# A retrospective analysis of sheep generated by somatic cell nuclear transfer

Ying Liu[A], Rusty Stott[A], Misha Regouski, Zhiqiang Fan, Iuri Viotti Perisse, Tayler Patrick, Jacob Keim, Qinggang Meng, Irina A Polejaeva

Department of Animal, Dairy and Veterinary Sciences, Utah State University, Logan, UT, USA;

[A] These authors contribute equally to this study.

Correspondence author:

Irina A. Polejaeva; Email: Irina.Polejaeva@usu.edu
or Ying Liu; Email: Ying.Liu@usu.edu

**Abstract**

Somatic cell nuclear transfer (SCNT) is one of the primary methods for production of genetically engineered sheep, which allows for gene editing or transgene introduction in somatic cells. The use of SCNT eliminates the risk of genetic mosaicism in embryos and animals that is commonly observed after zygote micromanipulations. This retrospective analysis of SCNT in sheep performed at Utah State University, spanning from 2016 to 2021, examined parameters that may impact pregnancy and full-term development, including donor oocytes (donor age), donor cell lines, SCNT parameters (time of oocyte activation following SCNT, number of transferred embryos, *in vitro* maturation and culture conditions), and recipients (surgical number and ovulatory status), as well as factors that may correlate with large offspring syndrome or abnormal offspring syndrome (LOS/AOS) in the fetuses and lambs. Our findings indicated that compared to prepubertal oocytes, the SCNT embryos produced from adult sheep oocytes had comparable *in vitro* maturation rates, pregnancy and full-term development rates, as well as SCNT efficiency. In addition, earlier activation time of SCNT embryos (e.g. 24-26 hours post maturation) was correlated to the early pregnancy loss rate, full-term rate, and SCNT efficiency. Compared to our standard serum-containing medium, commercial serum-free culture medium showed a positive correlation with the full-term development of sheep SCNT embryos. Transferring 15-30 embryos per recipient resulted in consistently good pregnancy rates. Surgical numbers and ovulatory status (having at least one follicle between 6-12mm in size or a corpus hemorrhagicum (CH)) of recipients did not affect pregnancy and full-term development rates. In summary, this retrospective analysis identified parameters for improving pregnancy and full-term development of SCNT embryos in sheep.

Keywords: nuclear transfer, adult and prepubertal oocytes, pregnancy, sheep

## 1. Introduction

Domestic sheep (*Ovis aries*) are a valuable livestock species that have been raised for the production of meat, milk and fiber. Additionally, due to their similarity in size, anatomy and physiology to humans, sheep have become an important model animal in biomedical research. Somatic Cell Nuclear Transfer (SCNT) is still one of the most efficient methods to produce genetically engineered (GE) livestock including sheep. This method allows for gene editing and/or transgene introduction in somatic cells and eliminates genetic mosaicism in the resulting offspring, which is commonly observed using zygote micromanipulation approach [1]. Since the birth of Dolly, numerous studies have focused on improving the efficiency of SCNT in sheep, however, the overall efficiency remains low, typically ranging between 1-5% (number of live births from the number of SCNT embryos transferred to recipients; [2, 3]).

There are many factors which impact overall SCNT efficiency in sheep. As reported in other species, the developmental competence of oocytes from prepubertal animals is lower than that of adult animals, e.g., pigs [4], goats [5], and sheep [6]. To our knowledge, there is no study directly comparing the oocytes from lambs and ewes on the SCNT efficiency in sheep. Besides the age of oocyte donors, oocyte activation is another critical factor directly affecting SCNT efficiency. In mammalian fertilization, oocyte activation is triggered by sperm-specific phospholipase C zeta (PLCζ), a fundamental event that initiates embryonic development, by releasing calcium in specific patterns within the oocyte [7]. A deficiency in oocyte activation underlies most cases of fertilization failure in mammals. Chemical activation is a crucial step to initiate development of cloned embryos. The activation protocol of reconstructed SCNT embryos typically starts with $Ca^{2+}$ ionophore to elevate intracellular $Ca^{2+}$ levels, then followed with the treatment of broad-spectrum protein synthesis inhibitor (cycloheximide, CHX) or protein kinase inhibitor (6-dimethylatminopurine, 6-DMAP) to block cyclin B from functioning and reduce the activity of maturation promotion factor (MPF) that is maintaining meiotic arrest [8]. Most studies reported the activation time in sheep between 26-29 hours post-onset of maturation (hpm) [9]. No studies have reported if the activation time is correlated with pregnancy and full-term rates.

*In vitro* maturation (IVM) and *in vitro* culture (IVC) have been widely used in various species to generate SCNT embryos and animals. Standard IVM medium typically contains hormones (including luteinising hormone (LH), follicle stimulating hormone (FSH), estradiol-17β) and fetal bovine serum (FBS). Synthetic oviductal fluid (SOF) supplemented with amino-acids and BSA or FBS is a common IVC medium that has been used for sheep *in vitro* fertilization (IVF) and SCNT embryo culture. Even though adding serum during embryo culture was found to improve blastocyst formation in cattle [10], serum also leads to large offspring syndrome (LOS) and increased organ size [11]. Large offspring syndrome is defined as the amalgamation of developmental defects of the fetus, placenta, and calves/lambs from *in vitro* produced and SCNT embryos. Later the term "abnormal offspring syndrome (AOS)" has been introduced to broaden the definition of abnormally developed fetuses and placenta observed after transfer of *in vitro* production (IVP) and SCNT embryos [12]. It had been reported more often in sheep and cattle, than in other species [13]. *In vitro* culture and serum-containing culture media have been reported as one of the main causes of LOS/AOS [11]. Recently, the use of a serum-free *in vitro* production system is becoming more popular, such as IVF Bioscience media, which has been used for IVF embryos in cattle, sheep, and goats [14-17]. To our knowledge, there are no reports about how this commercial serum-free system affects sheep SCNT embryo development *in vivo*.

Besides the parameters of the SCNT system mentioned above, the number of transferred embryos is also an important factor affecting SCNT outcomes, which has been reported in different species: pigs [18], goats [19] and sheep [20]. Depending on the SCNT embryo stage used for embryo transfers, the number of embryos transferred vary between different laboratories. Careful selection of recipients for embryo transfer is also important to achieve good success rates. With surgical embryo transfer, it is recommended that each recipient should not have more than two surgeries. All animal procedures must be approved by an Institutional Animal Care and Use Committee (IACUC). Surgical number of the recipient ewes may affect implantation of sheep SCNT embryos. Follicular development status of recipient ovaries is another factor that could impact SCNT efficiency, but there is limited information about their effects in sheep.

In this retrospective analysis, we evaluated several parameters which have the potential to improve SCNT efficiency, including age of donor oocytes, donor cell lines, SCNT parameters (*in vitro* maturation and culture conditions, timing of chemical activation), number of transferred embryos, and recipients (surgical numbers and ovulatory status), as well as other factors which may correlate with LOS/AOS outcomes in SCNT-derived lambs.

## 2. Materials and Methods

SCNT and embryo transfer work was performed from 2016-2021 at Utah State University. The *in vitro* data (oocyte numbers, maturation, fusion, and lysing rates) were available from 2017-2021 (*in vitro* data in 2016 were not collected), and the *in vivo* data were collected from 2016-2021. All animal procedures were approved by and conducted according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Utah State University (IACUC protocols #10089, #10126, 310238, #10240, #11498, #11908, #11910) and conformed to the National Institute of Health guidelines. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified.

### 2.1. Oocyte collection and *in vitro* maturation

Ovaries were sourced from prepubertal domestic sheep at a local abattoir (Springville, Utah) or from adult domestic sheep at a different local slaughterhouse (Monticello, UT) as well as USU's animal science farm, and then all ovaries collected by ovariectomy were transported to the laboratory at 20-27°C in 0.9% saline within 4 hours after collection. For super-stimulation of oocyte donors (ovaries collected from USU's animal science farm), the ewes were given 2 ml of FSH intramuscularly (IM) (40 mg/ml, Folltropin Vetoquinol) reconstituted in Map-5 (Hyaluronic acid 10 mg/ml, Vetoquiniol) 36 h prior to ovary collection. All ovaries were collected during sheep breeding season between October and January of each year.

The protocol for oocyte collection and IVM was reported previously [21]. Briefly, cumulus-oocyte complexes (COCs) were recovered from ovaries in modified TL-Hepes medium (Lonza, Walkersville, MD) supplemented with 1% fetal bovine serum (FBS; HyClone, Logan, UT), 100 U/mL penicillin/streptomycin and 30 μg/mL heparin using a slicing technique. The COCs were washed in standard maturation medium (TCM-199 (Gibco, Grand Island, NY), 10% FBS, 10 μg/mL LH, 5 μg/mL FSH, 1 μg/mL estradiol-17β and 0.05 g/L gentamycin) or BO-IVM (IVF Bioscience, Cornwall, UK) and were cultured in groups of 40-50 in 4-well dishes containing 500 μL of maturation medium for 21-22 h at 38.5°C in 5% $CO_2$ in air. Maturation status was assessed by the presence of the first polar body after 21-22h of culture. Oocytes at this stage are termed metaphase II (MII) oocytes, and only MII oocytes were used for SCNT.

2.2. Donor cell lines

Donor cells used in this study were sheep fetal fibroblasts or adult fibroblasts with genetic modifications or naturally occurring mutations generated for seven different projects ([22-26] and unpublished data)). Both male and female donor cell lines were used to generate genetically modified fibroblasts for five projects, while two projects only used male donor cell lines. Among all the projects, five employed CRISPR/Cas9 to generate genetically modified fibroblasts, one utilized random integration of the vector into the host genome, and one project employed fibroblasts derived from animals with natural mutations. Prior to SCNT, the donor cells were cultured in DMEM/high-glucose medium (HyClone, Logan, UT) supplemented with 15% FBS and 100 U/mL penicillin/streptomycin. The fibroblasts were grown to 80-90% confluence and used as nuclear donor cells for SCNT after 24-48 h of serum starvation (0.5% FBS supplemented DMEM medium).

2.3. Somatic cell nuclear transfer

The SCNT procedure was described in our previous report [21]. The oocytes from both serum-containing and serum-free IVM were manipulated under same condition until activation. Briefly, the first polar body and metaphase plate were removed from a denuded MII oocyte, and a single donor cell was subsequently injected into the perivitelline space of the enucleated oocyte in HSOF (20 mM HEPES-supplemented synthetic oviductal fluid (SOF) medium) containing cytochalasin B (CB, 10 μg/ml). Fusion of somatic cells with oocyte cytoplasm was performed in 0.31 M sorbitol fusion medium containing 0.1 mM calcium, 0.5 mM magnesium, 0.5 mM HEPES and 1 mg/mL BSA) by two DC electric pulses of 2.0 kV/cm for 40 microseconds each. Following fusion, embryos were incubated in modified SOF medium [27] with 2.5% FBS or BO-IVC (IVF Bioscience) supplemented with 7.5 μg/ml CB for 45 min to 1 h prior to activation. Reconstructed embryos were activated between 24 and 29.5 hpm by exposure to 5 μM ionomycin for 5 min followed by a four-hour incubation in 2 mM 6-DMAP and 10 μg/mL CHX. Following activation, the embryos were cultured under mineral oil in 40 μL droplets of either SOF or BO-IVC media (max. 40 embryos per drop) at 38.5°C in 5% $CO_2$ and 5% $O_2$ in air for 8-12 h prior to transfer into the estrus synchronized recipient ewes. Activation times post-maturation, ranging between individual hours, were categorized into integral hour. For example, activation times between 24.0h to 24.9h were marked as 24h.

2.4. Recipient synchronization, embryo transfer, pregnancy, and neonatal care

The age of our recipient ewes ranged from 2-5 year old, and all the ewes had lambs before being used for SCNT embryo transfer. Recipient synchronization and embryo transfers were conducted as previously described [21]. Briefly, with an appropriate disinfection and lubricant, SYNCRITE vaginal sponges (Animal Health Supplies) containing 40 mg flurogesterone acetate were vaginally inserted and left in place for 10 days. Additionally, 2 mL of EstruMate was given IM at the time of SYNCRITE Vaginal Sponge removal. Estrus occurred at 36–48 h after sponge removal with ovulation usually occurring 12–24 h after the event of estrus. On average, 14.9 ±2.7 one-cell stage embryos were transferred into the oviduct of each synchronized recipient that showed estrus within 12 h of the transfer time. The status of dominant follicle size or ovulation was evaluated before embryos were transferred into a recipient. The recipients with a follicle size of 6-12 mm or with a corpus hemorrhagicum (CH) were used for embryo transfer. All recipients with only a 5 mm dominant follicle were given Gonadotropin-Releasing Hormone (GnRH; Boehringer Ingelheim, 100 mcg IM) at the time of embryo transfer. Initial SCNT pregnancies were confirmed at 40±5

days of gestation by transabdominal ultrasonography and were checked again at 90±5 days (90-day pregnancies). Early pregnancy loss was defined as the loss occurring between initial pregnancy and 90-day pregnancy, excluding the early pregnancy terminations for sample/fetus collections. Late pregnancy loss was defined as the loss occurring between 90-day pregnancy and full-term. Recipients were allowed to lamb naturally or induced if gestation passed 148 days with 15 mg of dexamethasone which was given intramuscularly 24 hours prior to anticipated cesarean section surgery. The ewes underwent caesarean section surgery if they did not respond to the induction protocol. After delivery, the offspring remained with their dams and nursed freely until weaning at 2.5–3 months of age. LOS/AOS lambs were defined as having at least one of several malformations, including ventricular septal defects, hydronephrosis, enlarged umbilicus, angular limb deformities, and cleft palates, and with an excessive birth weight (above 7.7kg). The lambs born with only excessive birth weight (above 7.7kg) but healthy were not considered as LOS/AOS.

2.5. Statistical analysis

All embryo transfers were included in the initial pregnancy rate analysis, but recipients that had their pregnancies terminated for research purposes (e.g. for fetal sample collections) were excluded from 90-day pregnancy and full-term rates, and early and late pregnancy loss rates. The live birth rates were calculated by dividing the total number of lambs born alive by the total number of lambs developed to term. The survival rates at one month were calculated by dividing the total number of lambs alive at one month by the total number of lambs alive at birth. The SCNT efficiency was calculated by dividing the total number of lambs alive at birth by the total number of transferred embryos (excluding the embryos in the terminated pregnancies). The transfer with embryo from mixed of adult and prepubertal oocytes were excluded from the effect of donor age.

The number of oocytes per ovary and maturation rates were analyzed using a generalized mixed model with donor age and locations of ovary collection included as random effects. Fusion and lysing rates were analyzed using a generalized mixed model with donor age and locations of ovary collection included as random effects. Live birth rates, survival rates at one month, SCNT efficiency, and LOS/AOS rates were analyzed using a generalized mixed model with projects and donor age, locations of ovary collection, and surgery number in the same season included as random effects, when donor age was not considered a fixed effect. The model formula was

$$Y=X\beta+ Z_1b_1+ Z_2b_2+ Z_3b_3+ Z_4b_4 +\varepsilon$$

in which Y was the vector of observation, X was the design matrix for the fixed effects, β was the vector of fixed-effect coefficients. $Z_1$, $Z_2$, $Z_3$, and $Z_4$ were the design matrices for the random effects (donor age, cell line, locations of ovary collection, and surgery number of ewe within the season). $b_1$, $b_2$, $b_3$, and $b_4$ were the vectors of random-effect coefficients corresponding to the four random effects, ε was the vector of residual errors. Initial pregnancy, 90-day pregnancy and full-term rates were analyzed using a generalized mixed model with projects, donor age, locations of ovary collection, and surgery number in the same season included as random effects, when donor age was not considered a fixed effect. The model formula was

$$g(E[Y])=X\beta+Z_1b_1+Z_2b_2+ Z_3b_3 + Z_4b_4$$

$g(E[Y])$ is the logistic link function that relates the expected value of the response variable, X, β, $Z_1$, $Z_2$, $Z_3$ and $Z_4$, and $b_1$, $b_2$, $b_3$ and $b_4$ had the same meaning as the mixed model. The effect of donor age was analyzed using logistic generalized mixed model with projects, locations of ovary collection, and surgery number in the same season included as random effects. The effect of projects was analyzed using generalized mixed model with donor age and surgery number in the same season included as random effects. Odds ratio (OR) was calculated by probability of event

divided by probability of no event and was used to show the fold changes between groups. Multiple comparisons among different projects used post hoc test with Holm correction. All data were analyzed using Jamovi 2.3. A probability of $P < 0.05$ was considered to be statistically significant. Effect of superovulation of oocyte donors (from the USU farm) was not considered separately in the analysis due to the lower number of embryo transfers in the non-superstimulated donor group.

## 3. Results

### 3.1. Effect of donor age on the number of oocytes collected per ovary and maturation rates, pregnancy and full-term rates, and SCNT efficiency.

The number of oocytes collected per ovary in adult animals was comparable to that in prepubertal animals ($P>0.05$), and no differences were observed in maturation rate between the two groups (Table 1). As shown in Table 2, there were no differences in the initial and 90-day pregnancy rates, full-term rates, survival rates or SCNT efficiency between SCNT embryos produced from adult sheep oocytes and those from prepubertal sheep oocytes ($P>0.05$). In addition, both early and late pregnancy loss rates were comparable between two groups (adult vs. prepubertal: 28.5% vs. 35.3%, $P=0.561$; 20.4% vs. 9.1%, $P=0.383$, respectively).

### 3.2. Effect of projects and donor cells on fusion, pregnancy, full-term rates, and SCNT efficiency.

The data were collected from seven different projects aimed at producing genetically engineered animals. Fusion rates ranged from 78.8% to 88.7%, while initial pregnancy rates, full-term rates, and SCNT efficiencies ranged from 35.0% to 48.1%, 16.1 to 30.0%, and 0.4% to 2.0%, respectively. There were no differences in fusion rates, initial pregnancy rates, 90-day pregnancy rates, full-term rates, and SCNT efficiency among these projects (Table 3). Early pregnancy loss rates ranged from 14.3% to 53.8% while late pregnancy loss rates ranged from 0% to 40% among the projects but were not statistically significant. Additionally, no differences were observed between male and female donor cells in initial pregnancy rates (43.5% vs. 46.7%, $P=0.996$), 90-day pregnancy rates (29.2% vs. 28.9%, $P=0.454$), full-term rates (23.1% vs. 23.3%, $P=0.767$), survival rates (72.6±0.5% vs. 90.5±0.6% at birth, $P=0.174$, 30.6±6.5% vs. 62.5±10.8% at one month, $P=0.856$), and SCNT efficiency (1.3±0.2% vs.1.6±0.4%, $P=0.579$).

### 3.3 Effect of *in vitro* maturation and culture system on the maturation, fusion, lysing rates, pregnancy and full-term rates, and SCNT efficiency.

Two culture conditions were used in our study: a serum-containing system (control IVM +IVC) and a serum-free system (BO-IVM and BO-IVC). No differences were observed in the maturation, fusion and lysing rates between two culture conditions regardless of the oocyte source (adult or prepubertal animals) (Table 4). There were no differences in the initial pregnancy rates between these two culture conditions. However, we observed differences in the 90-day pregnancy and full-term rates, and the SCNT efficiency ($P= 0.036$, 0.011, and 0.001 respectively, Table 5), where serum-free system improved developmental outcomes compared to the serum-containing system. Additionally, early pregnancy loss rate was lower in serum-free system compared to the serum-containing system (5.6% vs. 32.6%, $P=0.020$), whereas late pregnancy loss rates were comparable between the two systems (11.8% vs. 20.7%, $P=0.260$). There were no differences in lamb survival rates both at birth and at one month of age between the two culture conditions.

When the age of oocyte donors was included in the comparison, no differences were observed between the serum-containing system and the serum-free system on pregnancy and full-term rates

when adult sheep oocytes were used for SCNT embryo production. On the contrary, SCNT embryos derived from prepubertal sheep oocytes cultured in BO-IVM and BO-IVC had greater 90-day pregnancy and full-term rates, compared to that from control IVM and IVC ($P$=0.025, 0.012, respectively; Table 6). The increased SCNT efficiency was observed in the serum-free system, compared to the serum-containing system, with both oocyte types derived from adult or prepubertal animals (adult: $P$=0.034; prepubertal: $P$ =0.003).

3.4 Effect of the SCNT embryo activation time on pregnancy, full-term rates, and SCNT efficiency.

The overall pregnancy and full-term rates among different activation times (between 24 and 29hpm) are presented in Figure 1A, while the early and late pregnancy loss rates are shown in Figure 1B. Compared to early activation time (24 to 26h), there was an increasing trend in early pregnancy loss rate from 27h to 29h post maturation. Consequently, all transfers were categorized into two groups based on activation time post maturation: 24-26h and 27-29h. Although no differences were observed between two activation time groups in initial pregnancy rates and 90-day pregnancy ($P$=0.895 and 0.074), the full-term rates were higher in the 24-26h group, compared to the 27-28h group ($P$= 0.031, Figure 2A). The early pregnancy loss rate was higher in the 27-29h group than in 24-26h group ($P$=0.008, Figure 2B), but no differences were observed in late pregnancy loss rates between the two activation time groups. Moreover, the SCNT efficiency was higher in the 24-26h group compared to the 27-29h group (1.5±0.3% vs. 0.8±0.2%, $P$=0.029).

Looking at SCNT embryos produced from adult sheep oocytes, there was a trend of increased 90-day pregnancy and full-term rates in the 24-26h group compared to the 27-29h group, although not significant ($P$=0.156 and 0.064), However, the transferred SCNT embryos in the 24-26h group had a lower early pregnancy loss rate (22.5% vs. 44.9%, $P$=0.025) and higher SCNT efficiency (1.7±0.3% vs. 0.9±0.2%%, $P$=0.028) than those in the 27-29h group (Figure 3A and 3B). Conversely, no differences were found in initial pregnancy, 90-day pregnancy and full-term rates, and early and late pregnancy loss rates, between the two activation time groups when SCNT embryos were generated using prepubertal oocytes (Figure 3A and 3B). SCNT efficiency was comparable between 24-26h and 27-29h groups when oocytes were derived from prepubertal animals: 1.0±0.5% vs. 0.5±0.3% ($P$=0.904).

3.5 Effect of the number of SCNT embryos transferred per recipient on the pregnancy, full-term rates, and SCNT efficiency.

As shown in Figure 4, there was a trend of increasing initial and 90-day pregnancy rates, full-term development rate, live birth rate, and survival rate at one month from the lower number group (10-14 embryos) to higher number group (20-30 embryos), although the differences were not significant ($P$>0.05). Similarly, there were no differences in early and late pregnancy loss rates among three groups (P>0.05). Furthermore, the 10-14 group showed a tendency of lower SCNT efficiency and live birth rate than that in the 15-19 and 20-30 groups but was not significant (SCNT efficiency: 1.2±0.2% vs. 1.5±0.3% and 2.0±0.6%, $P$ =0.056 and 0.056; live birth rate: 66.7% vs. 86.4% and 93.8%, $P$ = 0.078 and 0.244).

3.6 Effect of the surgical numbers and ovary status of recipients on the pregnancy, full-term development rates, and SCNT efficiency.

Recipient ewes were used for up to two SCNT embryo transfers during the same breeding season, or across multiple seasons. Compared to the first embryo transfer surgery, no differences were observed in initial and 90-day pregnancy rates and full-term development rates, as well as live

birth rate and survival rate at one month in second surgery, regardless if the second surgery was performed in the same season or across multiple seasons (Figure 5A and 5B). SCNT efficiency of the second surgery during the same season was slightly higher than that in the 1st surgery, but not significant (3.3±1.3% vs. 1.0±0.2%, *P*=0.097), while SCNT efficiency was comparable between the first surgery and second surgery across the seasons (1.0±0.2%, vs. 1.2±0.3%, *P*=0.999), and between the second surgery within same season or different seasons (*P*=0.169). As shown in Figure 6, no differences were observed in the initial pregnancy, 90-day pregnancy and full-term rates with regard to different follicle size or ovulation status (*P*>0.05).

### 3.7 LOS/AOS

Overall, 9% of lambs (9/100) were categorized as having LOS/AOS in our study. After excluding 4 lambs produced from mixed-age group oocytes, it was found that all LOS/AOS lambs were produced from adult oocytes, however, the differences were not significantly (adult vs. prepubertal, 12.2±3.8% (9/91) vs. 0% (0/9), *P*=0.059). Compared to the groups cultured in serum-containing media, groups cultured in serum-free media had a slightly lower LOS/AOS rate, but the difference was not significant (11.6±3.9% (8/83) vs. 7.1±7.1% (1/17), *P*=0.234). Additionally, the LOS/AOS rates varied among the projects, but the differences were not significant (*P* >0.05, Table 7). Notably, all the LOS/AOS lambs were males, and the LOS/AOS rate in males was significantly greater than that in females (14.5±4.5% (9/73) vs. 0% (0/27), *P*=0.033).

## 4. Discussion

The overall SCNT efficiency in sheep is affected by numerous factors. In the present study, a total of 6102 reconstructed embryos were transferred into 412 recipients and produced 100 cloned lambs, of which, 76 clones were born alive. Thirty-one clones were alive and healthy at one month of age, which some losses were related to the genetic modification(s) introduced. We evaluated factors which may impact pregnancy, full-term development, and overall SCNT efficiency.

Age of oocyte donors is one of the important factors that affects the oocyte recovery rate and developmental competence of oocytes [28-30]. In our study, the oocyte numbers collected per ovary in adult donors was comparable to that in prepubertal donors, which is consistent to previous report that no difference was observed in COC recovery rates between young and adult ewe ovaries [31]. Previous studies have reported that even though the maturation rates were comparable between oocytes from adult and prepubertal sheep, both the first meiotic division and ATP rise were delayed in prepubertal oocytes, compared to that in the adult oocytes [28]. Moreover, O'Brien et al found that the IVF blastocyst formation was significantly lower from oocytes derived from prepubertal sheep than for those from adult sheep, but there were no differences in the pregnancy rate and number of lambs born following transfer of blastocyst stage embryos derived from prepubertal and adult sheep [29]. Our results also showed that there were no significant differences in the pregnancy and the full-term development of SCNT embryo produced from adult or prepubertal oocytes. This is consistent with our previous reports on oocytes derived from large and small follicles in goats [21] and observations in cattle [32], where the majority follicles of prepubertal ovaries are less than 3mm. One possible reason may be that artificial activation induces calcium influx in SCNT embryos, which could rescue the developmental competence of prepubertal oocyte. These results suggest the full-term developmental potential of prepubertal sheep oocytes are comparable to that of adult sheep oocytes when used for SCNT. To our knowledge, this is the first study that performed a direct comparison of *in vivo* developmental

competences of SCNT embryos derived from oocytes recovered from either prepubertal or adult sheep.

After the fusion of a donor cell with an ooplasm, embryos are typically incubated in $Ca^{2+}$ ionophore for 5 min, followed by culture in 6-DMAP or CHX for 4-5 h for chemical activation. Our activation protocol uses both CHX and 6-DMAP after ionomycin treatment, which has shown positive results in ovine, caprine, and bovine SCNT embryo development [21, 23, 33]. Although there was no significant difference in initial and 90-day pregnancy rates, earlier activation time (24-26hpm) of SCNT embryos resulted in a greater development to full-term. It is known that the maturation promoting factor (MPF) activity remains high in MII oocytes and gradually declines without activation. In the case of SCNT, MPF activity declines rapidly with activation [34]. Our results suggest an earlier decline of MPF activity, e.g. 24-26h post maturation, in ovine oocytes may facilitate better reprogramming in the donor cell nucleus. This may be due to the activation of the somatic nucleus in high MPF environment (early activation in this case) leading to higher incidence of premature chromosome condensation (PCC) which believe to be beneficial for reprogramming [34]. Even though we did not find differences in pregnancy and full-term development rates between SCNT embryos produced from adult and prepubertal oocytes, the earlier activation correlated to higher SCNT efficiency only in SCNT embryos derived from adult oocytes, but not in those from prepubertal oocytes. As reviewed by Zhu et al., oocyte maturation from prepubertal animals was delayed (26-32h) compared to oocytes from adult animals (24-26h), therefore the fertile span is different between adult and prepubertal sheep oocytes [35]. Another study reported that prepubertal MII oocytes had significantly lower level of MPF activity than adult oocytes [36], which may delay the donor cell nuclear envelope breakdown [37]. This might explain why the early activation does not impact the development of SCNT embryos derived from prepubertal oocytes in our study. However, the number of transfers performed was fewer when using embryos produced with prepubertal oocytes than those produced with oocytes from adult sheep. More data are needed before drawing a final conclusion.

Serum-containing culture systems have reportedly led to early embryo loss, LOS and increased organ size in offspring. In this study, we compared the commercial serum-free IVM and IVC medium (BO-IVM and BO-IVC; IVF Biosciences) to our standard IVM and IVC culture medium. We did not observe differences in the maturation, fusion, and lysing rates between the two *in vitro* culture conditions. Although SCNT embryos were only cultured *in vitro* overnight prior to transfer to minimize a potentially negative IVC impact on embryonic development, we found the commercial serum-free medium improved full-term rate and overall SCNT efficiency for embryos produced from prepubertal sheep oocytes. This is the first study that compares the effect of commercial serum-free IVM and IVC media and serum-containing media on the development of sheep SCNT embryos. Our results suggested that BO-IVM and BO-IVC media improve the developmental competence of prepubertal oocytes, which resulted in higher full-term development of SCNT embryos. Interestingly, we didn't find a correlation between the culture conditions and the number of LOS/AOS lambs nor other factors of interest, e.g. oocyte donor age or projects cell lines used. The only factor correlated to LOS/AOS in our study was gender, with male cloned lambs having a higher LOS/AOS rate than females. Until now very few studies have reported effect of gender on LOS/AOS in cloned animals. One possible reason might be the different expression pattern of imprinted genes between male and female fetuses [38]. More data should be collected to conclude whether these factors impact LOS/AOS manifestation in these animals.

The number of transferred embryos per recipient is another factor impacting SCNT efficiency. A previous study reported that the transfer of 7-9 and 11-13 SCNT embryos resulted in significantly higher pregnancy rates than that of six embryos [20]. Consequently, it was not surprising that there was an increasing tendency of initial and 90-day pregnancy rates and full-term rates with the higher number of embryos transferred per recipient. Moreover, the SCNT efficiency was also higher in 15-19 and 20-30 groups, compared to 10-14 group, although not statistically significant. Taken together, our results indicate that transferring 15-30 SCNT embryos per recipient should result in consistently good pregnancy rates and full-term development.

Regarding the selection of recipients for embryo transfers, we investigated two factors: surgical number and ovulatory (follicle) status. All of our recipients had similar reproductive history prior to the first SCNT embryo transfer. Based on our observations, the pregnancy and full-term rates of recipients with second surgeries were comparable to that of recipients undergoing their first surgery, regardless of whether the second surgery happened in the same or a different breeding season. This finding could be helpful in reusing non-pregnant recipients for embryo transfer within the same season to save on the cost of purchasing new recipients. In addition, there was no effect of ovulation status (CH) or follicle size (6-12mm) on pregnancy and full-term rates, suggesting that recipients with a follicle size between 6-12 mm or with a CH are suitable for SCNT embryo implantation. Moreover, with follicles measuring 5 mm in size, giving recipients GnRH still resulted in comparable pregnancy and full-term rates compared to those with larger follicles. To our knowledge, this is the first study to investigate the effect of these two factors on SCNT efficiency in sheep. These findings will be beneficial for practical handling of recipient ewes for SCNT embryo transfers.

## 5. Conclusion

This study revealed that the age of oocyte donors does not impact *in vitro* maturation rates, pregnancy, full-term development and SCNT efficiency. Earlier activation time (24-26hpm) of SCNT embryos was correlated with a lower early pregnancy loss rate, and higher full-term rate and SCNT efficiency. Compared to our standard serum-containing medium, commercial serum-free culture medium showed a positive correlation with the full-term development of sheep SCNT embryos. Transferring 15-30 embryos per recipient should result in consistently good pregnancy and full-term rates. Surgical number and ovulatory status (having at least one follicle between 6-12mm or CH) of recipients would not affect pregnancy and full-term development rates.

## Author contributions

IAP and YL took part in conception and design, interpretation of data, preparation of manuscript; YL took part in data analysis; ZF and IVP generated cell lines; YL, QM, TP, ZF and JK generated SCNT embryos; RS and MR performed embryo transfer and pregnancy check, animal delivery, neonatal care, and acquisition of data. All authors have read and agreed to the published version of the manuscript.

## Declaration of interest

The authors declare no conflicts of interest.

## Acknowledgement

The authors thank Dr. Alexis Sweat for excellent veterinary care, Laura Adams, Renata Blocher and Ian Bunderson for technical assistance, and Haozhe Chen for assistance in the statistical

analysis. This work was supported in part by the Utah Agricultural Experiment Station (projects: UTA01343 and UTA01653), the USDA/NIFA multistate research project W-4171 (to IAP) and the Cystic Fibrosis Foundation (award POLEJA18G0).

## References

[1] Polejaeva IA. 25th ANNIVERSARY OF CLONING BY SOMATIC CELL NUCLEAR TRANSFER: Generation of genetically engineered livestock using somatic cell nuclear transfer. Reproduction. 2021;162:F11-F22.
[2] Loi P, Toschi P, Zacchini F, Ptak G, Scapolo PA, Capra E, et al. Synergies between assisted reproduction technologies and functional genomics. Genet Sel Evol. 2016;48:53.
[3] Vazquez-Avendaño JRA-G, D. A. ; Cortez-Romero, C. ; Trejo-Córdova, A.; Navarro-Maldonado, M. C. Current state of the efficiency of sheep embryo production through somatic cell nuclear transfer. Small Rumin Res. 2022;212:106702.
[4] Li J, Pedersen HS, Li R, Adamsen J, Liu Y, Schmidt M, et al. Developmental potential of pig embryos reconstructed by use of sow versus pre-pubertal gilt oocytes after somatic cell nuclear transfer. Zygote. 2014;22:356-65.
[5] Leoni GG, Succu S, Satta V, Paolo M, Bogliolo L, Bebbere D, et al. In vitro production and cryotolerance of prepubertal and adult goat blastocysts obtained from oocytes collected by laparoscopic oocyte-pick-up (LOPU) after FSH treatment. Reprod Fertil Dev. 2009;21:901-8.
[6] Ledda S, Bogliolo L, Calvia P, Leoni G, Naitana S. Meiotic progression and developmental competence of oocytes collected from juvenile and adult ewes. J Reprod Fertil. 1997;109:73-8.
[7] Kashir J, Ganesh D, Jones C, Coward K. Oocyte activation deficiency and assisted oocyte activation: mechanisms, obstacles and prospects for clinical application. Hum Reprod Open. 2022;2022:hoac003.
[8] Liu L, Ju JC, Yang X. Differential inactivation of maturation-promoting factor and mitogen-activated protein kinase following parthenogenetic activation of bovine oocytes. Biol Reprod. 1998;59:537-45.
[9] McLean ZL, Appleby SJ, Fermin LM, Henderson HV, Wei J, Wells DN, et al. Controlled Cytoplast Arrest and Morula Aggregation Enhance Development, Cryoresilience, and In Vivo Survival of Cloned Sheep Embryos. Cell Reprogram. 2021;23:14-25.
[10] Carolan C, Lonergan P, Van Langendonckt A, Mermillod P. Factors affecting bovine embryo development in synthetic oviduct fluid following oocyte maturation and fertilization in vitro. Theriogenology. 1995;43:1115-28.
[11] Sinclair KD, McEvoy TG, Maxfield EK, Maltin CA, Young LE, Wilmut I, et al. Aberrant fetal growth and development after in vitro culture of sheep zygotes. J Reprod Fertil. 1999;116:177-86.
[12] Farin PW, Piedrahita JA, Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology. 2006;65:178-91.
[13] Walker SKH, K. M. ; Seamark, R.F. . The production of unusually large offspring following embryo manipulation: concepts and challenges. Theriogenology. 1996;45:111-20.
[14] Mahdi AK, Medrano JF, Ross PJ. Single-Step Genome Editing of Small Ruminant Embryos by Electroporation. Int J Mol Sci. 2022;23.

[15] Mazzoni G, Salleh SM, Freude K, Pedersen HS, Stroebech L, Callesen H, et al. Identification of potential biomarkers in donor cows for in vitro embryo production by granulosa cell transcriptomics. PLoS One. 2017;12:e0175464.
[16] Shahzad Q, Pu L, Ahmed Wadood A, Waqas M, Xie L, Shekhar Pareek C, et al. Proteomics Analysis Reveals that Warburg Effect along with Modification in Lipid Metabolism Improves In Vitro Embryo Development under Low Oxygen. Int J Mol Sci. 2020;21.
[17] van der Weijden VA, Chen S, Bauersachs S, Ulbrich SE, Schoen J. Gene expression of bovine embryos developing at the air-liquid interface on oviductal epithelial cells (ALI-BOEC). Reprod Biol Endocrinol. 2017;15:91.
[18] Rim CH, Fu Z, Bao L, Chen H, Zhang D, Luo Q, et al. The effect of the number of transferred embryos, the interval between nuclear transfer and embryo transfer, and the transfer pattern on pig cloning efficiency. Anim Reprod Sci. 2013;143:91-6.
[19] Liu J, Li LL, Du S, Bai XY, Zhang HD, Tang S, et al. Effects of interval between fusion and activation, cytochalasin B treatment, and number of transferred embryos, on cloning efficiency in goats. Theriogenology. 2011;76:1076-83.
[20] Yuan Y, Liu R, Zhang X, Zhang J, Zheng Z, Huang C, et al. Effects of recipient oocyte source, number of transferred embryos and season on somatic cell nuclear transfer efficiency in sheep. Reprod Domest Anim. 2019;54:1443-8.
[21] Yang M, Hall J, Fan Z, Regouski M, Meng Q, Rutigliano HM, et al. Oocytes from small and large follicles exhibit similar development competence following goat cloning despite their differences in meiotic and cytoplasmic maturation. Theriogenology. 2016;86:2302-11.
[22] Davies CJ, Fan Z, Morgado KP, Liu Y, Regouski M, Meng Q, et al. Development and characterization of type I interferon receptor knockout sheep: A model for viral immunology and reproductive signaling. Front Genet. 2022;13:986316.
[23] Fan Z, Perisse IV, Cotton CU, Regouski M, Meng Q, Domb C, et al. A sheep model of cystic fibrosis generated by CRISPR/Cas9 disruption of the CFTR gene. JCI Insight. 2018;3.
[24] McGegor CGAB, G. W.; Fan, Z.; Davies, C. J.; Polejaeva, I. A. Genetically engineered sheep: A new paradigm for future preclinical testing of biological heart valves. The Journal of Thoracic and Cardiovascular Surgery. 2023.
[25] Van Wettere AJ, Leir SH, Cotton CU, Regouski M, Viotti Perisse I, Kerschner JL, et al. Early developmental phenotypes in the cystic fibrosis sheep model. FASEB Bioadv. 2023;5:13-26.
[26] Perisse IVF, Z.; Liu, Y.; Regouski, M.; Van Wettere, A.; Wang, Z.; Harris, A.; White, K. L.; Polejaeva, I. A. Towards the correction of meconium ileus with cystic fibrosis transmembrane conductance regulator (CFTR) intestinal expression in CFTR knockout sheep. Reproeduction, Fertility and Development. Reprod Fertil Dev. 2019;31:228-.
[27] Tervit HR, Whittingham DG, Rowson LE. Successful culture in vitro of sheep and cattle ova. J Reprod Fertil. 1972;30:493-7.
[28] Leoni GG, Palmerini MG, Satta V, Succu S, Pasciu V, Zinellu A, et al. Differences in the Kinetic of the First Meiotic Division and in Active Mitochondrial Distribution between Prepubertal and Adult Oocytes Mirror Differences in their Developmental Competence in a Sheep Model. PLoS One. 2015;10:e0124911.
[29] O'Brien JK, Catt SL, Ireland KA, Maxwell WM, Evans G. In vitro and in vivo developmental capacity of oocytes from prepubertal and adult sheep. Theriogenology. 1997;47:1433-43.

[30] Falchi L, Ledda S, Zedda MT. Embryo biotechnologies in sheep: Achievements and new improvements. Reprod Domest Anim. 2022;57 Suppl 5:22-33.
[31] Lorenzo-Torres A, Rangel-Santos R, Ruiz-Flores A, Ambriz-Garcia DA. In vitro embryo production from ewes at different physiological stages. J Vet Sci. 2023;24:e10.
[32] Piedrahita JA, Wells DN, Miller AL, Oliver JE, Berg MC, Peterson AJ, et al. Effects of follicular size of cytoplast donor on the efficiency of cloning in cattle. Mol Reprod Dev. 2002;61:317-26.
[33] Cuthbert JM, Russell SJ, Polejaeva IA, Meng Q, White KL, Benninghoff AD. Comparing mRNA and sncRNA profiles during the maternal-to-embryonic transition in bovine IVF and scNT embryosdagger. Biol Reprod. 2021;105:1401-15.
[34] Campbell KH, Loi P, Otaegui PJ, Wilmut I. Cell cycle co-ordination in embryo cloning by nuclear transfer. Rev Reprod. 1996;1:40-6.
[35] Zhu J, Moawad AR, Wang CY, Li HF, Ren JY, Dai YF. Advances in in vitro production of sheep embryos. Int J Vet Sci Med. 2018;6:S15-S26.
[36] Ledda S, Bogliolo L, Leoni G, Naitana S. Cell coupling and maturation-promoting factor activity in in vitro-matured prepubertal and adult sheep oocytes. Biol Reprod. 2001;65:247-52.
[37] Tani T, Kato Y, Tsunoda Y. Direct exposure of chromosomes to nonactivated ovum cytoplasm is effective for bovine somatic cell nucleus reprogramming. Biol Reprod. 2001;64:324-30.
[38] Nava-Trujillo H, Rivera RM. Review: Large offspring syndrome in ruminants: current status and prediction during pregnancy. Animal. 2023;17 Suppl 1:100740.

Legend to the figures

Figure 1. Initial pregnancy, 90-day pregnancy and full-term rates (A), and early and late pregnancy loss rates (B) at different activation times (24 to 29 hours post maturation). The total numbers of recipients are shown in the tables below. The differences in total number of transfers between the initial pregnancy and the 90-day pregnancy were caused by early pregnancy terminations for sample/fetus collections.

Figure 2. Initial pregnancy, 90-day pregnancy and full-term rates (A), and early and late pregnancy loss rates (B) in two activation time groups, 24-26h and 27-29h. The total numbers of recipients are shown in tables below. The differences in total number of transfers between the initial pregnancy and the 90-day pregnancy were caused by early pregnancy terminations for sample/fetus collections.

Figure 3. Effect of activation time of SCNT embryos and oocyte donor age on pregnancy and full-term development. (A) Initial pregnancy, 90-day pregnancy and full-term rates (B) early and late pregnancy loss rates in two activation time groups (24-26h and 27-29h) and donor age groups (adult and prepubertal). The total numbers of recipients are shown in the tables below. The differences in total transfer numbers between the initial pregnancy and the 90-day pregnancy were caused by early pregnancy terminations for sample/fetus collections.

Figure 4. Effect of number of SCNT embryo transferred per recipient on the pregnancy and full-term development. (A) Initial pregnancy, 90-day pregnancy and full-term rates; (B) early and late pregnancy loss rates; (C) live birth rates and survival rates at one month. The total numbers of recipients are shown in the tables below. The differences in total number of transfers between

639 the initial pregnancy and the 90-day pregnancy were caused by early pregnancy terminations for
640 sample/fetus collections.
641 Figure 5. Effect of surgery number of recipients on pregnancy and full-term development of
642 SCNT embryos. *P*>0.05. The total numbers of recipients are shown in the tables below. The
643 differences in total number of transfers between the initial pregnancy and the 90-day pregnancy
644 were caused by early pregnancy terminations for sample/fetus collections.
645 Figure 6. Initial pregnancy, 90-day pregnancy and full-term rates of different ovulary status of
646 recipients, follicle size between 5 to 12 mm or ovulation. CH= corpus hemorrhagicum. All the
647 pairwise comparisons showed *P*>0.05.
648
649
650
651 Table 1. Effect of donor age on the number of oocytes collected per ovary and maturation rate.
652

| Age of oocyte donors | Oocyte numbers per ovary ± SEM (no. of replicates) | Maturation rate ± SEM % (no. of oocytes) |
|---|---|---|
| Adult | 13.9±0.5 (31) | 69.4±1.6 (5079/7448) |
| Prepubertal | 10.8±0.7 (6) | 68.1±1.8 (1161/1740) |
| *P* value | 0.857 | 0.592 |

653 Replicates= no. of oocyte collection days
654
655
656 Table 2. Effect of the age of oocyte donors on the pregnancy and full-term development rates,
657 and SCNT efficiency in sheep
658

| Age of oocyte donors | Initial pregnancy rate (D40±5) [1] | 90-day pregnancy rate (D90±5) [1]* | Full-term rate [1] | Live birth rate ±SEM[2] | Survival rate at one month ±SEM [3] | SCNT efficiency, mean±SEM[4] |
|---|---|---|---|---|---|---|
| adult | 47.1% (165/350) | 31.5% (96/305) | 24.6% (75/305) | 77.0±4.4% (69/91) | 41.1±6.1% (29/69) | 1.5±0.2% (69/4510) |
| prepubertal | 29.8% (17/57) | 19.3% (11/57) | 17.5% (10/57) | 77.8±14.7% (7/9) | 28.6±18.4% (2/7) | 0.7±0.3% (7/853) |
| OR | 1.62 | 1.23 | 1.09 | 1.02 | 1.55 | 1.75 |
| *P* value | 0.405 | 0.769 | 0.875 | 0.392 | 0.671 | 0.266 |

659 1: no. of pregnant or full-term animals/no. of embryo transfers
660 2: no. of lambs born alive/ total no. of born lambs
661 3. no. of survived lambs at one month/ no. of live born lambs
662 4. no. of live born lambs /total no. of transferred embryos
663 * The differences in total transfer numbers between initial pregnancy and 90-day pregnancy were
664 caused by early pregnancy terminations for sample/fetus collections.
665 OR: odds ratio.
666
667

Table 3. Effect of projects on fusion, pregnancy, and full-term development rates

| Projects | Fusion rates ± SEM (replicates) | Initial pregnancy rates (D40±5) [1] | 90-day pregnancy rates (D90±5) [1]* | Full-term rates[1] | SCNT efficiency, mean ±SEM [2] |
|---|---|---|---|---|---|
| A | 84.6±1.0% (45) | 47.7% (103/216) | 31/8% (55/173) | 25.4% (44/173) | 1.6±0.2% (39/2497) |
| B | 85.4±2.3% (15) | 42.3% (22/52) | 26% (13/50) | 22.0% (11/50) | 1.4±0.4% (12/818) |
| C | 86.0±2.3% (9) | 48.1% (13/27) | 22.2% (6/27) | 22.2% (6/27) | 1.8±0.7% (8/436) |
| D | 78.8±2.8% (15) | 37.0% (17/46) | 26.1% (12/46) | 19.6% (9/46) | 1.0±0.4% (6/658) |
| E | 85.4±2.1% (12) | 35.5% (11/31) | 22.6% (7/31) | 16.1% (5/31) | 0.4±0.3% (2/466) |
| F | 88.7±2.4% (7) | 35.0% (7/20) | 30.0% (6/20) | 30.0% (6/20) | 2.0±1.0% (6/286) |
| G | 82.6±3.7% (6) | 45.0% (9/20) | 40.0% (8/20) | 20.0% (4/20) | 0.9±0.5% (3/282) |

Replicates= no. of SCNT experiment days

1: no. of pregnant or full-term animals/no. of embryo transfers

2. no. of live born lambs /total no. of transferred embryos

* The differences in total transfer numbers between initial pregnancy and 90-day pregnancy were caused by early pregnancy terminations for sample/fetus collections.

All the pairwise comparisons among 7 projects using post hoc test with Holm-Bonferroni correction showed *P* value was above 0.05.

Table 4. Effect of medium and donor age on maturation, fusion, and lysing rates

| Age of oocyte donors | Medium | Maturation rate ± SEM (no. of oocytes) | Fusion rate ± SEM | Lysing rate ± SEM |
|---|---|---|---|---|
| Adult | Control IVM +IVC | 68.9±1.9%(4570/6659) | 83.7±1.6% (3604/4314) | 2.1±0.4% (91/4394) |
| | BO-IVM+IVC | 71.9±3.0% (410/560) | 86.2±1.7% (351/408) | 3.6±0.6% (15/409) |
| | *P* value | 0.527 | 0.253 | 0.123 |
| Prepubertal | Control IVM +IVC | 68.3±2.8% (727/1098) | 86.2±1.8% (561/647) | 7.4±1.6% (54/689) |
| | BO-IVM+IVC | 67.6±0.4% (434/434/642) | 84.8±2.8% (343/406) | 5.0±1.4% (18/404) |
| | *P* value | 0.704 | 0.741 | 0.345 |

Table 5. Effect of *in vitro* culture systems on the pregnancy and full-term development rates, and SCNT efficiency

| Medium | Initial pregnancy rate (D40±5) [1] | 90-day pregnancy rate (D90±5) [1]* | Full-term rate [1] | Live birth rate ±SEM[2] | Survival rate at one month ±SEM [3] | SCNT efficiency, mean±SEM[4] |
|---|---|---|---|---|---|---|
| Control IVM +IVC | 44.3% (164/370) | 27.7% (90/325) | 21.5% (70/325) | 74.6±4.7% (61/83) | 41.1±6.5% (26/61) | 1.3±0.2% (61/4797) |
| BO-IVM+IVC | 42.9% (18/42) | 40.5% (17/42) | 35.7% (15/42) | 89.3±7.7% (15/17) | 34.6±13.1 % (5/15) | 2.4±0.6% (15/646) |
| OR | 1.02 | 0.46 | 0.38 | 0.31 | 0.79 | 0.18 |
| *P* value | 0.954 | 0.036 | 0.011 | 0.078 | 0.744 | 0.001 |

1: no. of pregnant or full-term animals/no. of transfers
2: no. of live born lambs/total born lambs
3. no. of survived lambs at one month/no. of live born lambs
4. no. of live born lambs /total no. of transferred embryos
* The differences in total transfer numbers between initial pregnancy and 90-day pregnancy were caused by early pregnancy terminations for sample/fetus collections.
OR: odds ratio.

Table 6. Effect of age of oocyte donors and culture systems on the pregnancy and full-term development rates

| Donor age | Medium | Initial pregnancy rate[1] | 90-day pregnancy rate[1]* | Full-term rate[1] | Live birth rate ±SEM[2] | Survival rate at one month ±SEM [3] | SCNT efficiency, mean±SEM[2] |
|---|---|---|---|---|---|---|---|
| adult | Control IVM +IVC | 47.4% (155/327) | 30.5% (86/282) | 23.8% (67/282) | 76.5±4.7% (60/80) | 41.8±6.6% (26/60) | 1.4±0.2% (60/4183) |
| | BO-IVM+IVC | 43.5% (10/23) | 43.5% (10/23) | 34.8% (6/18) | 81.3±13.2 % (9/11) | 35.7±18.0 % (3/9) | 2.9±0.9% (5/327) |
| | OR | 1.56 | 0.68 | 0.58 | 0.48 | 1.40 | 0.381 |
| | *P* value | 0.327 | 0.427 | 0.293 | 0.449 | 0.658 | 0.034 |
| Pre-puberta l | Control IVM +IVC | 23.7% (9/38) | 10.5% (4/38) | 7.9% (3/38) | 33.3±33.3 % (1/3) | 0% (0/1) | 0.2±0.2% (1/534) |
| | BO-IVM+IVC | 42.1% (8/19) | 36.8% (7/19) | 36.8% (7/19) | 100% (6/6) | 33.3±21.1 % (2/6) | 1.8±0.6% (6/319) |
| | OR | 0.427 | 0.195 | 0.147 | N/A | N/A | 0.048 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | *P* value | 0.157 | 0.025 | 0.012 | 0.018 | 0.720 | 0.003 |

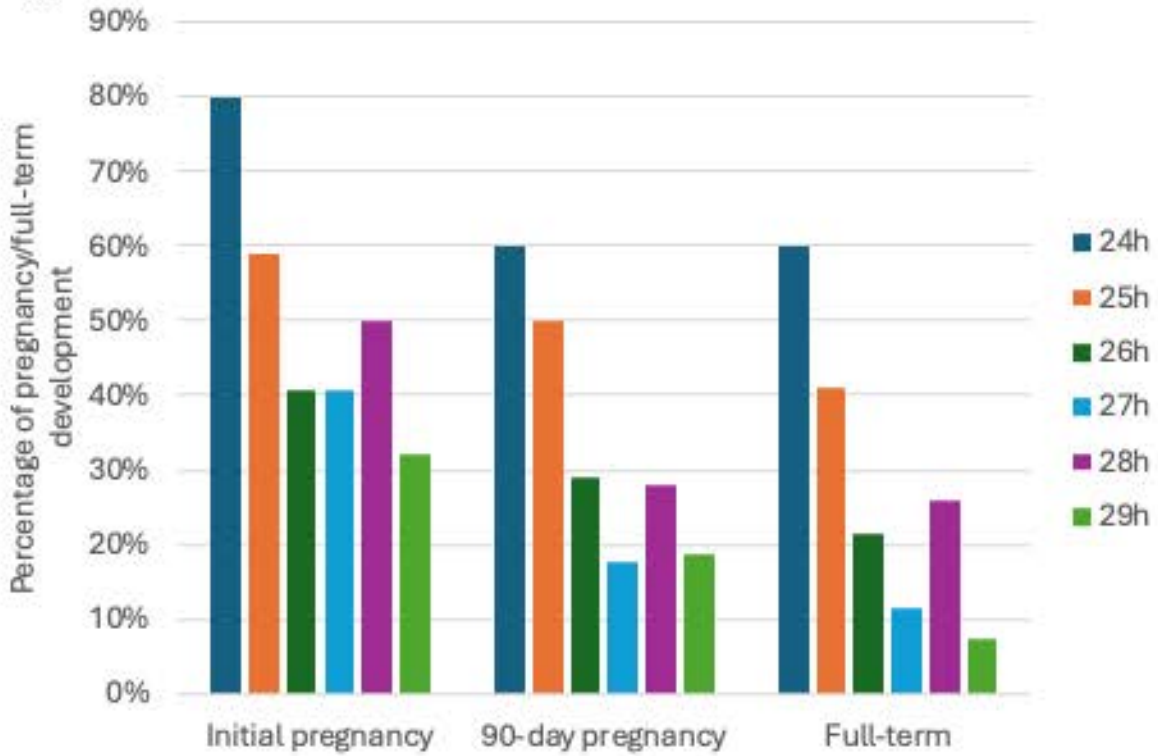
1: no. of pregnant or full-term animals/no. of transfers
2. no. of live born lambs /total no. of transferred embryos
* The differences in total transfer numbers between initial pregnancy and 90-day pregnancy were caused by early pregnancy terminations for sample/fetus collections.
OR: odds ratio.
N/A: no enough data to calculate OR.

Table 7. Effect of projects on LOS/AOS rates

| Projects | LOS/AOS rates |
|---|---|
| A | 9.3±4.5% (4/51) |
| B | 0% (0/13) |
| C | 0% (0/8) |
| D | 25±16.4% (2/11) |
| E | 20±20% (1/5) |
| F | 16.7±16.7% (1/8) |
| G | 25±25% (1/4) |

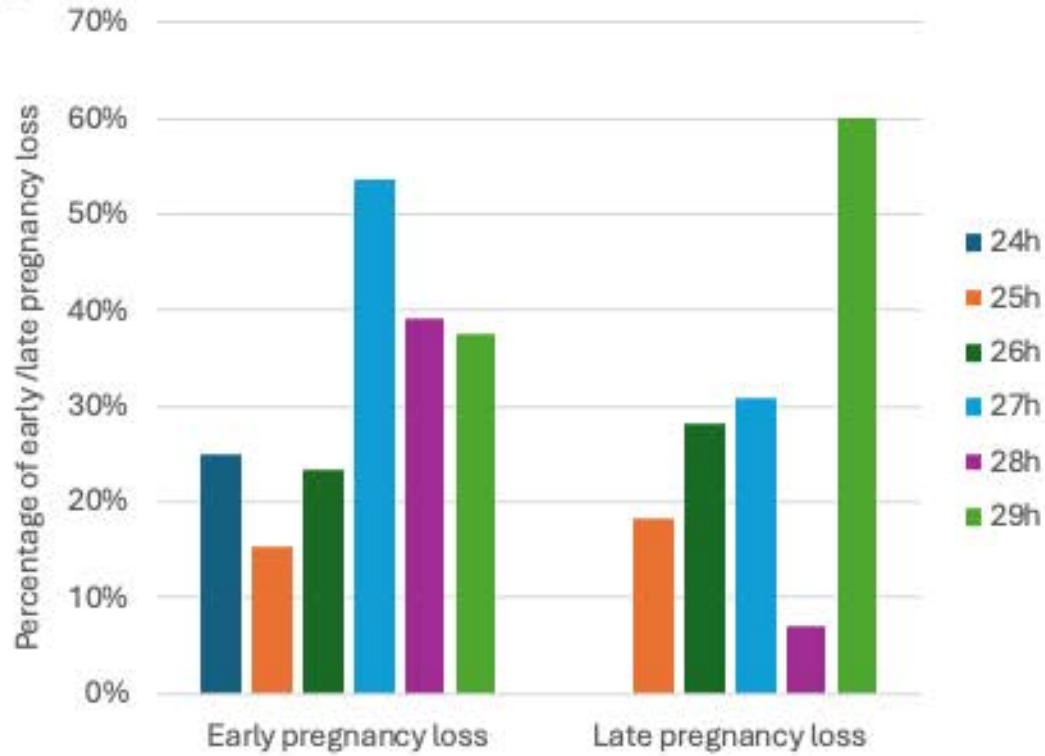
All the pairwise comparisons among 7 projects using post hoc test with Holm correction showed $P > 0.05$.

A



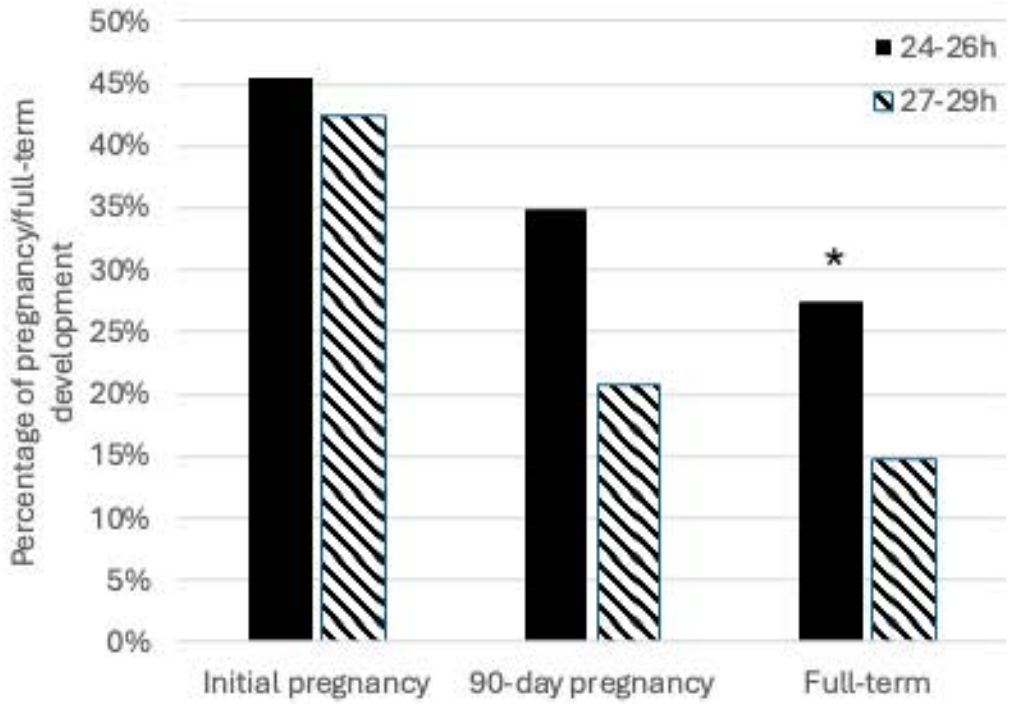

| Activation time | No. of recipients | | |
|---|---|---|---|
| | Initial pregnancy | 90-day pregnancy | Full-term |
| 24h | 5 | 5 | 5 |
| 25h | 22 | 22 | 22 |
| 26h | 86 | 79 | 79 |
| 27h | 118 | 91 | 91 |
| 28h | 58 | 50 | 50 |
| 29h | 28 | 27 | 27 |

B



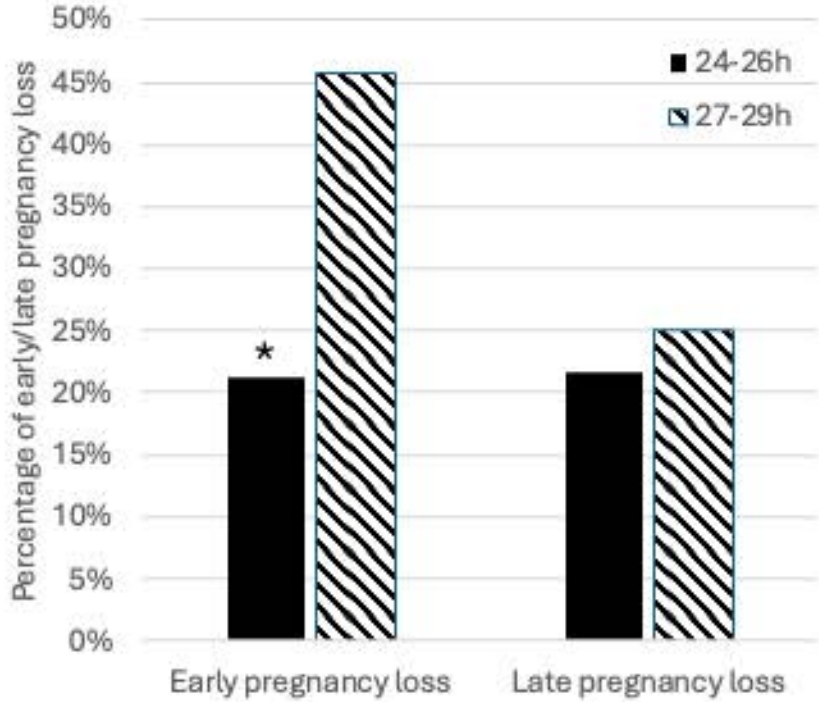

| Activation time | No. of recipients | |
|---|---|---|
| | Early pregnancy loss | Late pregnancy loss |
| 24h | 4 | 3 |
| 25h | 13 | 11 |
| 26h | 30 | 23 |
| 27h | 28 | 13 |
| 28h | 23 | 14 |
| 29h | 8 | 5 |

A



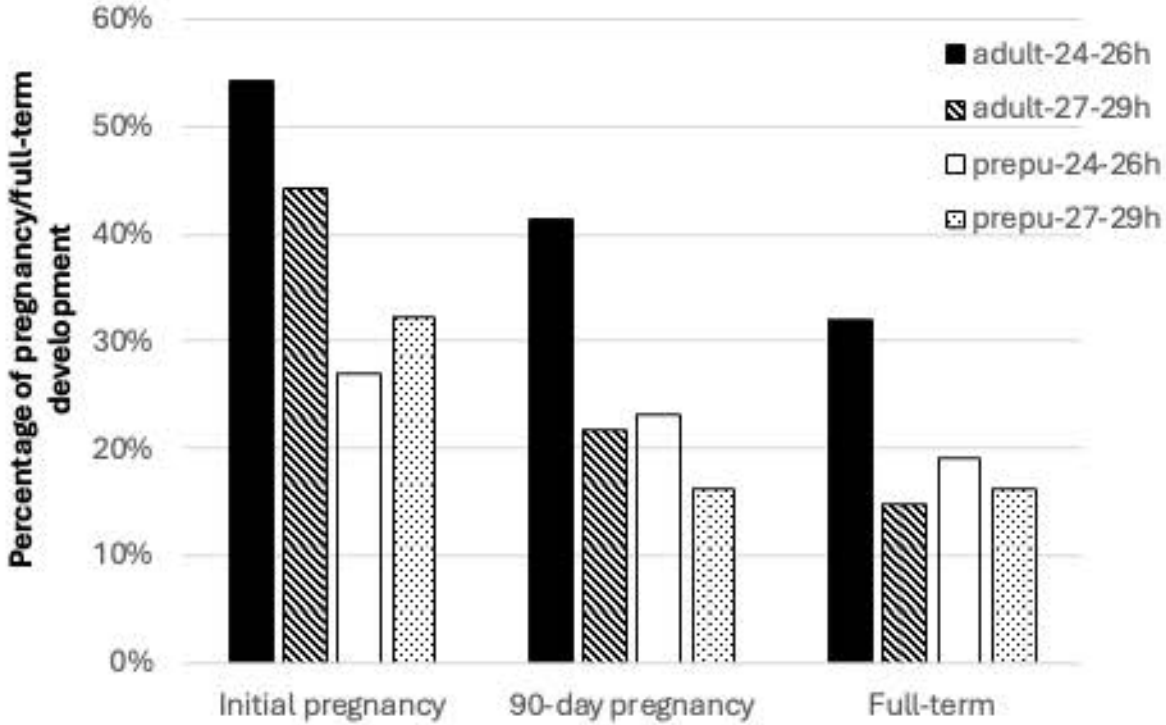

| Activation time | No. of recipients | | |
|---|---|---|---|
| | Initial pregnancy | 90-day pregnancy | Full-term |
| 24-26h | 112 | 106 | 106 |
| 27-29h | 205 | 169 | 169 |
| P value | 0.980 | 0.107 | 0.036 |

B



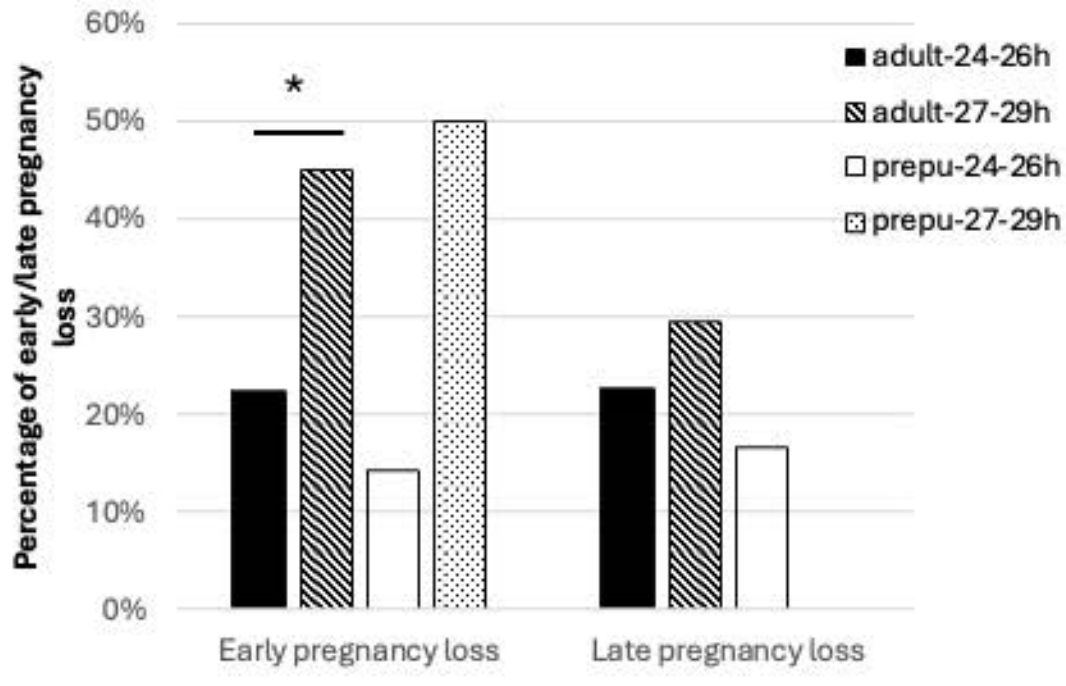

| Activation time | No. of recipients | |
|---|---|---|
| | Early pregnancy loss | Late pregnancy loss |
| 24-26h | 47 | 37 |
| 27-29h | 59 | 32 |
| P value | 0.008 | 0.686 |

A

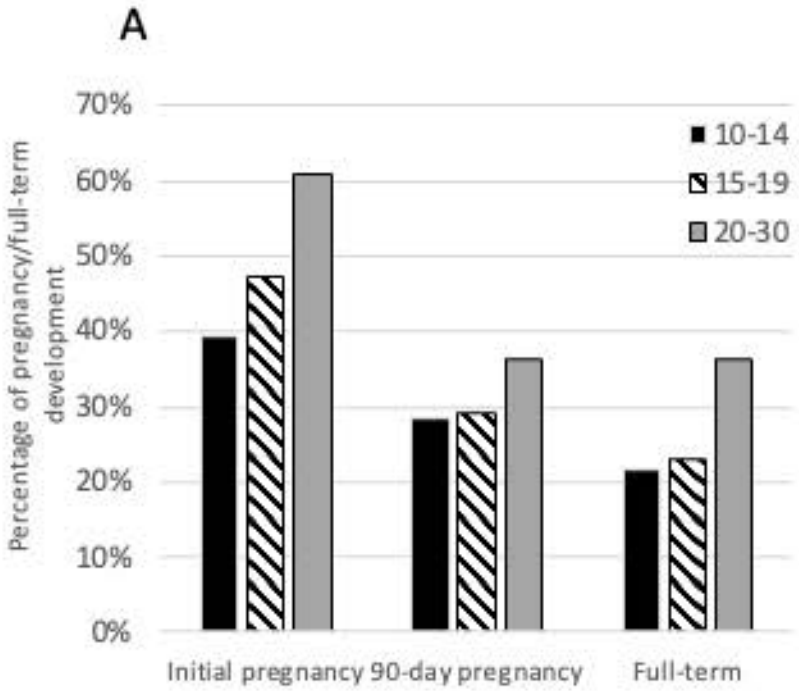


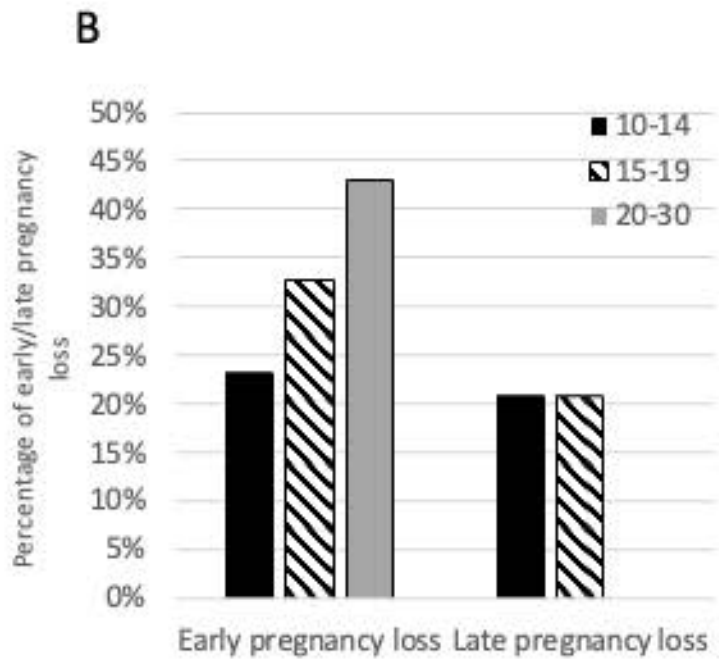

| Activation time | No. of recipients | | |
|---|---|---|---|
| | Initial pregnancy | 90-day pregnancy | Full-term |
| adult-24-26h | 81 | 75 | 75 |
| adult-27-29h | 174 | 138 | 138 |
| P value | 0.493 | 0.156 | 0.064 |
| prepu-24-26h | 26 | 26 | 26 |
| prepu-27-29h | 31 | 31 | 31 |
| P value | 0.661 | 0.510 | 0.759 |

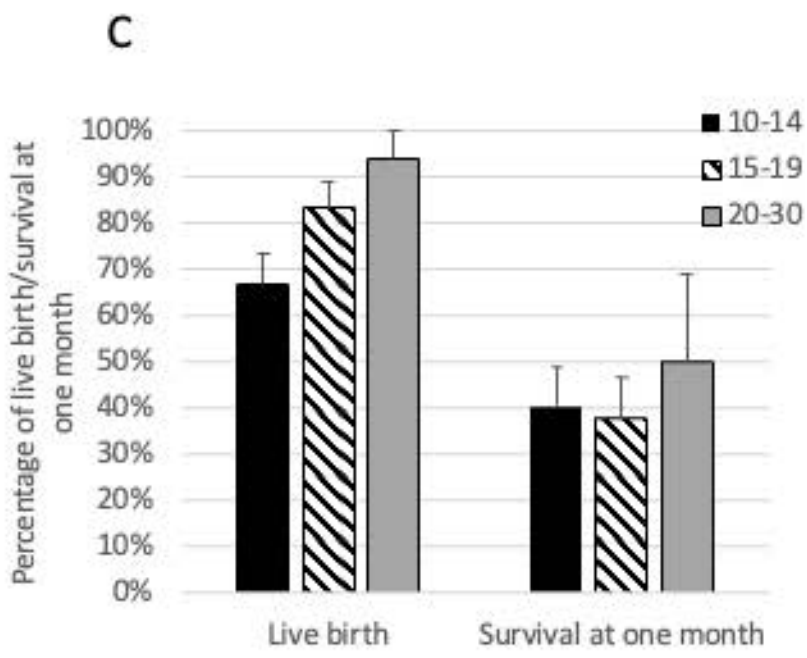
B




| Activation time | No. of recipients | |
|---|---|---|
| | Early pregnancy loss | Late pregnancy loss |
| adult-24-26h | 40 | 31 |
| adult-27-29h | 49 | 27 |
| P value | 0.025 | 0.486 |
| prepu-24-26h | 7 | 6 |
| prepu-27-29h | 10 | 5 |
| P value | 0.152 | 0.994 |



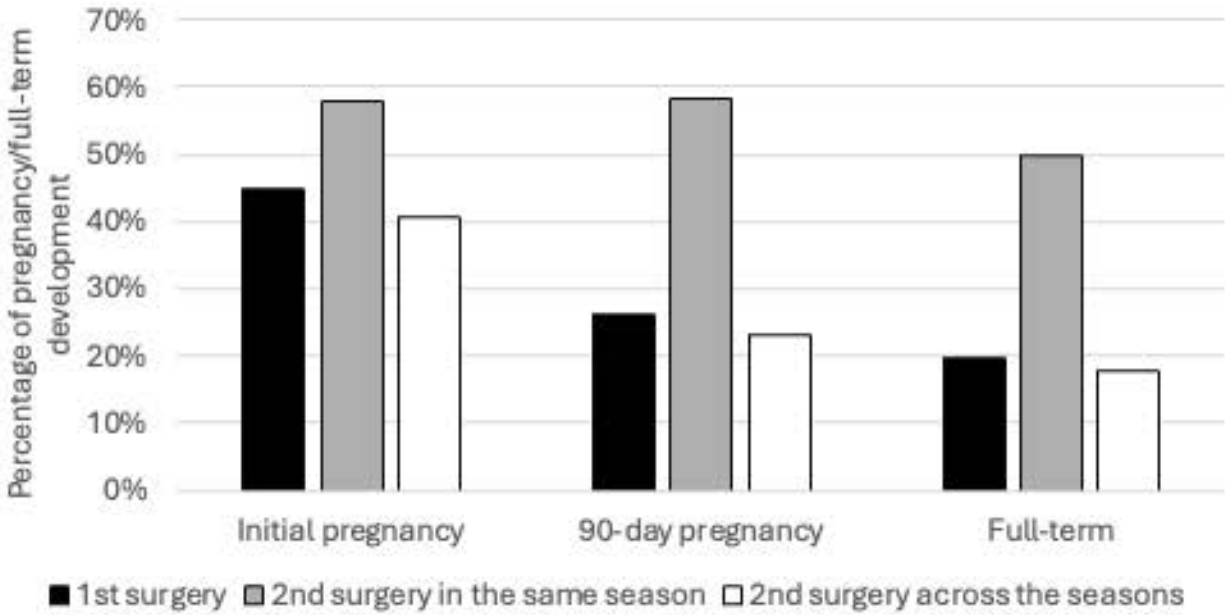




| No. of embryos transferred per recipient | No. of recipients | |
|---|---|---|
| | Initial pregnancy | 90-day pregnancy and full-term |
| 10-14 | 218 | 198 |
| 15-19 | 170 | 147 |
| 20-30 | 23 | 22 |

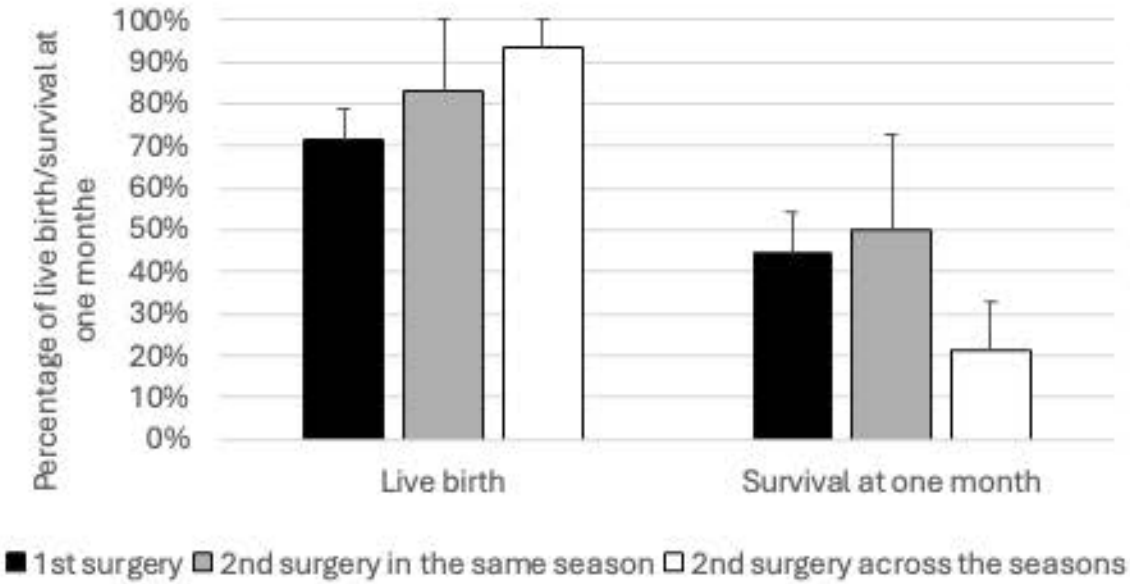
| No. of embryos transferred per recipient | No. of recipients | |
|---|---|---|
| | early pregnancy loss | late pregnancy loss |
| 10-14 | 69 | 53 |
| 15-19 | 64 | 43 |
| 20-30 | 14 | 8 |

| No. of embryos transferred per recipient | No. of live born lambs/total no. of born lambs | No. of survived lambs at one month/no. of live born lambs |
|---|---|---|
| 10-14 | 32/49 | 13/32 |
| 15-19 | 35/41 | 13/35 |
| 20-30 | 9/10 | 5/9 |

A

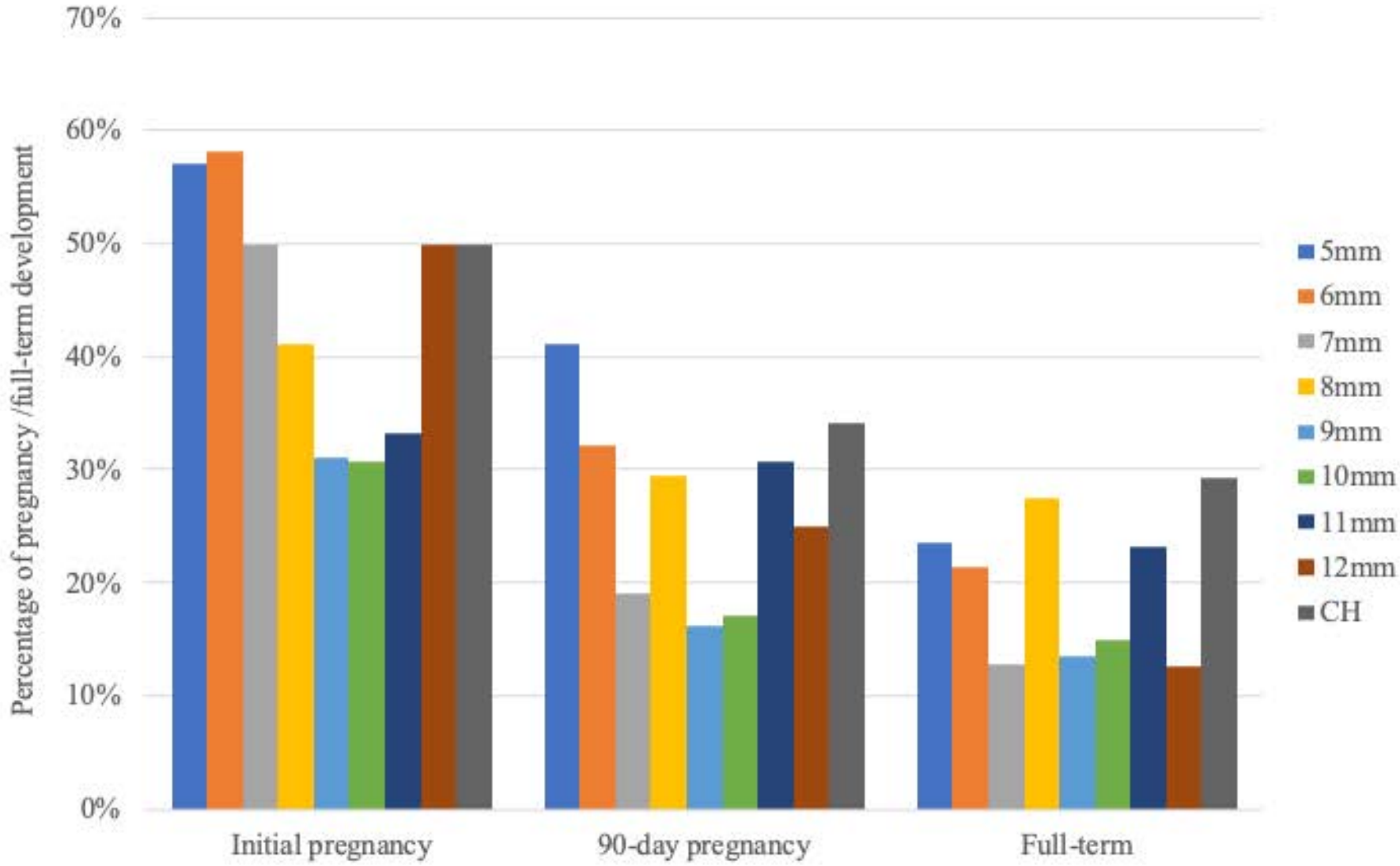



B




| Surgery no. | No. of recipients | |
|---|---|---|
| | Initial pregnancy | 90-day pregnancy and full-term |
| 1st surgery | 197 | 183 |
| 2nd surgery in the same season | 19 | 12 |
| 2nd surgery across the seasons | 113 | 91 |

| Surgery no. | No. of live born lambs/total no. of born lambs | No. of survived lambs at one month/no. of live born lambs |
|---|---|---|
| 1st surgery | 39/41 | 13/29 |
| 2nd surgery in the same season | 6/7 | 3/6 |
| 2nd surgery across the seasons | 16/17 | 4/16 |


70%
60%
50%
40%
30%
20%
10%
0%
Percentage of pregnancy /full-term development
Initial pregnancy
90-day pregnancy
Full-term
5mm
6mm
7mm
8mm
9mm
10mm
11mm
12mm
CH