motile sperm were collected after centrifugation at approximately $700 \times g$ for 20 min at room temperature. The motile fraction was washed once in HSOF and then resuspended in fertilization medium, a high Ca^{2+}-ion (5.0 mM) content version of SOFaaBSA, further supplemented with 20% heat-inactivated metestrous ewe serum and 0.2 μM penicillamine and 0.1 μM hypotaurine. Insemination was performed in 50 μl of fertilization medium (final concentration at insemination was 1×10^6 sperm/ml) in flat-bottomed microtiter plates (5 oocytes/well) over a 24-h period under the same conditions as described for oocyte maturation. After insemination, putative zygotes were removed and washed twice in HSOF before allocation to treatment groups.

In Vitro Culture of Embryos

After collection, in vivo-derived embryos from each donor ewe were randomly allocated within donor to either SOF + HS or SOFaaBSA medium. The BSA used for culture was the same as used for HSOF medium at a concentration of 8 mg/ml. In vitro-produced embryos (IVP) were pooled, then allocated randomly to either medium. Embryo culture was performed in 20-μl microdrops overlaid with paraffin oil (Squibb, Princeton, NJ) as described elsewhere [4]. Where possible, groups of 4 embryos per drop were maintained, although some groups of 2 were included. All embryos were incubated in chambers held at 39°C and continually gassed with humidified 5% CO_2:7% O_2:88% N_2. All embryos were transferred to fresh medium every second day, and culture ended on Day 5, approximately 120 h after start of culture. All embryos were assessed morphologically for cleavage and development to the compacted morula or blastocyst stage, and judged for suitability for transfer, according to criteria for "normal embryos" as described by Wintenberger-Torres and Sevellec [13]. If not suitable, they were stained with bisbenzimide 33342 (Sigma) and assessed for normal fertilization or cleavage.

Embryo Transfer

Eighty mature ewes (from the same flock as donor ewes, with body weights of 40–60 kg) were synchronized by a single 12-day CIDR treatment. At the time of CIDR withdrawal, each ewe received 400 IU eCG. A selected group of 16 ewes (balanced for age, weight, and breed) was placed with rams immediately after CIDR withdrawal. These rams were the same as those used for donor mating and in vitro fertilization. The group of 16 ewes provided control information on the fertility of the flock, lamb birth weight, and gestation length. The remaining 64 ewes were placed with vasectomized rams, and estrous onset was recorded in both groups during twice-daily observations. Approximately 2

days after estrus, all ewes, including controls, underwent laparoscopy to determine the ovulation rate, One ewe failed to ovulate and was therefore excluded. Ewes for embryo transfer were subsequently allocated into one of four groups, balanced for ovulation rate, age, weight, and breed.

Embryo transfer took place on two separate days, corresponding approximately to Day 6 after estrus of both donor and recipient ewes. Embryos were transferred in HSOF by a modified laparoscopic ("transpic") technique [14]. Each recipient received two embryos of similar stage of development ipsilateral to the preferred CL. All ewes, including controls, received a CIDR device at the time of embryo transfer (i.e., approximately Day 6 of pregnancy) that remained in place for 8 days.

Pregnancy Assessment and Progesterone Analysis

Blood samples were taken by jugular venipuncture on the day after CIDR device withdrawal (i.e., approximately Day 15 of pregnancy), and plasma was extracted and frozen (−20°C) until assayed. Progesterone concentrations were measured on duplicate (100 μl) unextracted plasma samples by RIA, by a method described elsewhere [15], and validated for sheep plasma. All samples were included in one assay. The within-assay CV was 7.2% to 7.8% for replicate samples over the range of 2.0–7.6 ng/ml. The minimum detectable concentration was 0.2 ng/ml. Ewes were considered "biochemically pregnant" if plasma progesterone concentration was above 1.0 ng/ml [16].

All ewes, including controls, were further assessed for pregnancy by ultrasound sonography 55 days after embryo transfer (on approximately Day 60 of pregnancy). The number of fetuses was also recorded. Pregnant ewes were then allowed to proceed to term, and lambing took place outdoors. Data on gestation length as well as birth weight, sex, and health of the lamb were recorded.

Electron Microscopy

A small number (2–3 from each medium treatment) of transferable-quality blastocysts that were not required for embryo transfer were processed for electron microscopy by fixation for 2 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. The embryos were then washed in two changes of buffer, postfixed in 1% OsO_4 for 30 min, and embedded in Spurr's resin. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and examined under a Phillips EM400 (Eindhoven, The Netherlands) transmission electron microscope at 80 kV.

Statistical Analyses

Unfertilized oocytes were excluded from all analyses apart from the comparison of cleavage rates. The proportion data for in vitro development of embryos were analyzed, after logit transformation, by use of the Generalized Linear Models (GLM) procedure within the GENSTAT statistical package (Lawes Agricultural Trust, Rothamsted, UK). In addition to the main treatment groups and their interaction, the data were also tested for random effects of day of surgical collection and culture dish-to-dish variations within day. Pregnancy and embryo survival rates were analyzed by contingency tables, with Pearson's chi-square statistics calculated. Lamb birth weight and gestation length were analyzed by Least Squares procedures within the GENSTAT program. The analyses tested the effects of the main treatment groups and their interactions, accounting for the occurrence of single or multiple births and differences due to sex when these caused significant variation. Also determined was whether differences in birth weight could be explained by differences in gestation length. The results presented are predicted values calculated after the appropriate models were fitted.

TABLE 1. Percentage of IVD and IVP putative zygotes that cleaved, and the percentage of cleaved embryos developing to the CM and BL stages or that were TQ following culture.*

Zygote	Medium	N	% CL	% CM/BL	% BL	% TQ
IVD	SOFaaBSA	90	90 ± 4	74 ± 6	55 ± 7[a]	68 ± 7
	SOF + HS	94	93 ± 4	81 ± 6	78 ± 6[b]	80 ± 6
IVP	SOFaaBSA	150	70 ± 5	37 ± 6	24 ± 6	35 ± 6
	SOF + HS	146	61 ± 6	38 ± 7	32 ± 7	32 ± 7

*Abbreviations: N = number, CL = cleaved embryos, CM = compact morulae, BL = blastocysts, TQ = transferable quality embryos. Proportions are back-transformed estimates. Values with different superscripts differ significantly ($p < 0.05$).

RESULTS

Embryo Development and Morphology

The percentages of putative zygotes cleaving and of cleaved embryos developing to at least the compact morula stage are described in Table 1. Significantly more in vivo-derived embryos cleaved than did IVP embryos ($p < 0.001$); however, there were no differences between the two media used. The proportion of embryos that developed to post-compaction stages was much greater for in vivo-derived embryos than for IVP embryos ($p < 0.001$). The proportion of embryos developing to the compact morula stage or beyond 5 days culture was not different between the two media, regardless of source ($p > 0.05$). However, the proportion of embryos that reached the blastocyst stage was significantly higher in SOF + HS (56 ± 5%) than for SOFaaBSA (42 ± 5%, $p < 0.05$, back-transformed estimates with a noninteractive model). Despite this, the proportions of embryos adjudged transferable were comparable (SOFaaBSA = 53 ± 5% vs. SOF + HS = 59 ± 5%, $p > 0.05$). There were no interactions for development between source of embryo and medium used.

Morphological examination of embryos under an inverted light microscope revealed that those cultured in SOF + HS were darker and had numerous "lipid-like" inclusions compared to those cultured in SOFaaBSA (Fig. 1, a and b). These lipid-like inclusions were identified as osmophilic

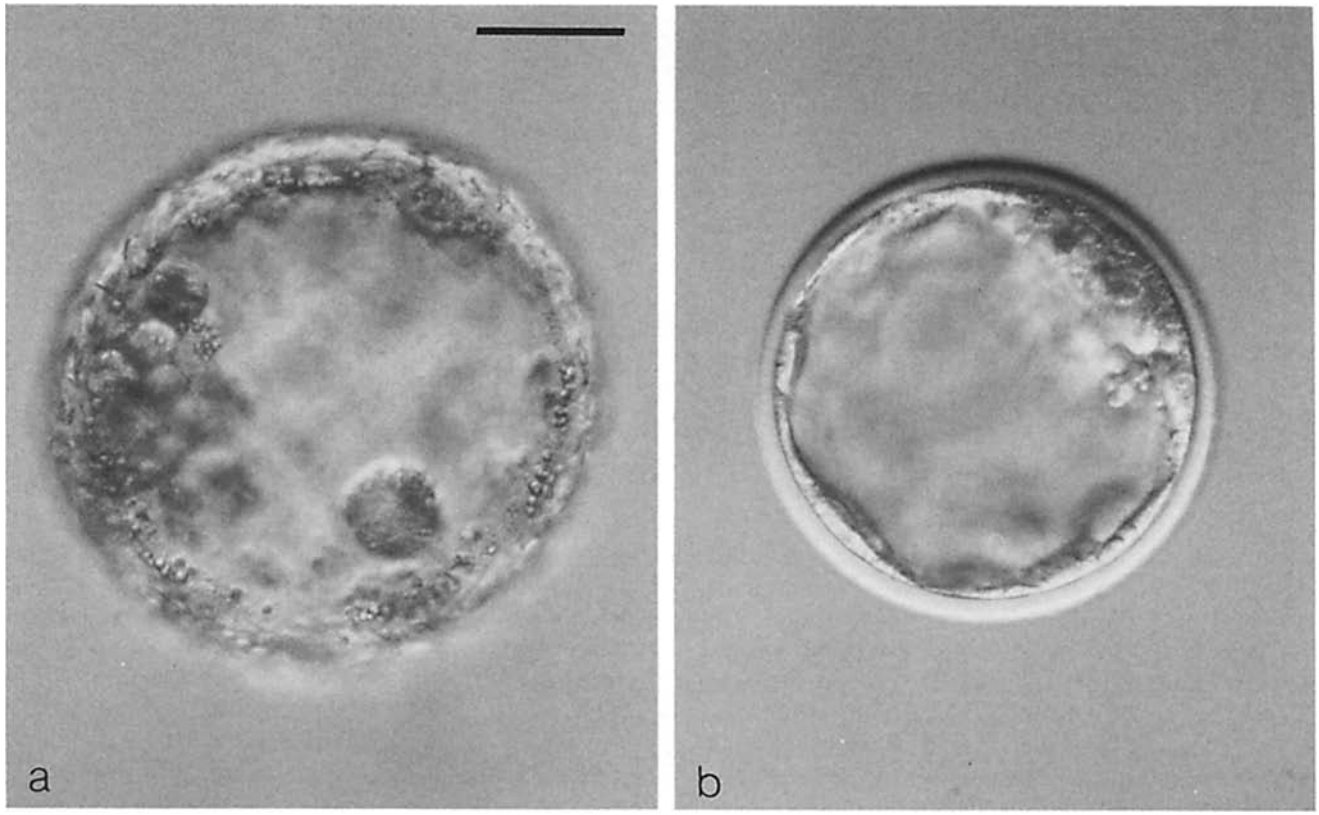

FIG. 1. Differential interference contrast microscopy of in vivo-derived ovine zygotes cultured in either SOF + HS (**a**) or SOFaaBSA (**b**) to blastocyst stage. The blastocyst cultured in SOF + HS (**a**) has numerous cytoplasmic droplets present, especially visible in trophectoderm cells (bar = 50 μm).

lipid droplets by transmission electron microscopy, indicating that they contained a significant proportion of unsaturated lipid (Fig. 2). No further morphological differences were identified, as numbers processed were small.

Pregnancy Rate and Embryo Survival

The proportion of recipient ewes with plasma progesterone samples > 1.0 ng/ml ("biochemical pregnancy") was 51 of 63 (81%, Table 2) and did not differ significantly from the corresponding proportion of the flock fertility controls (15 of 16 ewes, 94%). There were no differences in biochemical pregnancy rates for either source of embryo or culture medium treatment groups. At ultrasound sonography, the pregnancy rate of recipient ewes (41 of 63, 65%) was lower than the number of ewes assessed biochemically pregnant ($\chi^2_1 = 3.26$, $p < 0.1$) because of 10 ewes with plasma progesterone > 1 ng/ml subsequently scanned as not pregnant. This rate was significantly lower than that in the flock fertility controls, which remained at 94% ($p < 0.05$). Embryo survival recorded at ultrasound sonography was 61 of 126 (48%), and there were no significant differences between treatment groups. There was, however, an interaction ($p < 0.05$) between developmental stage and source of embryo: there was no difference in embryo survival between compact morula and blastocysts from in vivo-derived embryos (3 of 8, 38% and 24 of 58, 41%, respectively), but only 1 of 6 (17%) compact morulae from in vitro-derived embryos survived after transfer compared with 33 of 54 (61%) of blastocysts. The number of fetuses observed in the flock fertility controls was 22 from 35 ovulations.

TABLE 2. Pregnancy rate and embryo survival of transferable quality embryos in recipient ewes measured at approximately Day 15, Day 60 and at lambing.

	Pregnancy rate				Embryo survival			
	SOF + HS		SOFaaBSA		SOF + HS		SOFaaBSA	
	IVD	IVP	IVD	IVP	IVD	IVP	IVD	IVP
Day 15 (biochemical)	14/15	11/14	15/19	11/15	—	—	—	—
Day 60 (scanning)	11/15	10/14	10/19	10/15	16/30	14/28	15/38	16/30
Lambing	11/15	10/14	9/18	8/15	13/30	14/28	14/36	11/30

One pregnant recipient ewe, which had received in vivo-derived embryos cultured in SOFaaBSA, disappeared after ultrasound sonography; therefore her lambing data were unavailable. Another 4 ewes, scanned as pregnant, were barren at lambing. Two had received in vitro-derived embryos cultured in SOFaaBSA, one had received in vivo-derived embryos cultured in SOFaaBSA, and the fourth was one of the flock fertility controls. The remaining 38 recipient ewes and 14 flock fertility control ewes gave birth to 52 (Table 2) and 23 lambs, respectively. There was some minor deviance between fetal numbers at scanning and lambs born. Eleven neonatal deaths were recorded, which were

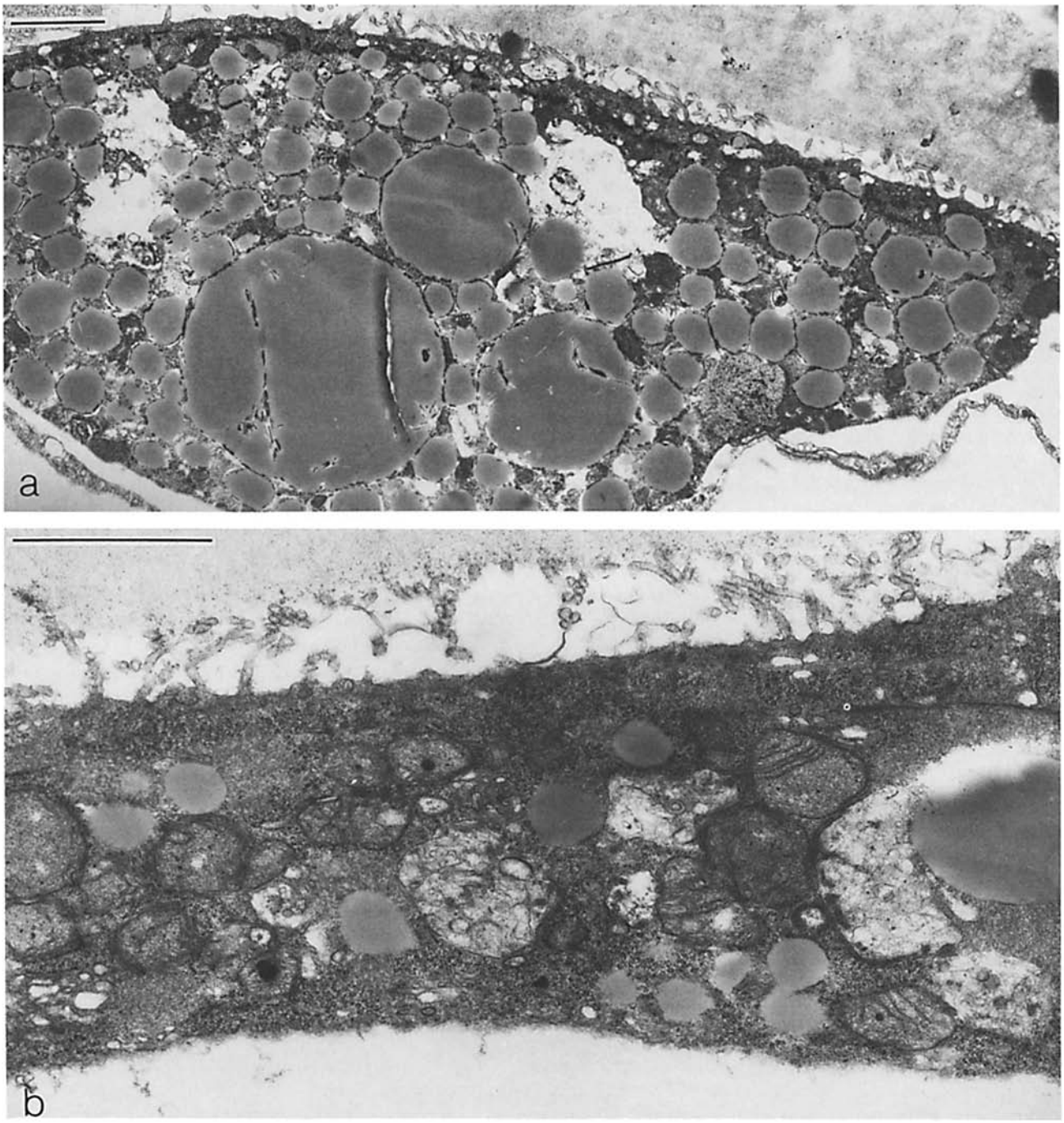

FIG. 2. Transmission electronic microscopy image of trophectoderm cell from blastocyst developed from in vivo-derived ovine zygote cultured in SOF + HS (a) and in SOFaaBSA (b). There were abundant lipid droplets (round gray structures) present in cytoplasm of embryos cultured in SOF + HS, compared to those cultured in SOFaaBSA (bar = 2 μm).

spread among all groups. This was due in part to two separate days of inclement weather during the lambing period: 8 of 11 neonatal deaths occurred on these days.

Gestation Length and Lamb Birth Weights

The gestation length was significantly longer ($p < 0.05$) for lambs from embryos cultured in SOF + HS (147 ± 1 days) than for those from embryos cultured in SOFaaBSA (145 ± 1 days). The gestation length of the control flock lambs (146 ± 1 days) was intermediate. Mean weight of lambs at birth from embryos cultured in SOF + HS was significantly heavier (4.2 ± 0.2 kg) than that of those from the control flock (3.4 ± 0.2, $p < 0.01$) or from embryos cultured in SOFaaBSA (3.5 ± 0.2, $p < 0.05$). These differences were both still significant ($p < 0.05$) when the analy-

sis accounted for differences in gestation length. There was no significant difference between weight of lambs from in vivo- or in vitro-derived embryos. There was also no interaction involving medium and source of embryo.

DISCUSSION

It has been well documented that lambs produced from embryos cultured in SOF + HS have a higher birth weight and longer gestation length than normally expected [6, 7]. However, the cause of these abnormalities has not been identified [6]. The evidence from this study suggests that it is not the period of in vitro culture but the culture system used that gives rise to these phenomena. Although we cannot conclude that the presence of human serum in ovine embryo culture medium is the specific cause for all the differences observed here, since more than one difference in composition exists between the two media tested, it is highly likely that the differences were due to serum. Nevertheless, although we suggest that serum causes these abnormalities, the mechanisms involved have yet to be understood. However, two observations made during in vitro development may relate to the observed high birth weight and extended gestation: 1) embryos cultured in SOF + HS were morphologically different from those cultured in SOFaaBSA; 2) by Day 5 of culture, significantly more embryos had reached the blastocyst stage when cultured in SOF + HS than in SOFaaBSA.

Our observation that embryos exposed to human serum have a markedly different morphological appearance from those incubated in its absence confirms previous reports [4, 8, 17]. Our results parallel closely the observation that embryos cultured in SOF + HS accumulate lipid [17], but we would add that at least some of the accumulated lipid is osmophilic, representing unsaturated forms. However, the question as to the source of lipid has yet to be answered. It is unlikely, with perhaps the exception of the rabbit embryo, that mammalian embryos require endogenous stores of energy substrates and obtain their energy requirements by uptake of substrates from their surrounding environment [18, 19]. It is most likely that the source of lipid accumulated in serum-exposed embryos is from the serum itself. It has been well documented that cells in culture can readily take up fatty acids, phospholipids, and triglycerides from serum-supplemented medium [20]. Most of the lipid is derived from triglycerides contained in serum lipoproteins, although free fatty acids constitute another important source [20]. If the medium contains a high concentration of lipid, as would be the case when supplemented with 20% human serum, most taken up would be stored as triglycerides in cytoplasmic droplets [20]. These results demonstrate that morphological appearance and, as Gardner et al. [4] found, metabolic activity of the ovine embryo can be manipulated if embryos are cultured under different conditions. This, in turn, may manifest itself in differences in birth weight and gestation length. If such a relationship could be demonstrated, then manipulation of the in vivo environment during early pregnancy may also result in the regulation of lambing birth weight and/or gestation length. The recent observation that progesterone administration during early pregnancy can increase fetal weight supports this hypothesis [21].

It is known that accelerated, or premature, blastulation is a characteristic of ovine embryos incubated in SOF + HS [6]. In this study, significantly fewer embryos had developed to blastocysts after 5 days culture in SOFaaBSA than in SOF + HS, although comparable rates of development to the morula stage were observed. This suggests that premature blastulation can be prevented by changing to a culture system devoid of human serum, a change likely also to prevent or reduce accumulation of lipid. Premature blastulation may, in turn, impart the embryo a property that results in extended gestation and increased birth weight (e.g., a shift in the ratio between inner cell mass and trophectoderm cells that could result in either large placentation or large fetal size). Although cell numbers were not reported here (because nearly all transferable-quality embryos were transferred), it is significant that when in vivo-derived ovine embryos were cultured for 6 days in SOFaaBSA, resulting blastocysts had more cells than those cultured in SOF + HS [4].

Despite the differences in embryo morphology and development rate, the pregnancy and embryo survival rates were remarkably similar for embryos judged transferable after culture in either SOF + HS or SOFaaBSA. Likewise, transfer of in vivo- or in vitro-derived blastocysts resulted in similar pregnancy and embryo survival rates. Furthermore, overall embryo survival was similar to that previously reported for cultured ovine embryos from our laboratory [22]. These data indicate that the observed differences in morphology and development have little influence on the survival of those embryos judged to be transferable and demonstrate the adaptability of ovine embryos to maintain viability despite differences in morphology and chemical composition. Ovine blastocysts derived in vivo also have a varied morphology, especially in terms of the size and numbers of lipid droplets [17]. Perhaps this flexibility allows embryos to maintain their viability under a wide range of environmental conditions, not only in vitro, but also within the reproductive tract itself. Death of cultured embryos following transfer occurs largely between approximately Day 15 and Day 60 of pregnancy, most likely around the time of implantation [23]. This is in agreement with other data from our laboratory [22] and suggests that, as for all post-compaction-stage embryos (regardless of source), the ability to elongate and begin the initial stages of organogenesis in conjunction with attachment to the endometrium is the most critical time for the developing ovine conceptus.

In conclusion, our data demonstrate that different culture systems can lead to differences in embryo morphology and

developmental rates, lamb birth weight, and gestation length. Some of these characteristics have previously been attributed to removal from the reproductive tract and to embryo culture in general, but we suggest that this notion requires redefining to emphasize difference in embryo culture systems. Finally, our data demonstrate that the viability of IVP, transferable-quality embryos grown under serum-free conditions is no more compromised than that of those grown in medium supplemented with human serum or from in vivo-derived zygotes cultured in vitro.

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