

# Embryo viability, duration of gestation and birth weight in sheep after transfer of *in vitro* matured and *in vitro* fertilized zygotes cultured *in vitro* or *in vivo*

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The influence of various *in vitro* procedures on embryo survival and the production of normal offspring was investigated in sheep. Zygotes produced from *in vitro* matured (IVM) and fertilized (IVF) oocytes derived from slaughtered Merino ewes were allocated to three culture treatments for 6.5 days. Two groups were cultured *in vitro* in the absence or presence of oviduct epithelial cells while the third group was cultured *in vivo* in the oviducts of synchronized ewes. A fourth group of zygotes obtained from superovulated Merino ewes was also cultured *in vivo* and served as controls. After culture, IVM–IVF morulae and blastocysts, and control embryos were transferred to final recipient ewes. Pregnancy was diagnosed at day 50 of gestation by ultrasonography and pregnancies were allowed to go to term. The survival to term of IVM–IVF zygotes cultured *in vitro* was reduced compared with both *in vivo* cultured IVM–IVF zygotes and control zygotes (25–35% versus 51–60%, respectively,  $P < 0.05$ ). Day 6.5 IVM–IVF morulae had a lower survival rate than did control morulae regardless of culture treatment ( $P < 0.05$ ), while survival rates of day 6.5 IVM–IVF blastocysts cultured *in vivo* did not differ from those of control blastocysts ( $P > 0.1$ ). Both the gestation period and birth weight of IVM–IVF lambs were increased when compared with controls, the former significantly in all groups (154.0–154.9 days versus 150.6 days;  $P < 0.01$ ), while the latter increase was on the borderline of significance (4.5–4.8 kg versus 4.0 kg;  $0.01 \leq P \leq 0.1$ , respectively) and dependent on the prolongation of the gestation period. It is concluded that *in vitro* maturation and fertilization compromise subsequent embryonic and fetal development in sheep irrespective of the subsequent *in vivo* or *in vitro* culture treatment. Subjecting IVM–IVF zygotes to *in vivo* culture for 6.5 days minimizes only some of these effects, thus leading to the aberrant production of some offspring.

## Introduction

Successful embryo culture plays an important role in the development of techniques involved in the multiplication of genetically superior or rare genotypes. These techniques can include *in vitro* maturation (IVM) and fertilization (IVF) of oocytes, use of co-culture of embryos with somatic cells (for example, oviduct epithelial cells) and collection of embryos from superovulated animals. However, the viability *in vitro* and *in vivo* of embryos produced or cultured *in vitro* is affected adversely (Czlonkowska *et al.*, 1991; Kelk *et al.*, 1992; Walker *et al.*, 1992a; Holm *et al.*, 1994a), and this is a major constraint to further developing the technologies. In addition, Walker *et al.* (1992a) observed that *in vitro* culture resulted in prolonged gestation, increased birth weight and increased perinatal death rate after embryo transfer. Similar findings have been reported in sheep (Thompson *et al.*, 1995) and in cattle (Behboodi *et al.*, 1995; Farin and Farin, 1995).

Revised manuscript received 18 March 1996.

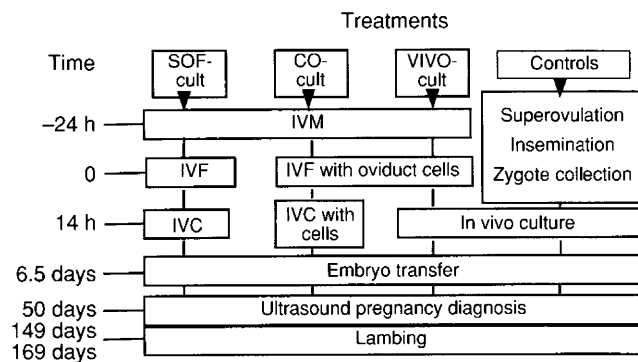
Factors responsible for either the reduced viability of embryos or the production of abnormal offspring after *in vitro* handling have not been identified. The purpose of the present study was to examine the effect of different *in vitro* procedures on the rates of embryo survival and the production of normal offspring after embryo transfer. Specifically, the effects of the following factors were compared: (1) source of embryos (IVM–IVF versus *in vivo* derived), (2) *in vitro* versus *in vivo* culture of *in vitro* derived zygotes and (3) co-culture versus non-co-culture of *in vitro* derived zygotes.

Preliminary results have been published earlier in abstract form (Holm *et al.*, 1994b).

## Materials and Methods

### Experimental design (Fig. 1)

In six replicates conducted from September to December 1992, zygotes produced by IVM–IVF were cultured for



**Fig. 1.** Experimental design illustrating the time of *in vitro* maturation (IVM), *in vitro* fertilization (IVF) with or without ovine oviduct epithelial cells, and *in vitro* culture (IVC) in synthetic oviduct fluid with (SOF-cult) or without (CO-cult) oviduct cells or *in vivo* culture (VIVO-cult) in oviducts of synchronized sheep for the three IVM–IVF treatment groups and one control group in which zygotes were collected from superovulated ewes 16–20 h after the expected median time of ovulation.

6.5 days under three different conditions; SOF-cult: *in vitro* culture in cell-free synthetic oviduct fluid (SOF; Tervit *et al.*, 1972) with 20% heat-inactivated human serum (HS), CO-cult: *in vitro* culture in SOF in the presence of ovine oviduct epithelial cells, and VIVO-cult: *in vivo* culture in non-ligated oviducts of synchronized ewes. *In vivo* derived zygotes (control) were subjected to culture *in vivo* for 5.5 days (approximately 6 days after the expected median time of ovulation). All embryos were transferred to synchronized recipients after the respective culture period. Recipient ewes were treated such that their expected median time of ovulation was within 12 h of the time of ovulation in donor ewes or the time of IVF.

#### Production of *in vivo* derived zygotes

South Australian Merino sheep located at Turretfield Research Centre were treated according to routine procedures for collection and transfer of embryos (Walker *et al.*, 1986). Briefly, donor ewes were treated with progestagen pessaries (60 mg medroxyprogesterone acetate; Upjohn, Rydalmere) for 12 days and 17 mg FSH (FSH-P; Heriot Agvet Pty Ltd, Melbourne, Victoria) administered as two daily injections i.m. approximately 12 h apart (5, 4, 3, 3 and 2 mg), starting 48 h before pessary removal. At 27 h after pessary removal, 25 µg synthetic GnRH (Intervet, Artamon, NSW) was injected i.m., followed 18 h later by laparoscopic insemination (Killeen and Caffery, 1982) of frozen–thawed semen pooled from three rams.

Zygotes were collected by mid-ventral laparotomy 12–15 h after the expected median time of ovulation. A Tom Cat catheter (Sherwood Medical, St Louis, MO) was inserted into the fimbriated end of the oviduct and 10 ml of Dulbecco's PBS with 2% sheep serum (mPBS) was flushed through the oviduct from the tip of the uterine horn. Zygotes were washed and stored in SOF + 20% HS for up to 4 h before being transferred to intermediate recipients.

#### Production of *in vitro* derived zygotes

Zygotes were produced using the procedures of Holm *et al.* (1994a). Briefly, ovaries were collected from nonpregnant South Australian Merino ewes slaughtered at a local abattoir. Immature oocytes were aspirated from 2–4 mm follicles and those with evenly granulated ooplasm surrounded by tight cumulus cells were selected for IVM. During aspiration and selection, oocytes were kept at 30–35°C in HEPES-buffered TCM-199 (Gibco, Melbourne, Victoria) supplemented with 2% heat-inactivated sheep serum (SS) and 25 iu heparin ml<sup>-1</sup> (H-3393, Sigma, MO). The oocyte–cumulus complexes were matured in bicarbonate-buffered TCM-199 supplemented with 15% heat-inactivated fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Victoria), 5 µg FSH ml<sup>-1</sup> (Folltropin-V; Vetrepharm Inc., Melbourne, Victoria), 5 µg LH ml<sup>-1</sup> (Lutropin-V; Vetrepharm) and 1 µg oestradiol ml<sup>-1</sup> (E-2257; Sigma) for 24 h. Thereafter, cumulus cells were removed by gentle pipetting in 0.1% (w/v) hyaluronidase (H-3506; Sigma) and ova were randomly allocated to three treatment groups for fertilization in SOF + 20% SS either without (experimental group SOF-cult) or with ovine oviduct epithelial cells (experimental groups CO-cult and VIVO-cult). Ova were incubated with frozen–thawed semen for 14 h at a final concentration of 1.5 × 10<sup>6</sup> motile spermatozoa ml<sup>-1</sup> in 50 µl drops of medium under paraffin oil (10–15 ova per drop) in an atmosphere of 5% CO<sub>2</sub>:5% O<sub>2</sub>:90% N<sub>2</sub>. The thawed semen pellets were subjected to a 1.5 h swim-up procedure and the motile fraction was added directly to the fertilization drops. The semen used for IVF was collected and pooled from the same rams that provided semen for laparoscopic insemination.

#### Culture procedures

After three washes in HEPES-buffered SOF + 20% HS, *in vitro* produced zygotes were cultured for 6 days either in SOF + 20% HS in the absence (SOF-cult) or presence of ovine oviduct epithelial cells (CO-cult) or in non-ligated oviducts of synchronized ewes (VIVO-cult). *In vitro* culture occurred in an atmosphere of 5% CO<sub>2</sub>:5% O<sub>2</sub>:90% N<sub>2</sub> at 38.5°C in 50 µl drops containing 12–15 embryos. *In vitro* culture medium and oviduct epithelial cells were changed every 48 h. At the end of the culture periods, morulae and blastocysts from *in vitro* produced zygotes and all recovered *in vivo* produced embryos were washed and held in HEPES-buffered SOF + 20% HS for up to 3 h at 38.6°C until transfer.

#### Preparation of ovine oviduct epithelial cell co-culture

Oviduct epithelial cell co-cultures were prepared as described by Holm *et al.* (1994a). In summary, oviducts of post-oestrous sheep were freed from ligaments and adjoining blood vessels and 1.5 cm of both the ampulla and isthmus regions were removed. Epithelial cells were expressed into a Petri dish with HEPES-buffered TCM-199 and segregated into small clusters by flushing through a 30 gauge needle. The cell suspension was then washed by centrifugation (three times 500 g for 5 min). After the final spin, the soft pellet was diluted 1:1 and 10 µl of this cell suspension were transferred to 1 ml of bicarbonate buffered TCM-199 + 20% SS and cultured overnight at 38.6°C

in 5% CO<sub>2</sub> in air. The cell clusters were then washed three times in either IVF- or IVC-medium and transferred to each of the IVF- or IVC-culture drops (approximately 150 cell clusters per 50 µl drop). The oviduct epithelial cells were added 6 h before the start of co-culture and new co-culture drops were made every 48 h from the primary cell culture.

#### Embryo transfer procedures

Intermediate and final recipients were treated with progestagen pessaries (60 mg; Upjohn) for 12 days and 400 iu pregnant mares' serum gonadotrophin (Intervet) was administered at the time of pessary removal.

Presumptive *in vivo* and *in vitro* zygotes were transferred by mid-ventral laparotomy to intermediate recipients approximately 12 h after the expected median time of ovulation. Transfer was to a nonligated oviduct ipsilateral to an ovary containing at least one corpus luteum; embryos were in groups of 10–30 per ewe. Embryos were subsequently recovered by mid-ventral laparotomy at the end of the culture period. A Foley catheter (size 10 FG, Sherwood Medical) was inserted into the uterine lumen and the oviduct and uterine horn were flushed with 20 ml mPBS. Recovered embryos were washed and stored in SOF + 20% HS for 2–4 h before being transferred to final recipients.

Embryos produced *in vivo* and *in vitro* were transferred laparoscopically on day 6–6.5 to the tip of a uterine horn ipsilateral to an ovary with at least one corpus luteum. Blastocysts produced *in vitro* were transferred in pairs or, in two instances, in groups of three. *In vitro* produced morulae were transferred in groups of 5–13 per recipient as previous studies have demonstrated unequivocally that embryos that had not developed beyond the morula stage after 5 days *in vitro* culture were not viable or of very low viability (S. K. Walker, unpublished). Consequently, by transferring these large numbers of embryos per ewe, worthwhile savings in the use of animals were made without the likelihood of producing litter-bearing pregnancies. All but one of the *in vivo* derived embryos were transferred in pairs. If too few recipients were available on a given day, *in vitro* derived embryos were selected for transfer so that the proportion transferred from each experimental group was similar to the proportion that had developed in that experimental group on that particular day.

All surgeries in this study were conducted under general anaesthesia induced by 10% (w/v) thiopenthan sodium (Pentothal; Boehringer Ingelheim, Artamon) and maintained by a mixture of halothane (May and Baker, Dagenham) and oxygen.

Animal experimentation was performed in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* and the *Prevention of Cruelty to Animals Act 1985* after approval was granted by the Animal Ethics Committees of both the South Australian Research and Development Institute and the University of Adelaide.

#### Pregnancy and lambing observations

Pregnancies and numbers of fetuses were determined at day 50 of gestation by ultrasonography with a Microimager 1000

sector ultrasound scanner equipped with a 3.5 MHz abdominal transducer (Ausonics, Sydney). Pregnancies were allowed to go to term. Each ewe was identified with a side-brand number. Individual ewe–lamb details were recorded at lambing. The number, date of birth, birth weight and sex of lambs born were recorded along with incidences of assisted births and perinatal deaths. The flock was observed in both the morning and afternoon of each day to minimize the likelihood of mismothering occurring during the recording period.

#### Statistical analyses

Chi-squared tests were used to examine differences in developmental rates of *in vitro* produced ova.

Pregnancy rates and fetal survival rates at 50 days of gestation and at term were analysed by a mixed linear model (SAS Institute Inc., 1992). The model included culture treatment, replicate, embryo stage at transfer (when applicable), pregnancy type (twin/single) and sex of lamb as fixed effects and final recipient nested within replicate, experimental group, type of pregnancy and sex of lamb as a random effect.

Durations of gestation and birth weights were initially analysed in the same way using same variants as fixed effects, but variants with no effect ( $P > 0.2$ ) were omitted in the final analysis. In addition, birth weight data were analysed both with and without duration of gestation as an additional fixed effect. If fixed effects were significant ( $P < 0.05$ ), linear contrasts were used to determine the significance ( $P < 0.05$ ) of the individual levels within the fixed effect. Results are expressed as least squares means (LS means)  $\pm$  SEM.

## Results

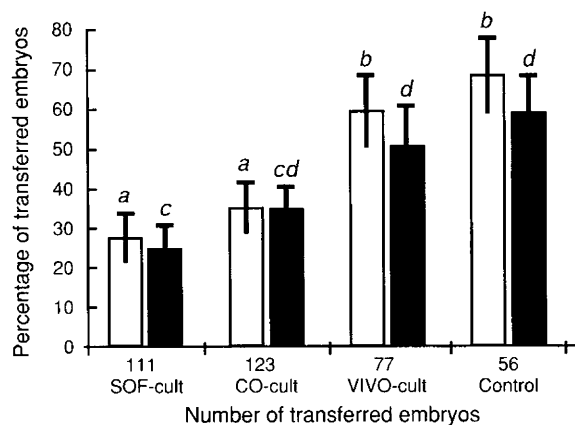
#### Embryo development and survival

A total of 328 IVM–IVF embryos were transferred to 110 recipient ewes. Ninety-four ewes received 190 blastocysts and 16 ewes received 138 morulae. Sixty-three *in vivo* embryos (24 blastocysts, 29 morulae and ten earlier stages) derived from ten donors were transferred to 32 recipients.

Seven recipients were excluded from the data. One recipient died a week after transfer of 13 *in vivo* cultured IVM–IVF morulae and six pregnant recipients were missing at term. The latter ewes received two *in vivo* cultured IVM–IVF blastocysts (one ewe), two *in vitro* cultured IVM–IVF blastocysts (one ewe) and seven *in vivo* control zygotes (four ewes).

Culture treatment did not affect the proportions of morulae (14–18%) and blastocysts (28–33%) that developed from IVM–IVF ova ( $n = 448, 503$  and  $284$  for SOF-cult, CO-cult and VIVO-cult, respectively) but a lower proportion of blastocysts had hatched *in vivo* by day 6.5 compared with *in vitro* (15% versus 19% versus 5% for SOF-cult, CO-cult and VIVO-cult, respectively;  $P < 0.001$ ). In contrast, none of the *in vivo* control blastocysts ( $n = 24$ ) had hatched and only 17% were expanded at the time of transfer.

The survival rates of morulae and blastocysts after transfer are shown (Fig. 2). The number of embryos surviving to day 50 of gestation and term were affected by culture treatment ( $P = 0.0002$  and  $P = 0.0025$ , respectively). The survival of



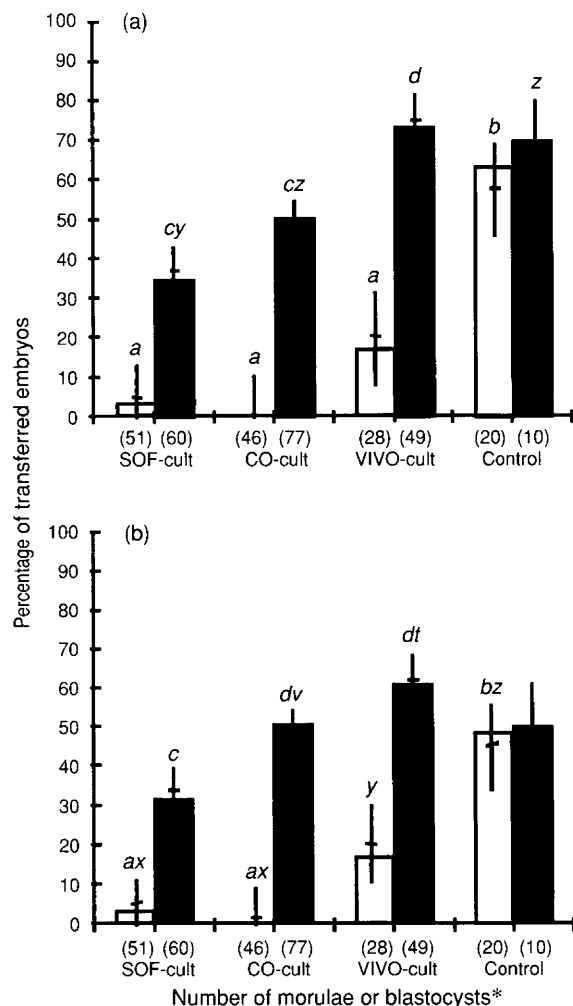
**Fig. 2.** Overall embryo survival rates (calculated as LS means  $\pm$  SEM) at 50 days of gestation ( $\square$ ) and at term ( $\blacksquare$ ) after transfer of IVM-IVF morulae and blastocysts cultured for 6.5 days after IVF in SOF in the absence (SOF-cult) or presence of ovine oviduct (CO-cult), or *in vivo* (VIVO-cult), or transfer of *in vivo* control embryos (control) at day 6 after the estimated median time of ovulation. Data are statistically analysed by a mixed linear model using culture treatment, replicate, embryo stage as fixed effects and recipient as a random effect. *ab* and *cd*:  $P < 0.05$  for different superscript within similar bars.

IVM-IVF zygotes cultured *in vitro* was significantly poorer than that of control zygotes, regardless of the presence or absence of oviduct epithelial cells. There was no significant difference between the overall survival rates of *in vivo* cultured IVM-IVF zygotes and *in vivo* produced zygotes.

As expected, the viability of IVM-IVF morulae was low with no signs of overcrowding of fetuses observed at ultrasound scanning at day 50 of gestation; two single lambs were obtained from 91 *in vitro* cultured morulae transferred in batches of 6–12 to each of 10 recipients, and three single lambs and one pair of twins were born from 28 *in vivo* cultured morulae transferred in batches of five to four recipients and eight to one recipient, respectively. However, the transfer of 20 *in vivo* derived morulae in pairs resulted in the birth of ten lambs. The transfer of earlier stage *in vivo* embryos as pairs ( $n = 2$ ) or accompanying an *in vivo* morulae or blastocyst ( $n = 8$ ) resulted in one single lamb and six lambs (e.g. four singles, one set of twins), respectively.

The developmental stage of embryos on the day of transfer influenced the survival rate of *in vitro* produced embryos ( $P < 0.001$ ) but not of *in vivo* produced embryos (Fig. 3); the viability of day 6.5 IVM-IVF morulae was reduced compared with that of the IVM-IVF blastocysts ( $P < 0.008$ ). Yet, analysis of the viability of specific stages of IVM-IVF blastocysts (Table 1) revealed that all blastocyst stages survived equally well to term when cultured *in vivo* or in the presence of oviduct cells while early (that is, delayed) blastocysts cultured *in vitro* in the absence of oviduct cells survived poorly compared with expanded and hatched blastocysts within the same treatment group. Furthermore, in all IVM-IVF treatment groups, hatched blastocysts survived equally well, while this was not the case for early and expanded blastocysts.

The fetal loss after day 50 of gestation varied substantially among treatment groups (SOF-cult: 9% (2 of 23) versus CO-cult: 0% (0 of 39) versus VIVO-cult: 15% (6 of 41) versus control: 20% (7 of 35)). The fetal loss in the control group was



**Fig. 3.** Embryo survival (a) at 50 days of gestation and (b) at term, after transfer of IVM-IVF morulae ( $\square$ ) and blastocysts ( $\blacksquare$ ) cultured for 6.5 days after IVF in SOF medium in the absence (SOF-cult) or presence of ovine oviduct cells (CO-cult), or *in vivo* (VIVO-cult), or transfer of *in vivo* control embryos (control) at day 6 after the estimated median time of ovulation. The pin-bars represent LS means  $\pm$  SEM calculated by a mixed linear model using culture treatment, replicate, embryo stage as fixed effects and recipient as a random effect.  $P < 0.05$  for *ab* and *cd* and  $P < 0.1$  for *vt* and *xyz* within similar bars. \*Transferred in pairs or groups of identical embryonic stages.

high compared with the approximately 10% usually seen in comparable experimental groups at Turretfield Research Centre.

#### Gestation period and birth weight of lambs

A total of 95 IVM-IVF lambs and 28 control lambs were born and the sex ratio (females:males) for each group was 0.51 (48:46) and 0.46 (13:15), respectively. One lamb was predated before sex and weight was determined. Birth weights and duration of gestation of individual lambs are shown (Fig. 2).

The number of single and twin births in the four experimental groups were, respectively, 15 and three for the

**Table 1.** Embryo survival rates (least squares means %  $\pm$  SEM) at term after transfer of ovine IVM–IVF derived morulae and blastocysts cultured in cell-free medium (SOF-cult), co-cultured with ovine oviduct cells (CO-cult) or cultured *in vivo* (VIVO-cult)

Culture treatment	Stage of blastocysts at transfer			Probability for effect of stage
	Early	Expanded	Hatched	
SOF-cult	6.7 $\pm$ 11.0 (16) <sup>a</sup>	39.4 $\pm$ 8.5 (26) <sup>c</sup>	48.8 $\pm$ 10.4 (16)	$P < 0.02$
CO-cult	38.6 $\pm$ 15.2 (19) <sup>ab</sup>	40.5 $\pm$ 7.8 (36) <sup>c</sup>	60.0 $\pm$ 7.9 (22)	$P > 0.05$
VIVO-cult	69.4 $\pm$ 17.7 (6) <sup>b</sup>	67.5 $\pm$ 7.4 (36) <sup>d</sup>	44.5 $\pm$ 30.4 (7)	$P > 0.05$

(*n*), number of blastocysts transferred in six replicates as singles or in pairs of same developmental stage. Different superscript within columns:  $P < 0.05$  for ab and cd.

Statistics: least squares mean-values are calculated with a mixed linear model using culture treatment, replicate, embryo stage as fixed effects and recipient within these as a random effect. Replicate effect was not significant.

**Table 2.** Duration of gestation and birth weight of lambs born after transfer of day 6.5 IVM–IVF embryos cultured in cell-free medium (SOF-cult), co-cultured with ovine oviduct cells (CO-cult) or cultured *in vivo* (VIVO-cult), and of *in vivo* control embryos (Control)

Treatment group	Duration of gestation (LS mean)		Birth weight (LS means)									
	Overall		Overall		Singles		Twins		Females		Males	
	<i>n</i>	days	<i>n</i>	kg	<i>n</i>	kg	<i>n</i>	kg	<i>n</i>	kg	<i>n</i>	kg
SOF-cult	21	154.9 $\pm$ 0.4**	21	4.7 $\pm$ 0.3*	15	5.2 $\pm$ 0.3*	6	4.4 $\pm$ 0.5	11	4.5 $\pm$ 0.2	10	5.1 $\pm$ 0.3
CO-cult	39	154.0 $\pm$ 0.5**	39	4.5 $\pm$ 0.2 <sup>†</sup>	17	5.5 $\pm$ 0.3**	22	3.5 $\pm$ 0.3	18	4.4 $\pm$ 0.2	21	4.6 $\pm$ 0.3
VIVO-cult	35	154.2 $\pm$ 0.5**	34 <sup>a</sup>	4.8 $\pm$ 0.2**	18	5.2 $\pm$ 0.3*	16	4.4 $\pm$ 0.3	19	4.5 $\pm$ 0.3	15	5.1 $\pm$ 0.3
Control	28	150.6 $\pm$ 0.5	28	4.0 $\pm$ 0.2	22	4.5 $\pm$ 0.2	6	3.9 $\pm$ 0.5	13	3.8 $\pm$ 0.2	15	4.5 $\pm$ 0.4
Probability for treatment effect		$P < 0.01$		$P = 0.05$		$P = 0.05$		$P = 0.12$		$P = 0.10$		$P > 0.20$

*n*, number of lambs.

\*Different from control group by  $P \leq 0.05$ ; \*\*Different from control group by  $P < 0.01$ ; <sup>†</sup>Different from control group by  $P = 0.1$ . The significant effect of treatment on birth weight is not seen if duration of gestation is included as co-variate in the statistical model.

<sup>a</sup>One newborn lamb predated weighing.

Statistics: LS mean values are calculated with a mixed linear model using culture treatment, sex of lamb and single/twin birth as main effects and randomizing recipient effects within these. No interactions were found between tested parameters.

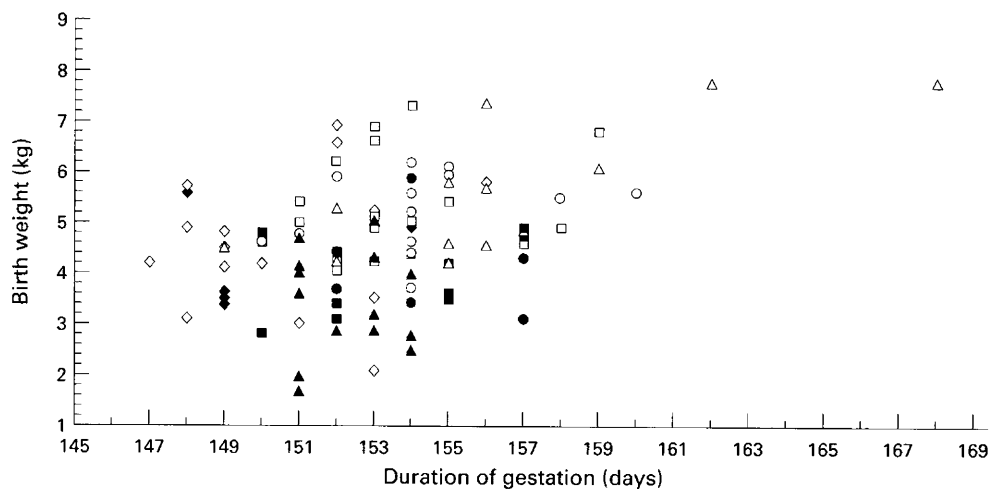
SOF-cult-group, 17 and 11 for the CO-cult-group, 18 and eight for the VIVO-cult-group and 22 and three for the control group.

The rates of assisted births and perinatal death of lambs were 0 of 21 and 0 of 21 for the SOF-cult-group, 6 of 39 and 8 of 39 for the CO-cult-group, 1 of 34 and 2 of 34 for the VIVO-cult-group and 1 of 28 and 0 of 28 for the control group, respectively.

The mean gestation period in the SOF-cult, CO-cult and VIVO-cult treatment groups did not differ significantly (154.0–154.9 days). However, the mean gestation period in the control group was 4 days shorter (150.6 days;  $P < 0.01$ ) (Table 2). The mean duration of gestation did not vary significantly between dams with either female or male lambs (153.0 days for both sexes). Single and twin lambs were born after 153.5  $\pm$  0.3 days and 152.5  $\pm$  0.4 days, respectively ( $P = 0.09$ ). Neither day of transfer, nor developmental stage of the embryo significantly influenced gestation length ( $P > 0.50$ ).

The birth weight of lambs was influenced by single/twin pregnancy, sex and culture treatment. The overall mean weights (kg) of single versus twin lambs and female versus male lambs were 5.1  $\pm$  0.2 versus 3.9  $\pm$  0.2 ( $P < 0.01$ ) and 4.3  $\pm$  0.2 versus 4.7  $\pm$  0.1 ( $P = 0.04$ ), respectively.

All IVM–IVF lambs other than those derived from co-cultured embryos were significantly heavier than *in vivo* control lambs ( $P \leq 0.05$  and  $P = 0.1$ , respectively); the mean birth weights in the SOF-cult, CO-cult, VIVO-cult and control treatment groups were 4.7  $\pm$  0.4, 4.5  $\pm$  0.2, 4.8  $\pm$  0.2 and 4.0  $\pm$  0.2, respectively. Separate analysis of birth weights of singles and twins or females and males revealed significant differences between all treatment groups and controls for single lambs and tendencies for female lambs, respectively, while results were less clear for twin and male lambs, respectively (Table 2 and Fig. 4). There was no significant effect of culture treatment on birth weights when duration of gestation was included as fixed effect in the statistical model.



**Fig. 4.** Duration of gestation and birth weights of new-born single and twin lambs developed from IVM-IVF zygotes cultured for 6.5 days in synthetic oviduct fluid in the absence (singles: ○; twins: ●) or presence of ovine oviduct cells (singles: △; twins: ▲) or *in vivo* in nonligated oviducts of synchronized sheep (singles: □; twins: ■), or from control zygotes cultured *in vivo* as above (singles: ◇; twins: ◆).

## Discussion

The *in vitro* procedures used in this study influenced not only the overall survival of embryos after transfer but also the duration of gestation and birth weight, thus supporting similar observations (Walker *et al.*, 1992a, b; Thompson *et al.*, 1995).

As in earlier studies (Holm *et al.*, 1994a), the proportions of IVM-IVF zygotes that developed to the morula and blastocyst stages were not influenced by the absence or presence of oviduct cells or by the *in vivo* culture conditions. However, the *in vitro* environment influenced development, as more IVM-IVF blastocysts hatched during the culture period compared with *in vivo* culture and none of the *in vivo* derived control embryos hatched during their culture period. Similar findings have been reported by Walker *et al.* (1992a). This finding may be a result of the high serum contents in the culture medium, as recent publications have shown that the use of serum as the protein source in the culture medium may accelerate the early embryonic development when compared with albumin (Thompson *et al.*, 1995; van Langendonck *et al.*, 1996).

Overall, *in vitro* culture of IVM-IVF zygotes for 6.5 days reduced embryo viability by 15–25% compared with *in vivo* culture; 25–35% of IVM-IVF-IVC morulae/blastocysts survived to term. These survival rates are higher than most comparable reports (Czlonkowska *et al.*, 1991; Kelk *et al.*, 1992), but lower than those reported by Thompson *et al.* (1994) who mainly transferred day 6 blastocysts. The oviduct and uterine environment of the synchronized intermediate recipient clearly improved the overall viability of IVM-IVF blastocysts, which was not different from that of *in vivo* produced control blastocysts. Our results indicate that slowly developing blastocysts (those that had just become blastocysts at day 6.5 after IVF) benefited most from an oviduct environment. Even the addition of oviduct cells to SOF tended to improve the viability of late forming blastocysts after transfer. This corresponds to the observations in sheep that the number of cells in blastocysts cultured *in vitro* are particularly low in the slowly

developing blastocysts (Walker *et al.*, 1992a; Holm *et al.*, 1994a). There was also a tendency for a better survival of IVM-IVF morulae when they were cultured *in vivo* compared with *in vitro*, although they did not survive as well as their *in vivo* control morulae and no effects of adding oviduct cells to the SOF-medium were found. Previous studies of prolonged culture of *in vivo* produced zygotes (S. K. Walker, unpublished) suggested that IVM-IVF morulae produced after 6.5 days of culture also would be nonviable or have very low viability, and indeed we did not see any signs of fetal overcrowding at ultrasound scanning on day 50. Thus the results supported this presumption; only 30% of recipients became pregnant, and less than 5% of the IVM-IVF morulae survived to day 50 of gestation and to term.

The production of zygotes *in vitro* prolonged the average duration of gestation by 4 days compared with *in vivo* controls, irrespective of whether IVM-IVF embryos were cultured *in vitro* or *in vivo*. The overall mean birth weights of lambs derived from IVM-IVF embryos cultured either *in vitro* without oviductal cells or *in vivo* were also increased significantly compared with controls. Lambs derived from co-cultured IVM-IVF embryos tended to be heavier than controls, but only the mean birth weights of singles in this group were significantly heavier than controls. The increase in mean birth weights was not statistically independent of the duration of gestation, nor could the unusual heavy lambs derived from IVM-IVF zygotes be characterized as statistical outliers, as their gestation period also tended to be unusually long (Fig. 4). Therefore, the present results do not conclusively support the observations of Walker *et al.* (1992b) and Thompson *et al.* (1995) regarding independent increases in the duration of gestation and in birth weights after the *in vitro* culture of ovine zygotes in SOF with 20% serum.

However, the present data show that IVM-IVF procedures alone alter the duration of gestation, and as the birth is triggered by the fetus (Silver, 1990), the prolongation of gestation alone is likely to be a result of physiological changes in the fetus.

The use of an oviduct epithelial cell co-culture system did not evidently normalize gestation length and birth weight. This finding is supported by studies in cattle (Behboodi *et al.*, 1995; Farin and Farin, 1995). Also, the *in vivo* culture of *in vitro* produced zygotes failed to reverse these effects, although blastocyst survival rate was normalized. A parallel may be drawn to more radical manipulations to the oocyte (for example, nucleus transfer) that also cause disturbances in fetal growth and duration of gestation which are not normalized by *in vivo* culture (Willadsen *et al.*, 1991; Wilson *et al.*, 1995). Furthermore, unusually large offspring derived from IVM-IVF zygotes cultured in sheep oviducts have been reported in cattle, although the observed mean birth weight of the eight calves derived from this culture group did not differ from the 64 control artificial insemination calves (Behboodi *et al.*, 1995).

Thompson *et al.* (1995) showed that duration of gestation and birth weight of lambs derived from both *in vitro* and *in vivo* produced zygotes were affected when the embryos were cultured in SOF with 20% human serum, but not in SOF in which sera were substituted with BSA and amino acids. This finding suggests that high contents of human serum in culture medium is a possible factor causing aberrant fetal development. All zygotes in the present study were subjected to high contents of human serum for at least a short period after fertilization. However, several other *in vitro* conditions may influence cytoplasmic and genetic factors in the oocyte and embryo important in fetal development (for review see Walker *et al.*, 1996), and also the way in which the oocyte or embryo is manipulated may influence the final outcome. Exposure of early preimplantation embryos to aberrant conditions *in vivo* such as an asynchronous uterus (Wilmot and Sales, 1981) or to the oviducts of progesterone treated ewes (Kleemann *et al.*, 1994) causes enhanced fetal growth. In cattle, *in vitro* procedures also affect fetal growth (Behboodi *et al.*, 1995; Farin and Farin, 1995), but not duration of gestation (Behboodi *et al.*, 1995), while cloning by nucleus transfer affects both (Willadsen *et al.*, 1991; Wilson *et al.*, 1995). The numbers of lambs and variation in birth weights in this study do not allow further conclusions regarding the possible relations between the type of manipulation, birth weight and duration of gestation.

The observation of physiological changes (for example abnormal metabolic regulation) in newborn nuclear transfer calves irrespective of their birthweight have been correlated with reduced neonatal survival (Garry *et al.*, 1996). *In vitro* culture has also been linked with increased rates of perinatal death in both sheep (Walker *et al.*, 1992a, b) and cattle (Behboodi *et al.*, 1995). In the present study, we observed high incidence of perinatal death only in the group of lambs derived from co-cultured IVM-IVF zygotes. This group also had a high incidence of assisted births, an event that at least in part may explain the reduction in perinatal survival.

In conclusion, the results of our study indicate that duration of gestation and birth weight of lambs are increased when oocytes are subjected to IVM and IVF procedures. These effects are manifested before first cell division and influence development, regardless of the subsequent culture treatments. Neither the oviduct environment nor oviduct cells in culture were able to reverse these effects even though the survival rate of IVM-IVF produced blastocysts was normalized.

The authors thank B. A. Petersen and R. J. Ashman, University of Adelaide, for their surgical assistance and H. Callesen, Embryo Technology Center, for his critical review of this manuscript. This work was supported by a grant from the Meat Research Corporation of Australia.

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