Health Assessment and Care for Animals Involved in the Cloning Process[1]

A consensus recommendation from the International Embryo Transfer Society
15 May 2008

New discoveries in the cloning process and in care of surrogates and neonatal clones are steadily improving the outcomes for animal clones, much as was observed when IVF was first adopted. These animal care guides serve as a place where these discoveries can be shared and the entire scientific cloning community can be quickly informed; for researchers new to the field to learn from those with more experience in order to improve animal health and welfare. Small cloning facilities, such as found at some University laboratories, may not have permanent veterinary staff dedicated to managing animals involved in the cloning process. Animal care guidance will assist contract or newly assigned staff become prepared quickly for what would ordinarily be low frequency adverse health events.

The Risk:
Animal cloning can be associated with a risk of adverse health impacts in the surrogate dams carrying a clone fetus to term and/or for newborn and juvenile clones. These outcomes are not unique to cloning and were first identified in other assisted reproductive technologies; however their incidence may be higher at present.

Some of the reasons why IETS believes it prudent to provide these guides include:

1. To serve as a means to compare experiences across labs world-wide and improve the overall success rate.
2. Adverse health outcomes observed in cloning vary from species to species. Lessons learned in one species are not necessarily helpful when attempting to clone a different species. As facilities experienced in one species expand their scope to include additional species, animal care guidance arranged by species will assist veterinary staff in planning for care for clones and surrogates.
3. Regular updating of these guides is expected for at least the next several years, or whenever a new species is added to the list of animals being cloned.

Risk Management Objective:
To share information and expertise among cloning practitioners to minimize the frequency and impact(s) of risks to the health of animals involved in the cloning process.

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[1] Document elaborated by the IETS Health And Sanitary Advisory Committee (HASAC) - thanks to all the contributors and members of HASAC - and approved by the Board of Governors of IETS.

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A. RECOMMENDATIONS FOR THE VETERINARY CARE OF CATTLE CLONES

i. Selection and Conditioning of recipient oocytes

Oocytes are frequently collected from animals in good body condition that are slaughtered for food. Whole ovaries can be harvested and oocytes collected at the arrival at the laboratory, or the oocytes may be collected directly from the follicles at the time of ovary collection and transported in maturation medium to the laboratory. Whole ovaries are washed in disinfectant solution and transported in a heated (38°C) box in phosphate buffered saline (PBS) with antibiotics added. Ovaries have also been successfully transported in normal saline (0.9% NaCl) in a thermos flask at ~30ºC without antibiotics or disinfectant.

ii. Selection and preparation of recipients dams

The selection of recipient dams should follow the IETS guidelines for selecting dams for embryo transfer.

Both heifers and cows have been used successfully according to multiple sources. Heifers are sometimes preferred due to their higher potential to become pregnant after regular embryo transfer. Intended recipients/surrogates for clone embryos are initially selected based on their potential to deliver a larger calf through the birth canal and a known history of successful, uncomplicated delivery (for cows). Large dams are preferred (cross beef breed or beef breed).

A complete veterinary examination should be performed that includes:

- serology (repeated 2x) to determine infectious status;
  - recipient dams must be negative for B*rucella abortus*, Enzootic Bovine Leukosis (EBL), and Bovine Virus Diarrhea (BVD) antigen - to confirm negative persistently infected (PI) status, *Mycobacteria bovis*, Infectious Bovine Rhinotracheitis (IBR), and *Leptospira* spp. (Leptospirosis);
  - optionally, and according to the local standard of herd health management, also rule out *Neospira* spp., *Chlamydia psittaci*, Bovine Herpes Virus 4 (BHV4), *Coxiella burnetti* (Q-fever), *Anaplasma spp.*, and *Mycobacteria paratuberculosis* (paratuberculosis, Johne's disease).

Potential recipients are also examined for healthy normal mammary glands, structural soundness, and their reproductive organs are carefully examined to rule out adhesions, overly large cervices or uteri, and for acyclic ovaries.

Recipients should be in good body condition and fed to maintenance levels. Feeding is preferably increased during the period of estrous synchronization but reduced to maintenance levels after embryo transfer.

Standard estrous synchronization includes a 10-12 day treatment with controlled release progesterone device (e.g., CIDR-B (controlled internal drug release), PRID (progesterone

releasing intravaginal device or Crestar implants) or prostaglandin injections. In the case of using an intra-vaginal device, strict aseptic technique is observed during insertion of the device to reduce the risk of inducing vaginitis. After estrous behavior is observed (≈95% of recipients), the presence of a CL should be confirmed by rectal palpation. Animals with poor CL formation and/or suspected cystic follicles should be excluded. Potential recipients which fail to synchronize, or those with a cervix that is excessively difficult to traverse with the transfer pipette are culled.

After embryo transfer, recipient cattle are monitored as they near 14 days post-transfer to identify any short-cycling animals. This indicates that these animals did not achieve estrous synchrony.

iii. Selection and transfer of embryo clones

Only grade 1 or 2 embryos (IETS guidelines) are transferred, generally as fresh embryos, though some teams have transferred frozen embryo clones successfully. In some laboratories, the stage of the embryo is matched with the heat timing of the recipient. For example, a morula would be put in a recipient that had a heat 7 days ago while an expanded blastocyst would be put in a recipient that had a heat 7 ½ or 8 days ago. The choice between single or double transfer has not been shown to significantly impact pregnancy rates.

iv. Assessment of fetal clone and recipient health, including criteria for terminating pregnancy

The first pregnancy check is performed by measuring maternal blood progesterone 20 days after recipient heat, or with ultrasound performed 30 days after recipient heat (23 d after embryo transfer).

Ultrasound and rectal palpation evaluation of the fetus and placenta

Ultrasound evaluation of the first trimester fetus and placenta has been reported using a standard veterinary ultrasound machine, used routinely to diagnose pregnancy in cattle, equipped with a 5 MHz rectal probe (for rectal examination) or a 2.5 to 3.5 MHz probe (for transabdominal examination). Alternatively a more sophisticated ultrasound machine (e.g., Toshiba Nemio 20), designed to follow human pregnancies, can be used.

First trimester:

Regular pregnancy checks are performed by transrectal ultrasound from 30 to 35 days up to 90 to 120 days depending on the breed of cattle. Beyond 120 days transabdominal ultrasound is required to visualize the fetus because the weight of the growing fetus pulls the uterus ventrally in the abdomen. Rectal ultrasound remains beneficial as it provides visualization of part of the placenta.

By gestational day 35, ultrasound observations of fetal size, heart beat, fluid volume, amniotic membranes and echogenicity are possible. Animals which still have the original CL and trophoblast remnants seen by ultrasonography, but are not pregnant, are sometimes observed. Generally, any intended recipient which fails to become pregnant following three consecutive rounds of synchronization and embryo transfer should be culled. Reports from several teams indicate that transfer of somatic cell nuclear transfer (SCNT) embryos results in good early pregnancy rates, so few animals are culled for this reason.

Repeated transrectal ultrasound examinations are recommended at 10-15 day intervals, until day 70, because the time of early placental development (30 to 70 days) is a major period of pregnancy loss in SCNT pregnancies. Recipients are scanned again at Day 70 to assess for placentome development and the presence of a live fetus. Those animals in which 0, 1 or 2 placentomes are observed often fail to maintain the pregnancy beyond Day 100. In case of a dead fetus the pregnancy may be terminated with prostaglandin injections. Any of these early failing recipients are eligible to be resynchronized and re-used after examination to confirm successful termination of pregnancy.

Fetal and placental measurements during the first trimester are useful to identify those recipients that will lose their pregnancy before 90-100 days (see table 1). More work is needed to improve our ability to reliably identify those dams that will develop hydrops in later stages.

Table 1: Significant ultrasound measurements for predicting pregnancy outcome in recipients of cloned embryos

Stage of pregnancy	**Measurement**	**Threshold value**	**Significance**	**Reference**
Day 30	Crown-rump length and Heart Rate	CRL<7.5 mm and HR<150 b/min	fetal death <90 days	(Panarace, Garnil et al. 2006)
Day 62	Crown-rump length	<36.6 mm (< normal measurement - 2SD)	fetal death <90 days	(Chavatte-Palmer, De Sousa et al. 2006)
Days 114-226	Abdominal circumference (surface in mm^2)	D114: > 278.2 mm^2 D157: > 470.5 mm^2 D184: > 593.8 mm^2 D212: > 704.1 mm^2 D226: > 738.8 mm^2 (> normal measurement in clones +2SD)	Fetal death before term	(Panarace, Aguero et al. 2006)

	Edematous, hyperechogenic placentomes, speckled allantoic fluid	At least two observations at > 3 days interval	Development of hydrops	(Heyman, Chavatte-Palmer et al. 2002; Chavatte-Palmer 2003)

Second and third trimester:

Pregnant animals are examined by rectal palpation and transabdominal ultrasound examination at days 100, 120, and then every fourteen days until day 240, or as required. Examinations should be made early in the day when the rumens are less full. Very little information can be gained from transrectal ultrasound scans following day 100 since the uterus drops ventrally into the abdomen.

Approximately 25% of recipients will develop hydrops*, with wide variations in rate and severity between genotypes. Hence, regular physical and ultrasound examination of the recipients is essential in order to enable early detection of hydrops and termination of the affected pregnancies well before the hydropic fetus poses a risk to recipient dam.

Clinical observations

All pregnant cattle are evaluated clinically (e.g., appetite, activity, abdominal size). Observation of the abdominal girth of the recipient from back view and side view are helpful in detecting hydrops. This assessment should include consideration of gestational age, and whether the pregnancy is singleton or twin. When hydrops is suspected, repeated measurement of the abdominal circumference (at the place of largest circumference), at the same time daily, and prior to meal time, is a reliable indicator of the rate of progression of hydrops. An increase of >10 centimeters per week signals an abnormally rapid increase in abdominal volume.

Rectal palpation

Rectal palpation allows assessment of the uterine wall thickness, tone and the relative size and shape of the uterus. Serial assessments enable detection of any rapid fluid accumulation. When hydrops is present the increased uterine pressure and the presence of a fluid-filled uterus can be palpated within the rectal cavity. This can become evident as early as Day 120, signaling possible development of hydroallantois. By around day 200, placental structures, fetal activity and relative fetal placement are noted. The presence of any unusually large and heavy placentomes and placental "mats", resulting from adventitious placentation, should be noted. These structures can develop to cover the internal os of the cervix during parturition and, at term,

* Hydrops of the allantois and the amnion are the most common cause of dropsy of fetal membranes. Rectal palpation of pregnant cattle in last trimester will reveal excessive fluid, inability to palpate fetus and cotyledons and over-distension of both sides of abdomen. Trans-rectal ultrasonography can confirm the same.

can impede calf delivery. Mammary gland development is also assessed at the time of each rectal palpation.

Transabdominal ultrasound

Cows are clipped and/or shaved from the sternum to the mammary gland and on the lower right flank. They must be restrained gently in a crush chute where they can be comfortable (especially when they become large in late gestation) and where they can remain calm. This is achieved by habituating the animals through regular training prior to the pregnancy, and by keeping the atmosphere quiet with dim lighting, and allowing them to settle down in the room (30 minutes in optimal conditions) prior to the examination (Figure 1). In each examination, the general appearance of placentomes and allantoic and amniotic fluid are observed and fetal movements are noted.

The most relevant measurements to be used for detection of abnormalities are listed in Table 1. Fetal heart rate or aortic diameter did not prove useful for an early detection of onset of Large Offspring Syndrome (LOS) or hydrops. Because it appears that placental abnormalities precede fetal abnormalities, it is important to pay close attention to the fetal membranes. Measuring placentome size has proven impractical due to the large variation in placentome size in clone pregnancies and the difficulty in choosing which placentome to measure. In contrast, detection of placentome edema before 15 days prior to term appears to be a very reliable indicator of the onset of LOS (Young et al., 2002), abnormal offspring (Farin et al., 2006) or "Large Placenta Syndrome" (Constant et al., 2006) and is usually associated with increased echogenicity of the placentome (Figures 2 and 3). At least two consecutive observations at several days interval are needed to confirm these observations.

Monitoring by maternal blood sampling

Endocrinology

To date, there is no known routine haematologic or serologic marker, or other biomarker that has been shown to reliably predict the onset of hydrops in pregnancies of clone fetuses. Elevated concentrations of the pregnancy associated glycoproteins (PAG), such as Pregnancy Specific Protein 60 (PSP60) or Pregnancy Specific Protein Bovine (PSPB), observed in consecutive blood samples in the second and early third trimester of pregnancy are thought to be indicative of LOS and can be viewed as supporting the diagnosis of LOS. Due to the wide variety of antibodies available for assaying PAG throughout the world, investigators should become familiar with the range of normal values for their relevant breed and gestational age, as measured by their local laboratory, before using this marker as a diagnostic tool. Furthermore, it is recommended that a trend of increasing PAG levels be observed through serial testing rather than relying on a single data point.

Biochemistry

After 200 days gestation maternal blood analyses for beta hydroxy butyrate (BOH), magnesium (Mg) and calcium (Ca) are done to identify and manage any deficiencies or metabolic problems.

Figure 1 : Examination of the fetus and placental membranes by transrectal ultrasonography using a portable, veterinary ultrasound machine and a 3.5 MHz probe (Patrice Laigre, INRA).

Figure 2 : Ultrasound appearance of normal placentomes (A) and edematous, hyperechogenic placentomes in a recipient carrying a clone fetus with development of hydrops (B). (Patrice Laigre and Pascale Chavatte-Palmer, INRA)

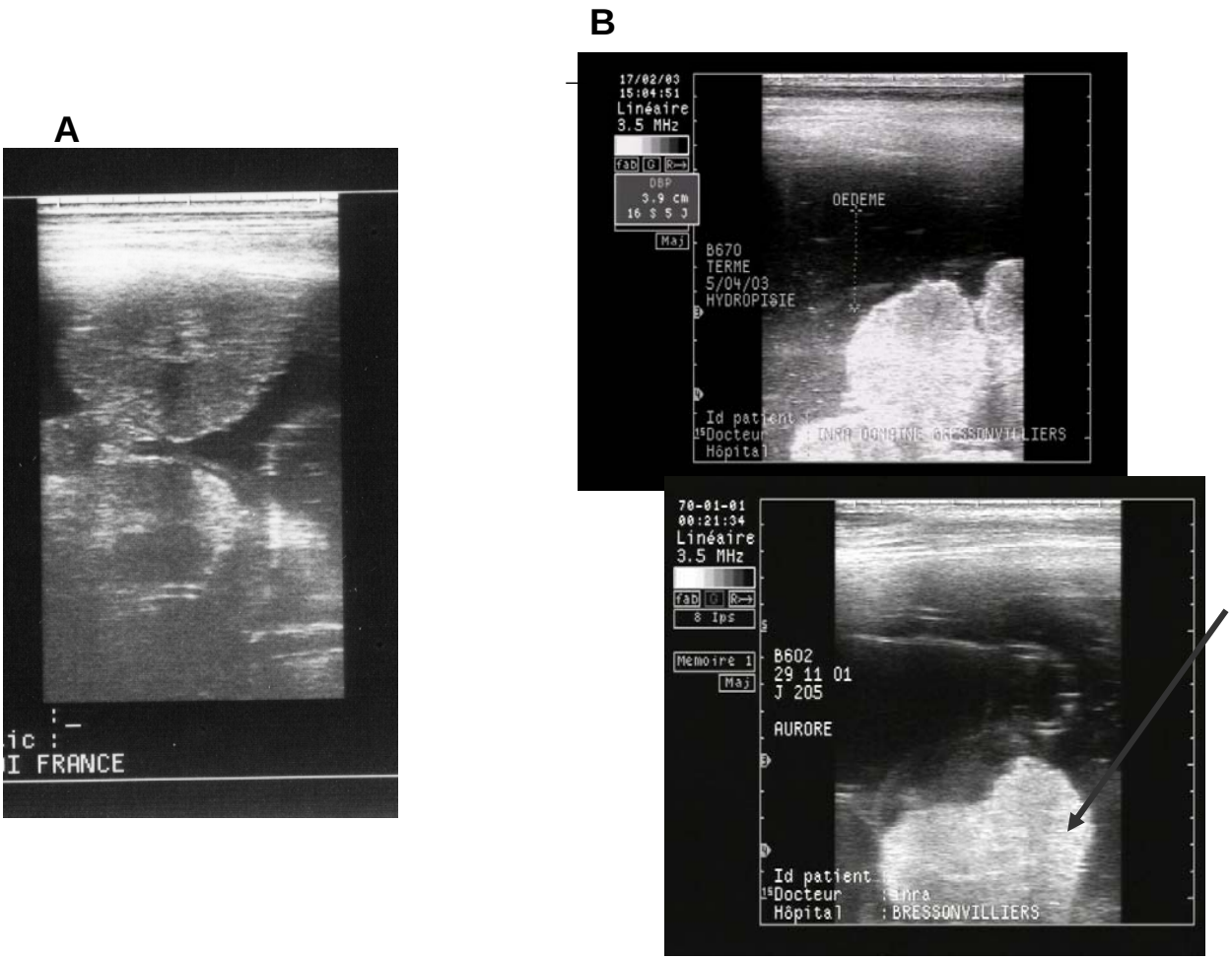
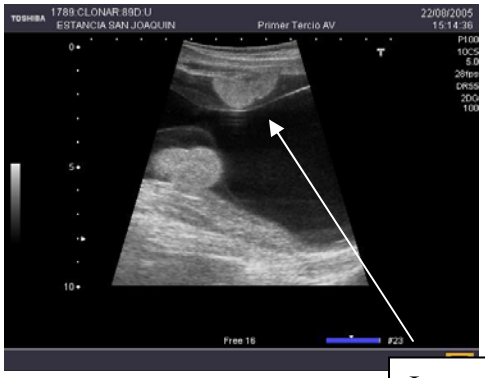


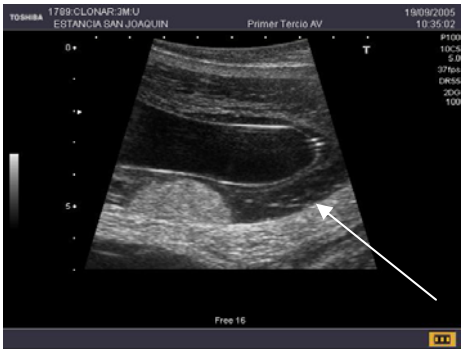
Figure 3 : Ultrasound images obtained from abnormal pregnancies of cloned fetuses. (M. Panarace, Goyaike)

Abnormal placenta 90 d of gestation



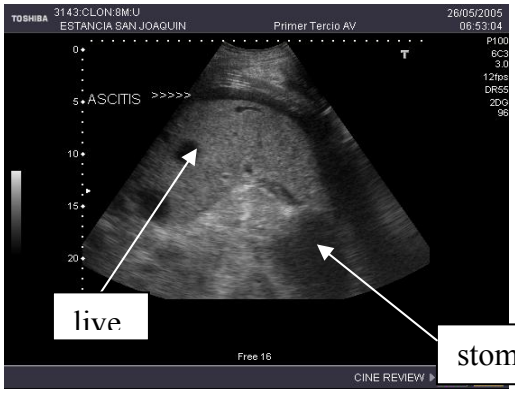
Increased thickness showing placental edema

Abnormal placenta 120 days of gestation



Increased thickness showing placental edema

Hydrops fetalis at 8 month of pregnancy



Termination of hydrops pregnancies

The interval from detection of abnormalities to fetal death is variable, ranging from 11 to 127 days. However, generally, once abnormalities are detected, fetal death is inevitable.

When hydroallantois is suspected, early pregnancy termination is recommended before the condition becomes life-threatening for the recipient. Clinical hydroallantois is diagnosed when the uterus becomes firm and tight and the fetus can no longer be felt at palpation. Allowing the recipient to progress to a grossly distended state is not ethically acceptable. When two or more of the following are observed termination of pregnancy should be strongly considered:

- Enlarged fluid filled uterus on rectal palpation
- Abnormally large, hyperechoic, edematous placentomes observed by rectal ultrasound
- Rapid increase of the abdominal circumference (>10 cm/week)
- Clinical signs such as abdominal distension, difficult ambulation, anorexia.

Alternatively, usually much later in gestation (gestation > 250 days), excessive accumulation of amniotic fluid, hydramnios, may occur. *The condition is characterized by a **gradual** accumulation of **thick**, viscid fluid during the last half of gestation. The cow has a pear shaped caudal view.* In this condition the fetus is usually palpable because the allantoic fluid volume is "normal" but the fetus appears positioned higher than normally expected since it is lying on membranes heavy with highly viscous, jelly-like amniotic fluid and large placentomes. This condition is harder to diagnose. *The pregnancy usually goes to term, and frequently a small, deformed fetus is delivered. Postpartum metritis is uncommon. The prognosis is good for life and fertility. No treatment is required. The cow may be allowed to go to term or induced to calve.*

A decision to terminate suspected hydroallantoic pregnancies should be made at the earliest time possible. Hydroallantoic pregnancies commonly present with overgrown fetuses, often with distended abdomens. Prolonging the pregnancy in an attempt to salvage the fetus is not indicated since the fetus is likely to have multiple problems. Moreover, calves from hydrops cows that are removed early (<250 days gestation) have not been viable despite intensive neonatal care. Allowing fetal death and subsequent necrolysis makes the fetus more difficult to extract and increases the risk of metritis. Hydrops pregnancies may also present significant risk to the recipient dam. Carrying the oversized fetus to term may result in severe complications including abdominal muscle tears, uterine injury, *Clostridial* infections, toxic shock, hypovolemic shock and metritis.

Hydrops pregnancies diagnosed prior to gestation day 150 are usually terminated with intramuscular prostaglandin injections administered at luteolytic doses. Repeated doses may be required to achieve CL regression.

After Day 150, pregnancies can be terminated medically by induction of parturition with corticosteroids. Five ml of dexamethasone (e.g., Dexavet, 5 mg/ml) is injected in the neck muscles on Day 0 and again 5 days later. Dilation of the cervix is initiated with the topical administration of 1 ml of prostaglandin F2α (e.g., Estroplan) on the cervix. Topical application

of prostaglandin F2α is repeated, twice per day for 2-3 days until the cervix is sufficiently soft and dilated enough to allow the fetus to be removed. The allantoic membranes are ruptured to drain the allantoic fluid away. The fetus can be grasped and pulled out approximately one hour later. When the allantoic membranes are thick and gelatinous, a scissors is required to incise the membranes. Although the uterus may be atonic, abdominal pressure will help lift the calf after the fluid is drained. A calf presented breech with a grossly distended abdomen is impossible to pull because the rib cage will expand outwards as the abdominal organs are pushed against it if pulled breech and will not pass through the cervix. The calf should be manually repositioned to allow the shoulders to dilate the cervix first. After Day 150, a fetus is capable of surviving for some time after extraction. For this reason the fetus should be humanely euthanized immediately with a lethal dose of pentobarbital.

Following termination and fetal extraction, it has been suggested that the recipient cow is administered 2 ml of prostaglandin F2α followed by systemic antibiotics and uterine antibiotic pessaries (El-Azab et al, 1998) every 3 days and allowed ad-lib grazing. In the event fetal membranes and/or the placenta is retained aggressive treatment with systemic anitbiotics is indicated for up to 2 weeks due to the degree of exposure to infection created by enlarged, deeply invasive cotyledons and thickened membranes often associated with these pregnancies. It is important to ensure adequate feed intake during this time as a depressed appetite can result in metabolic emergencies. A cow may reflexively and continually strain in an attempt to deliver the placenta. Epidural anesthesia can help interrupt this cycle and allow her to graze. Cattle are allowed a recovery period of at least 60 days before being considered for re-use as a recipient. Many of these animals have successfully carried subsequent pregnancies to term.

v. Delivering Clones, Criteria for Selection of C-section versus Natural (Vaginal) Birth

One common problem with clone pregnancies is that dams may be physiologically unprepared for parturition, and they have prolonged gestations with increased risk of dystocia resulting from an oversized fetus, when gestation is not induced at term. For these reasons the timing of calving is managed by inducing parturition between Days 270 and 282 of gestation. Parturition may be induced as early as Day 265 with successful outcome.

Inducing fetal maturation and calving

Several induction protocols have been used successfully by experienced veterinarians in the authors' teams. These are outlined below. Veterinarians that are not experienced in cattle cloning should begin with the simpler protocols used at term.

1. Induction at the due date (preferred for teams not experienced with cattle cloning):
   a. **Single injection of dexamethasone** (INRA) - When spontaneous calving has not occurred by Day 282, a single dose of 20 mg dexamethasone is given and Cesarean section (C-section) is performed 24 hours later.

   b. **Combination of prostaglandins and dexamethasone** (Goyaike) - When spontaneous calving has not occurred by Day 282, parturition is induced after an additional 3-5 days

by giving IM injections of 2 mL of PGF2α (Bioprost®, Biotay, Buenos Aires, Argentina or Estrumate®, Mobay Corp., Shawnee, KS, USA) and 30 mg of dexamethasone

2. Induction prior to due date:
   a. **With repeated injections of dexamethasone** (Ag Research) - Nine days before the scheduled calving date, cows are injected (day 0) in the neck region with 5 ml of long-acting dexamethasone (Dexavet, 5 mg/ml dexamethasone trimethylacetate) along with 1 ml/100kg of Hideject (vitamin A, D_3 and E) to prevent milk fever and to build up resistance to infection. On the morning of day 7, 25 mg of short-acting corticosteroid (Dexone 5, dexamethasone sodium phosphate (DSP)) is injected intramuscularly into the neck region. Calving is expected the following day, peaking around 43 h after the DSP injection. If there are no signs of cervical softening and dilation after 48-60 hours, another dose of DSP is administered. Some cows will begin calving after receiving the first long-acting dexamethasone injection.

      Cows are palpated at the first long-acting dexamethasone injection, then each evening after the dexamethasone injection to assess progress to calving. Cervical dilation and softening, mucus production and consistency are monitored. Calf size and positioning are assessed by rectal palpation.

   b. **With a combination of prostaglandins and dexamethasone** (Hematech) - Due to the importance of the calves, all recipients have scheduled C-sections performed 7-8 days prior to due date (or 275-277 days gestation). Recipients carrying twins are delivered between 264 and 266 days. Induction protocols include: dexamethazone (30mg IM) at hour 0, prostaglandin F2α (15 mg IM) at hour 0 and hour 12, C-section at hour 24.

<u>Calving or C-section management</u>

Whenever possible the cow is allowed to calve naturally. This is not always possible, for example, when 24 hour surveillance is not available, or when research priorities (e.g., collection of fresh term placental samples) make it impossible to let the cows calve on their own.

1. Natural Calving:
   a. If no limbs appear within one hour after the amnion has ruptured, the cow is examined to determine if the calf is normally positioned, and whether it is too large to pass through the birth canal. When necessary the calf's presentation position is manually corrected and the cow allowed to continue with labor. The cow should be continually observed for any signs of distress or lack of progress. Assistance is rendered by a veterinarian as needed. The option of Caesarian section delivery should be considered when the calf is too large to pass through the pelvis, abnormally presented, or when the cow has not responded to the attempts at medical induction. Traction is applied only after verification that the calf is correctly presented. Traction is applied simultaneously with the cow's uterine contractions.

2. Caesarian section:

a. Caesarian section is performed by the attending veterinarian, ideally with the cow standing. Aseptic conditions should be maintained throughout the procedure and broad-spectrum prophylactic antibiotics and anti-inflammatory drugs may be administered prior to the procedure. In case of hydrops condition fluids should be administered to the dam to compensate for the severe fluid loss during the C-Section. The umbilical vessels should be torn manually rather than clamped to collapse vessels, thus reducing the risk of infection. Oxytocin injections and oral energy drench should be given to the cow. In particular, recipients that were diagnosed with hydrops, are given an oral drench of fluids with electrolytes via stomach tube to replace the fluid loss. A non-steroidal anti-inflammatory drug (e.g., flunixen meglumine, diclofenac, ketoprofen) should be administered for post-operative pain and to expedite the cow's return to function. It has been suggested that the minimization of maternal distress tends to promote calf bonding when the cow is left with her calf. Antibiotics are continued for three days following surgery.

   Retained placenta may be a problem even following Cesarean section. Recipients are palpated at 72-96 hours post surgery if the placenta and fetal membranes have not passed. Extended use of antibiotics have proved helpful, along with IV treatment of Dextrose, NSAIDS and/or administering fluid and electrolytes into the rumen via stomach tube. In all cases, the cow is checked for any uterine or vaginal tears by palpation and given 30-50 U of oxytocin intramuscularly and drenched with 1 L of Starter plus energy drench with Ca and Mg. Antibiotic pessaries can be inserted in the uterus. Oxytocin is given 12 hourly for 48 h until entirely clear of placental membranes[*]. Manual removal of the membranes may be required but is generally not recommended for the risk of tear. Prophylactic antibiotics are given during the first week after calving to prevent septicemia in cases of retained fetal membranes. The cow should be observed for metabolic problems and mastitis.

Care of the calf at birth

In all cases animal care staff should be on hand and ready to catch the newborn calf when it is expelled (calving) or extracted from the uterus (C-section) to prevent the umbilicus from tearing too near the calf's abdomen. Newborn calves may breathe normally and lack any outward appearance or clinical signs of problems at birth. For those animals minimal care will be required. However, some animals will require more attention. Some studies have indicated that if the calf does not breathe on its own but has an auscultable heartbeat, 0.25 ml of doxapram HCl (Dopram, 20 mg/ml) is administered IV, immediately followed by another dose orally, directly under the animal's tongue. Epinephrine can also be used as a bronchodilator and as a cardiac stimulant when the heart rate is slow. Stimulation of the nasal passages and removal of fluids from around the nasal and oral cavities often helps, particularly when aspiration of amniotic fluid or meconium interferes with respiration. When respirations are not spontaneous, an endotracheal tube is passed and the calf is manually ventilated using an ambu bag. Artificial respiration is continued until the calf begins to breathe on its own, or until the calf has been determined to be

[*] The uterus is deemed to be clear of placental membranes, when there is no discharge (mal-odorous or infected fluid) from the vagina and the animal does not show any systemic signs of infection (fever etc.).

nonviable. Humidified oxygen may be supplemented via a face mask until the calf's mucous membranes become pink and the animal is breathing normally on its own. Supplemental oxygen can also be given via a nasal catheter that has been securely glued or sutured into one narus. This method will supply oxygen at a lower dose as appropriate, for example, when a calf is experiencing hypoxia but is breathing on its own. When oxygen is supplemented for greater than 30 minutes the oxygen supply should be humidified by passing the gas through a distilled water humidifier. Calves receiving continued supplemental oxygen should be weaned from it gradually by decreasing the rate over a period of time. The calf's serum glucose level should be checked rapidly (within half an hour) and IV glucose administered in a drip if needed and a broad-spectrum antibiotic treatment started immediately. Heart rate is also monitored during the first minutes of life (or more, if warranted). Manual stimulation (e.g., rubbing briskly with a towel) will increase the heart rate and should be continued to help the calf respond to its own environment. Thoracic and cardiac auscultation should be performed to detect heart murmurs. Tincture of iodine may be used on all four hooves at birth to prevent a possible spread of bacteria through the soft tissue of the hooves.

- When the calf is delivered naturally (vaginally):

A 10 % iodine solution is applied to the umbilicus and the umbilical vessels are checked for closure. The calf can be weighed once it has become steady on it legs. It should be returned as soon as possible to bond with the cow. In case of enlarged umbilicus, the umbilical cord should be clamped or resected immediately as recommended below for Caesarian section.

- When the calf is delivered by C-section:

Allowing the calf to stay for the first 1-2 minutes with head lower than the body position, while vigorously stimulating the calf by rubbing with a towel, helps the calf expel fluids in the lung and respiratory airways. It is recommended to place the calf in sternal recumbancy so as to inflate both lungs.

Calves delivered by C-section do not experience the normal stretching of the umbilical cord as a calf born by vaginal delivery. Some producers mimic the stretching of the umbilicus manually as the calf is removed during surgery. This promotes contraction of the umbilical vessels and reduces blood loss. In some cases the umbilical vessels are enlarged and will need to be clamped to prevent excessive blood loss. Sterile plastic clamps can be placed on the vessel and are removed after 3 to 7 days, after the umbilicus has dried. In cases when the umbilical structures are excessively large surgical resection (opening the abdominal cavity, separating and ligating each vein and artery and closing the abdominal cavity above the ligated vessels) of the umbilical cord is indicated in the first couple of days to minimize blood loss and to prevent infection. This surgery may be performed using local anesthesia. General anesthesia should be avoided in newborn calves.

vi. Neonatal Health Assessment and Care

Ideally the calf should be housed together with the dam. Surrogate dams that deliver prematurely by C-section will not produce colostrum at the time of calving. An alternative source of colostrum / immunoglobulins must be provided at that time. The calf is observed regularly after

birth to note the time when it stands and when it suckles. Attempt to deliver 2 L of colostrum through encouraged suckling. If there is no suckling response, colostrum is administered through a calf tube or esophageal feeder. Once the calf is able to suckle colostrum, administer 30 ml of any commercially available colostrum product (e.g., Colozen ™) orally to help calves resist infections. Some producers report also administering antibodies to *E.coli* and *Coronavirus* (e.g., First Defense ® oral bolus) prior to the first feeding. Draw blood and evaluate serum immunoglobulin (Ig) levels to ensure adequate passive transfer of immunity. The serumIg level should be >1600 mg/dl by 48 hours of age.

On the first day after birth calves are administered supplemental iron, Vitamin A, D and E and selenium by injection. Selenium is recommended particularly if area forages are low in selenium. Calves can also be given *Clostridium perfringens* antitoxin at birth.

When a calf cannot be kept with the dam, it should be fed orally with colostrum 5 times a day for the first 2-3 days and then 4 times a day. The amount of colostrum needed is about 10 % of body weight of colostrum per 24 h. From 7-10 days after birth, 3 feeds per day are recommended. Reconstituted milk powder is gradually introduced from Day 4, increasing to entirely reconstituted milk powder by Day 6. Solids are introduced in the form of roughage or chaffage as for normal hand-rearing.

Broad spectrum antibiotics should also be administered prophylactically for the first 5 days. During the first 2-3 days the animal's attitude, posture, serum glucose, and body temperature must be checked regularly (at least three times a day) to rapidly start treatment if necessary. If the calf is weak, systemic glucocorticoids (e.g., dexamethasone) can be administered rapidly after calving and once a day for the first 1-2 days.

Local disinfection of the umbilicus with diluted iodine solution or Novalsan is continued at least twice a day (more is better) for at least 5 days, until the umbilicus is dry.

vii. Correction of Neonatal Health Problems and veterinary care

The animals should be attended by a licensed veterinarian and all drugs should be administered only with the approval of the attending veterinarian. Some of the drugs mentioned in this Guide are not approved for use in cattle and their extra label use is the responsibility of the attending veterinarian.

When a calf is unable to stand on its own, pads or bolsters should be used to maintain the calf in a position of sternal recumbency. Supplemental heat may be supplied, as necessary, to maintain the animal's body temperature in the normal range. In order to avoid skin ulcers, soft bedding (e.g., sheep skin) should be used to prop the calf.

In case of prolonged health problems, the administration of ranitidine chlorhydrate (Zantac®) may be indicated to prevent abomasal ulcers.

The level of monitoring varies between teams, from complete blood counts (CBC) and biochemistry daily in some teams versus relatively low monitoring in others. In any case, under farm conditions, two analyses that can be done easily and inexpensively include: hematocrit (aka. packed cell volume, or PCV), and total protein (by refractometry). Both of these parameters are helpful in quantifying hydration, and assessing animals for onset of pulmonary edema or metabolic disorders (e.g., liver compromise in chronic infections). The white blood cell count (WBC) is also essential in assessing animals for infections, and in assessing the effectiveness of antibiotic treatments. Refractometer readings of 6.0 g/dl or greater in a normally hydrated calf are consistent with successful passive transfer.

Some common problems encountered in the neonatal period include:

Umbilical infection: A wet, un-healing umbilicus acts as a wick that promotes ascending infection (e.g., peritonitis, septic arthritis, and septicemia). This is a relatively common and serious problem in newborn calf clones, especially those delivered by C-section when the resection of the umbilical cord is not performed. Incidence rates reported by various authors range from 20-40% of newborns. Infections are most often limited and superficial, and can be managed with systemic antibiotic treatment along with topical application of iodine solution. An appropriate antibiotic is selected on the basis of culture and sensitivity of the microbial pathogen. Antibiotics reported as effective include amoxicillin / clavulanic acid, tetracycline, procaine penicillin G, cephalosporins, and trimethoprim sulpha combinations, in that order. Despite aggressive treatment, many cases will progress as bacterial pathogens enter the animal's bloodstream and seed localized infections. For example septic arthritis, referred to as "joint ill", is commonly reported as a sequel to umbilical infection in any newborn calf. While joint ill can be treated with joint lavage and systemic antibiotics, this clinical problem bears a very poor prognosis. Therefore, when the umbilicus is observed to be enlarged and oozing fluid at one week of age, surgical resection must be considered. Medical treatment (systemic antibiotics) of complicated cases has been unrewarding.

Flexor tendon contraction:

Some calves with light contraction will recover with exercise alone. Other, more severe cases must be treated as soon as possible by splinting to enable the animals to ambulate and feed. Splints can be made with lengths of PVC tubing or leg splints. Splinted calves require regular monitoring and frequent re-splinting (e.g., every 2 days) to avoid development of skin lesions. Splinted legs should receive physical therapy by manually extending and flexing each affected leg to stretch the tendons and to promote joint laxity and range of motion. An appropriate analgesic (e.g., flunixin meglumine, diclofenac, ketoprofen) is given prior to splinting to reduce discomfort and to reduce the calf's tendency to reactively flex its limbs in response to the manipulation. When the limb is contracted at the carpal joint, it can be much more difficult to achieve leg extension. Treatment with oxytetracycline for 3 days at 40 mg/kg may help loosen the tendons by binding to serum calcium, lowering serum calcium levels and reducing the tone of contracted muscles. Oxytetracycline should be given as a slow IV bolus, diluted with sterile saline. The oxytetracycline therapy works best in the first few days after parturition. Splints should be retightened during treatment. Surgical treatment, tenotomy (surgical incision of the superficial and deep flexor tendon), may be required to straighten the legs and allow splinting. If

the legs still do not straighten after performing a tenotomy, the prognosis is guarded. In some cases the flexure may be the result of contraction of the entire joint capsule or the bones of the carpus may be malformed.

Joint laxity: Laxity is sometimes found in pasterns and knee joints, and is usually associated with premature birth. The problem is self-limiting and will improve with time. When joint laxity causes the calf's pastern joint to contact the ground, a pastern splint may be used to enable the animal to ambulate on its hooves and to help strengthen and protect the leg.

Hypoglycemia: Is life threatening and can be treated with appropriate IV fluids and supplemental glucose.

Paradoxical hyperthermia : Occasionally paradoxical hyperthermia is observed without any other clinical signs. While ruling out infection (CBC and differential), animals should be swabbed with wet towels (if temperature <40°C or 104°F) or alcohol or water baths (temp >40°C or 104°F). Non-steroidal anti-inflammatory drugs (NSAIDs) may be considered to manage animals with pyrexia.

Infection: Pneumonia and diarrhea are common in young calves. Animals should be clinically monitored for problems, and they should be treated with fluids, antibiotics, and supportive care, immediately and aggressively as they occur. Milk substitute should be replaced with appropriate oral rehydration solutions until recovery.

Enlarged left ventricle, tachycardia and pulmonary edema: Suggested treatments include: diuretics (e.g., furosemide (Lasix®)), angiotensin converting enzyme (ACE) inhibitors (e.g., benazepril chlorhydrate (Fortekor®)), and bronchodilators (e.g., theophylline).

Bloating: Occurs commonly and has been treated with metoclopramide (e.g., Primperid®, Reglan®).

Internal hemorrhage: Must be detected very rapidly in order to try and treat with fluids and surgery.

viii. Criteria and Methods for Euthanasia when Health Problems cannot be corrected.

Calves that are not able to breathe (after resuscitation attempts have failed), not able to ambulate following appropriate treatments, unable to eat on their own, or are severely deformed are humanely euthanized. Decisions to euthanize a calf are made only after consultation with the attending veterinarian. Severe conditions that adversely impact the welfare of the calf and that are not resolved through appropriate medical or surgical treatment support the decision to euthanize a calf clone.

Useful references

Chavatte-Palmer, P., Constant, F., Heyman, Y., Laigre, P. (2003) Images échographiques de gestations pathologiques après clonage, exemples du syndrome du gros veau. Proceedings des Journées Nationales des Groupements Techniques Vétérinaires, Nantes.

Chavatte-Palmer, P., N. De Sousa, Laigre, P., Camous, S., Ponter, A.A., Beckers, J.F., Heyman, Y. (2006). Ultrasound fetal measurements and pregnancy associated glycoprotein secretion in early pregnancy in cattle recipients carrying somatic clones. Theriogenology **66**(4): 829-840.

Constant F., Guillomot M., Heyman Y., Vignon X., Laigre P., Servely J.L., Renard J.P., Chavatte-Palmer P. (2006) Large offspring or large placenta syndrome? Morphometric analysis of late gestation bovine placentomes from somatic nuclear transfer pregnancies complicated by hydrallantois
Biol. Reprod. **75** (1): 122-130.

El-Azab MA, Whitmore HL, Kakoma I, et. al. (1998). Evaluation of uterine environment in experimental and spontaneous bovine metritis. Theriogenology. 29: 1327.

Farin P.W., Piedrahita J.A., Farin C.E. (2006) Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology **65**(1): 178-191

Heyman, Y., P. Chavatte-Palmer, et al. (2002). "Frequency and occurrence of late-gestation losses from cattle cloned embryos." Biol. Reprod. **66**: 6-13.

Hill, J. R. and P. Chavatte-Palmer (2002). Pregnancy and neonatal care of cloned animals. Principles of Cloning. J. Cibelli. San Diego, Academic Press**:** 247-266.

Panarace, M., J. I. Aguero, et al. (2007). "How healthy are clones and their progeny: 5 years of field experience." Theriogenology. 67: 142-151

Young L. E.; Sinclair K. D.; Wilmut I. (1998) Large offspring syndrome in cattle and sheep. Reviews of Reproduction **3**(3): 155-163

B. RECOMMENDATIONS FOR THE VETERINARY CARE OF SWINE CLONES

General Comment: Except in rare cases when high value neonatal animals may receive extra help for a short period of time, all aspects of clone health assessment and care are the same as would be performed for artificial insemination (AI), or any other means of natural or assisted reproduction practices.

i. Selection and Conditioning of Surrogates (pre-implantation up to and including implantation)

Surrogates are selected and conditioned in exactly the same manner as per any standard embryo transfer procedure at the farm. Surrogates are mostly gilts that have cycled at least 2-3 times. Sows have also been used successfully as surrogates and may be the preferred choice for non-surgical transfer protocols. Surrogates are synchronized using a combination of synthetic progesterone (e.g., Matrix®) and human Chorionic Gonadotropin (HCG). The reproductive tract of the surrogate is evaluated (number of ovulations, corpus luteum (CL), cysts, adhesions, etc.) prior to embryo transfer (ET). Surrogates with cystic ovaries or surrogates in the wrong stage of the estrous cycle will not yield pregnancies. Embryos are typically implanted into the surrogate's oviduct via surgical mid-ventral laparotomy. The number of embryos utilized varies according to protocol.

ii. Assessment of Surrogate Health (post-implantation) including criteria for terminating pregnancy

Recipient animals are evaluated following embryo ET surgery for the following:

- 1-2 hrs post ET – stable vital signs (temperature, respiration)
- Day 1 post ET – eating, signs of infection, incision site appearance, swelling, drainage, dehiscence, etc.

Daily clinical assessments of the suture site and animal disposition continue for at least four weeks. Surrogates are checked for pregnancy status starting 28 days following ET and weekly thereafter. Pregnancy status can be checked by several methods, including visual signs of return to estrus, or by ultrasound. The Aloka 500 ultrasound machine provides goods results yielding a visual description of the piglet(s) and information about litter size. Pregnancies are terminated in cases in which the sow's health is in jeopardy. To date there are no reported cases of terminated pregnancies for this reason.

iii. Assessment of Clone Condition (pre-partum)

Weekly pregnancy checks are performed via rectal ultrasound. Initial ultrasound imaging is performed at day 28 post ET. The fetal dimensions, heart rates, and number are recorded. Most pregnancy losses occur between day 35-45, and a second smaller wave of losses occurs day 60-70. Pregnancies of 1-12 clone piglets have been observed to go to term with an average of 4-6 piglets per litter.

iv. Delivering Clones, Criteria for Selection of C-section versus Natural (Vaginal) Birth

With the exception of more frequent and stringent monitoring, farrowing is identical to standard practices with natural (vaginal) delivery as the preferred method. Two weeks prior to the estimated delivery date (day 114) the animal is moved to the farrowing barn. The normal gestation period is estimated to be between days 114-122, depending on the cell line. Animals are checked for milk letdown and vulvar dilation at least twice daily. The size of the fetus and fetal heart rate are checked daily via ultrasound. If the fetal size is too large or fetal heart rates exceed 190-200 bpm the animal is induced to farrow. Induction consists of a first set of injections: cloprostenol, 250µg/mL (Estrumate®) (2 ml IM) and dexamethasone (12 ml IM) followed by a second injection of cloprostenol (1 ml) 24 hrs later. Pigs will farrow within 48 hrs of first injection. Oxytocin may be administered at farrowing to induce uterine contractions. Piglets may be manually pulled if needed. If the pregnant animal is in full labor and her cervix is not fully dilated or she has not passed any piglets within 48 hours of induction, then Cesarean section is indicated. The animals are re-checked as many times as needed during the birthing process.

v. Neonatal Health Assessment and Care

Newborn piglets are toweled dry and their umbilicus is dipped in an iodine solution shortly after birth. At least one heat lamp may be needed to be placed in the farrowing pen to keep the piglets warm. A supplemental source of heat (e.g., water-circulating heating blanket) is also recommended during the winter months. Piglets should be monitored to make sure they have nursed successfully. Colostrum feeding within the first hour of life is important to ensure sufficient passive immunity from pathogens. During the first days of life piglets are injected with iron, vitamins, and antibiotics if needed. Ear notching and tail docking are done at this time. Vaccinations are administered according to farm herd health status. Piglets are weaned at 4-5 weeks of age to allow for optimum health and growth.

vi. Correction of Neonatal Health Problems

In those rare cases when neonatal problems occur, in some the health issues cannot be overcome, in which case the animals are humanely euthanized. By providing supportive clinical care a proportion of poor-doing neonates can be helped through critical stages and saved. Small and poor-doing animals may be placed in an incubator for a period of time, and injections of dextrose (IP) or stomach tube feeding provide energy to help piglets gain strength. As these animals recover they become able to nurse from their mother and are ultimately placed back with littermates. High value animals can be sent to veterinary hospitals and kept in intensive care units for a period of time for supportive care or antibiotic therapy.

vii. Criteria and Methods for Euthanasia when Health Problems cannot be corrected.

Piglets that are not able to eat or breathe on their own or are severely deformed are humanely euthanized. Piglets are not kept alive through extraordinary measures if it is not humane to do so. Approved standard protocols for humane euthanasia of piglets are used when required.

C. RECOMMENDATIONS FOR THE VETERINARY CARE OF GOAT CLONES

All aspects of clone health assessment and care are the same as are performed for artificial insemination (AI), or any other means of naturally or artificially bred animals.

i. Selection and Conditioning of Surrogates (pre-implantation up to and including implantation)

Young does that have already successfully kidded are selected and conditioned. This eliminates any problems that might be associated with a first pregnancy and parturition. Recipients are selected for implantation when their natural estrus corresponds with that of a donor female. Natural estrus is preferred over induced estrus because does in natural estrus tend to produce a superior corpus luteum (CL) that is less likely to regress prematurely as is sometimes the case when animals are hormonally manipulated. When hormones are used to synchronize donors and recipients, and no good CL can be seen, laparoscopy is performed to visually confirm the status of the ovary prior to surgical implantation of embryos. Does with uterine adhesions are not used as recipients. To minimize the risk of developing uterine adhesions, no animal is subjected to more than three embryo transfer procedures, and animals are given ample time to recover completely before being re-used.

ii. Assessment of Surrogate Health (post-implantation) including criteria for terminating pregnancy

Goat embryos are surgically transferred into oviducts of surrogate dams at the 1 to 4 cell stage. This is a major difference between cow and goat embryo transfer since cow embryos are cultured *in vitro* to the morula/blastocyst stage and transferred transvaginally making surgical implantation unnecessary. A total of 4 to 6 embryos are transferred to each recipient. All pregnancies are monitored weekly, by ultrasound imaging, beginning 28 days following the embryo transfer procedure.

iii. Assessment of Clone Condition (pre-partum)

All pregnancies are monitored weekly, by ultrasound imaging, beginning 28 days following the embryo transfer procedure. Fetal dimensions, heart rates, and number are recorded. In rare cases placental or fetal abnormalities necessitate pregnancy termination by induction of parturition using dexamethasone and prostaglandin F2 alpha (PGF2α). Abnormalities observed to date include large placentomes, hydrops allantois, hydrops amnios (enlarged umbilical cord, hydronephrosis, enlarged heart), or fetal death. A dead fetus is delivered by induced parturition in cases of singleton, but carried to term in cases of twins and triplets. Developing fetuses are evaluated for stress during late pregnancy. When stress is observed, and the pregnancy is beyond 143 days Cesearean section is indicated.

iv. Delivering Clones, Criteria for Selection of C-section versus Natural (Vaginal) Birth

The normal gestation period is estimated between 145-155 days. When no abnormalities are observed through clinical examination or ultrasound imaging dams are allowed to kid naturally (vaginally). In rare cases when there is evidence of placental insufficiency, parturition is induced with dexamethasone and PGF2α, and Cesearean section is performed 24 hours later.

v. Neonatal Health Assessment and Care

A normal goat kid is able to hold its head up immediately after birth and vocalize. Kids normally start trying to stand 10-20 minutes after birth. Umbilical cords are clipped to 5 cm in length, clamped with a hemostat, and dipped in either tincture of iodine or chlorhexidine solution. Kids are separated from the dam and kept in a warm environment for the first 24 hours. Colostrum that has been heat-treated (to prevent transmission of Mycoplasma and Caprine Arthritis-Encephalitis virus) and stored frozen is thawed, warmed, and bottle fed according to weight (5 feedings in the first 24 hours), making sure that kids receive the first colostrum within an hour of birth.

vi. Correction of Neonatal Health Problems

Newborn goat kids are assessed for respiration and heart beat. When a newborn kid is not breathing attempt to clear the airway using suction, or lowering the head down to drain the fluids. Fluid in the airways has been observed in goat clones. When the airway is cleared, if that animal does not begin to breathe spontaneously, introduce a pediatric endotracheal tube and inflate the lungs using positive pressure ventilation. Other approaches have been used in different studies e.g. Doxapram hydrochloride (Dopram) is administered orally, sublingually, to attempt to stimulate respiration, and oxygen may be supplemented. In cases when the heart beat is slow or weak, atropine can be administered IM. In cases of cardiac arrest epinephrine is administered either via the endotracheal tube, or intracardiac injection.

vii. Criteria and Methods for Euthanasia when Health Problems cannot be corrected.

A newborn goat kid that is not able to breathe or eat on its own is humanely euthanized. When an animal exhibits a strong heart beat it may often be salvaged using artificial respiration (e.g., ambu bag with face mask, endotracheal tube with ambu bag / oxygen, gentle rib compression). When there are severe respiratory problems cardiac arrest often follows, so euthanasia becomes unnecessary. In those cases that exhibit severe congenital anomalies (e.g., atresia ani, schistosoma reflexus, co-joined twins, etc.) humane euthanasia is indicated (e.g., sodium pentobarbital (Beauthanasia®) i.v., or i.c.).

D. RECOMMENDATIONS FOR THE VETERINARY CARE OF SHEEP CLONES

Summary

When livestock are cloned from embryonic or somatic cells, an increased standard of care is required to initiate and maintain pregnancies and then to produce viable offspring. This high standard of care will then complement the rigorous quality control required by the nuclear transfer laboratory. Selection and care of ewes at all stages of the cloning process is critical to achieve good outcomes in terms of results and animal welfare.

The welfare of the recipient ewes and their valuable fetuses remains a high priority throughout pregnancy and after birth. If the lamb clones survive the transition out of the *in utero* environment, they then face a higher incidence of disease and structural abnormalities that challenge their ability to thrive.

Much lower efficiencies in producing viable offspring from a clone embryo transfer program compared with results from natural service or artificial breeding (AI or ET) are expected and these are illustrated by reductions in:

- Pregnancy rates at Day 30
- Embryo survival from Day 30-60
- Fetal survival to term
- Neonatal viability.

As abnormalities are expected in clone pregnancies and in neonatal clones, it is *extremely valuable to generate control pregnancies* (non-clones) so that pregnant ewes and then lambs are available at similar stages of gestation or ages for comparison of clones against controls.

The standard of animal care and management required exceeds that expected from standard breeding programs. These skills are in:

i. Selection and Conditioning of Recipient Oocytes
ii. Selection and Conditioning of Surrogates (pre-implantation up to and including implantation)
iii. Assessment of Surrogate Health (post-implantation) including criteria for terminating pregnancy
iv. Assessment of Clone Condition (pre-partum)
v. Delivering Clones, Criteria for Selection of C-section versus Natural (vaginal) Birth
vi. Neonatal Health Assessment and Care
vii. Correction of Neonatal Health Problems
viii. Criteria and Methods for Euthanasia when Health Problems cannot be corrected.

i. Selection and Conditioning of Recipient Oocytes

The cloning process begins with high quality oocytes. These are obtained from either abattoir ovaries or from super ovulated donors. Following construction of nuclear transfer embryos, short term culture to blastocyst occurs either in vitro or in the oviduct of temporary recipient ewes. Selection of highest quality blastocysts then occurs prior to transfer of embryos into the recipient ewe.

ii. Selection and Conditioning of Surrogates (pre-implantation up to and including implantation)

Key skills required

- Animal husbandry
- Estrous synchronization
- Ultrasonography

Recipient preparation

For optimal results from embryo transfer (ET) programs, recipient ewes should be maintained on a consistent plane of nutrition for the 1-2 months prior to ET with a goal of achieving body condition score of 3 (out of 5) at time of transfer, i.e., neither too fat nor too thin. Ewes should be acclimatized to feeding and handling methods in the weeks prior to transfer. Their frame and body size should be adequate to cope with larger than normal lambs during gestation and at birth. Birth weights for lamb clones have a wider range than for nonclone births (1.9-11kg) and tend to be heavier than normal [1-4].

The number of embryos transferred will determine the proportion of multiple pregnancies. In general 1 or 2 embryos are transferred into each recipient ewe. Ultrasonographic determination of fetal numbers and fetal size will greatly assist nutritional management of ewes during pregnancy. It is vital that body condition and energy balance are maintained particularly in the third trimester of pregnancy when pregnancy toxemia (ketosis) may occur. Multiple lambs or large single lambs are prime risk factors for pregnancy toxemia.

Health status

Select recipient ewes from flocks of known incidence for abortion. Many infectious causes of abortion in small ruminants are dangerous to people. Although non-infectious causes of abortions are far more likely than infectious causes in pregnancies with clones, care should be taken that aborting ewes or does are separated from the rest of the flock. The most common infectious causes of abortion in sheep are Toxoplasmosis, Chlamydiosis, Campylobacteriosis and Q-fever (*Coxiella burnetii).* Recipient ewes should be vaccinated against infectious abortion, pasteurella and clostridial infections. Consideration should be given to vaccinating them with erysipelothrix and E.coli to afford protection to the lamb via colostrum against neonatal infections. *NB Availability of these vaccines may vary nationally.

Synchronization of estrus and embryo transfer

Recipients are synchronized as per standard procedures using a progestagen intravaginal implant (sponge or CIDR) for 12-14 days together with an injection (250-400 iu) of equine chorionic gonadotropin (eCG or PMSG) at the time of implant withdrawal. Transfer of embryos into the uterus is accomplished via laparoscopic assistance or via laparotomy. The ewes are under general anesthesia (intravenous or inhalation) or heavy sedation (e.g., xylazine) plus local anaesthetic infiltration. The surgical site is prepared on the belly (clipped and sterilized), the laparoscope is used to view the uterine horns and identify the horn ipsilateral to a corpus luteum. Embryo transfer is accomplished laparoscopically or by exteriorization of the horn through a small skin incision. Once the embryo(s) have been transferred into the uterine lumen, the body wall incisions are closed and antibiotics/analgesics administered. Recipient ewes should return to their familiar environment with minimal handling and interventions for the next 3 weeks.

Pregnancy diagnosis

Initial pregnancy diagnosis using transrectal or transabdominal ultrasonography can be performed from around Day 25 of pregnancy. Confirmation of embryonic viability is by observation of fetal heartbeat. Following transfer of clone embryos, Day 30 pregnancy rates are highly variable but usually range from 30-60% per recipient (2 embryos transferred) which corresponds to a 15-30% per embryo transferred[5]. After this initial pregnancy diagnosis, embryonic losses vary, but may be up to 50% [3, 6-13]. The fetuses that are destined to die become progressively more undersized for their age but it is not easy at this stage to predict which fetuses are destined to die. A reasonable rate for ultrasonography is every 7 days, beginning from Day 25-60 then every 2 weeks to term.

Embryonic death in the first 60 days will usually result in gradual resorption of the embryo and placenta. Evacuation of uterine contents and return to estrus can be hastened by administration of prostaglandins (5mg $PGF_{2\alpha}$ or 100 μg cloprostenol) to the ewe once fetal death has been confirmed.

iii. Assessment of Surrogate Health (post-implantation) including criteria for terminating pregnancy

Key skills required

- Gestational monitoring via ultrasononography
- Skilled assessment of pregnancy abnormalities – fetal and maternal
- Prevention of maternal metabolic diseases.

Pregnancy monitoring

During the second and third trimesters there are sporadic losses of clone fetuses, often accompanied by the development of major placental abnormalities such as hydroallantois. The focus is therefore on assessment of maternal health via *changes* in feed intake, body weight, and condition score and via ultrasonography of the fetus(es) and their placenta.

Diagnosis of hydrallantois is difficult in the early stages as the initial increase in fluid volume is gradual. However, by weekly monitoring and comparison with other pregnant ewes, the increased abdominal size will become apparent. When in doubt, further investigations are warranted (e.g., close analysis of feed intake, respiration, heart rate, abdominal circumference). I*t is extremely valuable to have control pregnancies (non-cloned,) at a similar stage of gestation, available for comparison in order to detect any abnormalities that occur.*.

The risk of maternal morbidity and mortality is higher for clone than conventional pregnancies. This risk results from late gestational fetal loss, increased size of the fetus, abnormal placentation, pregnancy toxemia, and most notably, hydrallantois or hydramnios. Excessive fetal fluids are often associated with fetal abnormalities, and, when severe, with maternal distress.

Expected gestational abnormalities associated with cloning

- Fetus: Increased embryonic and fetal losses, fetal death, abortion, oversized fetuses
- Placenta: Structural placental changes (as observed in cattle) leading to reduced placental efficiency and hydrallantois
- Dam: Long gestation length, short gestation length, pregnancy toxemia, retained placenta

Preventive actions to maintain surrogate health

- Monitor feed intake, abdominal circumference, body condition, body weight
- Monitor fetal viability, fetal size, placentome size, placental fluid accumulation
- Increase quantity, quality (energy content) of feed available for the last half of pregnancy

Criteria for terminating pregnancy

- Fetal death
- Hydrallantois where increasing placental fluids result in the dam becoming recumbent, respiration is labored and feed intake is markedly reduced.
- Pregnancy toxemia where the dam is recumbent, feed intake is markedly reduced.

Recipient health

Ewes should be weighed and condition scored weekly. Abdominal circumference can be measured as an aid to diagnosing hydrallantois. Feed intake is a sensitive indicator of maternal health. In pasture fed animals, the measurement of feed intake is subjective and relies on careful observation of behavior. This is easier to assess where pasture fed animals are fed supplemental grain, pellets or hay.

Measurement of urinary ketones will reveal the onset of pregnancy toxemia. Many ewes carrying multiple lambs will have a degree of ketonuria in the last few weeks of pregnancy – an indication that subclinical ketosis can progress if feed intake is restricted by feed quantity or quality.

If hydrallantois develops, feed intake is reduced due to rumen displacement. Special care needs to be taken with these ewes to ensure they are receiving a high energy ration. These animals are difficult to manage and require constant skilled monitoring and veterinary treatment. Where hydrallantois develops assessment of severity and speed of onset will determine whether the ewe will be nursed to term, the pregnancy terminated or, in severe cases the ewe is euthanized. These decisions are based on the health of the ewe (feed intake, changes in abdominal circumference,

body weight, management of ketosis), fetal viability (heart rate, size) and placental measurements (quantity of placental fluid, onset of placental edema).

In third trimester clone pregnancies, placental abnormalities such as edema and hydrallantois may occur at a similar rate to that seen in co-cultured IVF generated fetuses (Hill et al., 1999; Sinclair et al., 1999). A decreased number of enlarged placentomes may be observed indirectly using transabdominal and transrectal ultrasonography or directly following birth. The reduction in placentome numbers may not harm fetal viability if the total surface area for nutrient exchange remains constant by hypertrophy of the remaining placentomes (Bazer et al, 1979; Hill et al, 2001). As a result of incomplete placental development, a delicate balance exists between the capacity of the placenta to supply nutrients and the demands of a rapidly growing fetus.

Pregnancy termination is by administration of prostaglandins and or dexamethasone. A viable fetus is needed for dexamethasone to work. A cesarean section can also be performed to recover the fetus. Pregnancy termination by either method carries a higher risk of retained fetal membranes which can result in endometritis and toxemia.

Detection of high risk pregnancies

High risk pregnancies can be identified by external examination and clinical observations in conjunction with ultrasonographic imaging (e.g., abnormal edematous placentation, increased allantoic fluid) and clinical tests (e.g., urinary ketones). The presence of placental edema or excessive allantoic fluid often indicates a poor outlook for the fetus.

Monitoring of body weight, feed intake, abdominal circumference, heart/respiratory rate, body temperature provides valuable data to assess the health of the recipient animal. The presence of ketonuria is not necessarily a bad prognostic sign, but it does warn of potential for trouble. Significant reduction in feed intake or large changes in body weight (rapid gain indicates hydrallantois) are important signs.

iv. Assessment of Clone Condition (pre-partum)

Key skills required

- Fetal measurement by transabdominal ultrasononography
- Skilled assessment of pregnancy abnormalities – fetal and maternal

Placental pathology

In conventionally conceived animals, early fetal losses may be due to abnormalities of the embryo or its placenta, alterations in maternal uterine environment or feto-maternal interactions (Wilmut et al., 1986). However, first trimester clone pregnancies display a wide variety of placental morphologies [14-16]. In sheep pregnancies with clones, De Sousa et al. observed fetal (enlarged livers, dermal hemorrhages) and placental (poor vascularization and cotyledon development) abnormalities.

Fetal viability

A healthy, normal fetus clone has fetal and placental measurements within the normal range – i.e., a predicted birth weight of 3-5kg, normal number (50-80) and size (2-3 cm) of placentomes with normal quantities of allantoic and amniotic fluids. This can be assessed pre-term via ultrasonographic measurements of the fetus and placenta. Heart rates vary during pregnancy: Day 30-60 (~200 bpm), Day 120 (160 bpm). Prediction of fetal size is via crown rump length, crown-nose length, femur length and abdominal circumference. All of these measurements require a very high standard of ultrasonography. The most practicable test is for heart rate – observation of low heart rates (<100 bpm) imply poor fetal viability.

Maternal health

Although at present there is no useful therapy for improving the prognosis for severe cases of hydrallantois, less severe cases may be nursed through gestation by paying careful attention to the feed intake and metabolic status of the dam. Ruminants in particular are very prone to metabolic upsets (ketosis and fatty liver) due to the increased uterine volume and reduced rumen capacity that accompany hydrallantois. A differential diagnosis for enlarged abdomen is always multiple fetuses when multiple embryos have been transferred. Rapidly growing fetus clones may easily cause metabolic problems in ruminants and regular monitoring of urinary ketones will allow early intervention. The later the onset of the pathology the more likely the fetus may reach term. In severe cases, however, the survival of the offspring is most often compromised. Poorly viable third trimester fetuses may have significant circulatory abnormalities (hypoxia, liver congestion, placental edema).

Fetal lesions previously observed at necropsy (see Table 1)

Together with hydrallantois, fetal lesions including omphalocele, ascites, cardiac enlargement, liver steatosis and asynchronous growth of organs have been observed. Detailed observations from the Roslin Instiute illustrate the wide variety of major structural defects observed in lamb clones that died at or just afterbirth [17, 18]. Prominent systems affected were renal (e.g., massive bilateral hydronephrosis), cardiac (e.g., right ventricular hypertrophy), pulmonary (severe pulmonary hypertension), hepatic (few bile ducts) and musculoskeletal (contracted tendons).

v. Delivering Clones, Criteria for Selection of C-section versus Natural (vaginal) Birth

Key skills required:

- High standard of obstetrical care – for Cesarean or vaginal birth
- Knowledge of parturition induction

Induction: Selecting the day of birth

Many clone-bearing pregnancies progress beyond their due date. This may not be a problem in itself and may suggest that clones require more time *in utero* for full maturation. However prolonged gestations are usually associated with increased birth weight and increased risk of

dystocia. Rapidly increasing fetal demands, possibly associated with inadequate nutrient supply from a suboptimal placenta argue against letting the gestation go beyond term [19].

Thus, induction is commonly performed around the anticipated due date [8, 20-22]). Induction also helps to ensure that birth occurs when there is maximum skilled assistance available. Induction protocols utilizing either a single injection of dexamethasone or prostaglandin $F_{2\alpha}$ 24 hrs prior to cesarean section have been shown to induce final pulmonary maturation in bovine fetuses prior to birth [23]. This promotes production of lung surfactant that is critical to permit lung inflation. However it has become apparent that when routine induction protocols are used for cloned pregnancies poor lung maturation may still occur. It is unclear if this is the result of the induction protocol or from abnormal physiology of the fetus clone fetus or placenta.

Induced parturition follows the protocols developed in sheep as a model for inducing pulmonary maturity in premature human infants [24]. Administer Dexamethasone trimethylacetate (5mg) (TMA), 9 days before predicted birth date, followed by dexamethasone phosphate (5mg) 7 days later. For example this would mean injecting dexamethasone TMA on Day 138, dexamethasone phosphate on Day 145, with birth expected around 2-3 days later on Days 147-148. Round the clock monitoring from Day 135 is absolutely vital as the ewes can still lamb early! Betamethasone is an expensive, but very good, alternative to dexamethasone [25].

Cesarean or natural (vaginal) birth

A variety of pathologies have been described in clones (Table 2). Neonatal clones are at risk of being incompletely prepared for the critical transition to breathing room air. The most controlled method of delivery for the neonate is usually by cesarean section. However vaginal delivery remains the preferred option if there is skilled assistance available and the neonate is known to be of normal size and has a very high likelihood of passing quickly through the pelvic canal. Vaginal delivery should not be attempted with very large fetuses which will not pass easily through the pelvic canal.

Where cesarean section is necessary, it should ideally begin during the 2^{nd} stage labor, when the cervix is fully dilated. Waiting until second stage labor gives the fetus the maximum opportunity to be ready for birth. Take care to ligate the umbilicus prior to its tearing during removal of the lamb from the uterus. The umbilicus is often enlarged in clones and when broken the 2 umbilical arteries may not constrict. If the cord is not clamped, blood loss may be life threatening. Surgical removal of an enlarged abnormal umbilical stump immediately following delivery may also be necessary to avoid hemorrhagic and infectious incidents.

Retained placenta may occur, particularly in cases of hydrallantois. Resultant metritis should be treated with systemic antibiotics, anti-inflammatory drugs, and uterine stimulants (oxytocin, prostaglandins).

vi. Neonatal Health Assessment and Care

Key skills required:

- Neonatal and post natal care
- neonatal resuscitation
- medical or surgical procedures
- Early detection of disease or abnormal development from birth to weaning then to adult

Management of high risk neonatal clones

Lamb clones are generally less vigorous at birth. Without supportive care this lack of viability can rapidly result in mortality. Unlike the birth of conventionally conceived lambs, management of neonatal clones should be proactive. As the majority of clones will be less viable, it is better to over treat the neonates than try to play catch up after many have become hypoxic and acidotic in the first few hours after birth.

In general, the majority of lamb clones should receive treatments normally reserved for high-risk neonates [19, 26, 27]. This may be modified according to clinical experience. Over half of the neonatal clones will need extra care for the first 1-5 days of life. They may be hypoxic, acidotic, lacking in vigor, or have a weak suckle reflex. To prevent these already compromised neonates from spiraling into respiratory and cardiovascular collapse, provide ventilation support (supplemental oxygen) immediately after birth. Administer oxygen by face mask as soon as lambs are born. If higher concentrations of oxygen are required, insert an endotracheal tube and gently provide positive-pressure ventilation using an ambu bag. A nasal catheter can be inserted for longer term supplemental oxygen administration. In most cases, oxygen therapy should be continued for at least 1 hour. The safest option is to maintain every lamb clone on 0_2 for the first 24 hrs.

When possible arterial blood gases or oxygen partial pressure (pulse oximeter) should be measured within 15 min after birth and, ideally, should be monitored at least hourly until satisfactory 0_2, $C0_2$, $HC0_3$, and pH levels have been achieved. A conservative approach is to continue intensive care unless the lamb has made very vigorous efforts to rise, has successfully stood on its own, has a very vigorous suckle reflex and has an arterial blood 0_2 >60 mmHg, $C0_2$ <50 mmHg and pH of > 7.3.

This preventative therapy is aimed at preventing or reducing the effects of hypoxic pulmonary hypertension which is the equivalent of persistent fetal circulation. Persistent pulmonary hypertension of the newborn is characterized by sustained elevations of pulmonary vascular resistance after birth, leading to right-to-left shunting of blood across the ductus arteriosus or foramen ovale and resulting in severe hypoxemia. Therapies aimed at improving pulmonary function include oxygen, positive pressure ventilation, bronchodilators, pulmonary arterial vasodilators, and pulmonary surfactant. They should be used when one suspects pulmonary hypertension from the clinical examination.

Additional supportive therapy includes maintenance of body temperature (which may rapidly drop or increase), precautionary antibiotics (e.g., penicillin, aminoglycosides- not approved for animals that will enter the food chain), colostrum fed by bottle or nasogastric tube, iv fluids, glucose and possibly serum transfusion to provide an immediate source of antibodies. Colostrum should preferably be fed by bottle although suckling strength is often inadequate.

vii. Correction of Neonatal Health Problems

Postnatal monitoring

Lamb clones that progress through the early neonatal period have a high rate of survival. Depending on management conditions, these animals may be challenged by a variety of environmental pathogens that may produce septicemia, gastrointestinal or respiratory disease. There are published reports of diseases, occurring in the post-neonatal period, thought to be related to the cloning procedure (e.g., blood cell aplasia, cardiac disease) [20, 28, 29]. In the authors' experience there appears to be a higher than expected incidence of gastrointestinal disease as well. Infection of the umbilical stump also occurs in lamb clones. Plasma cortisol levels have been shown to be significantly elevated in neonatal lamb clones [2]. It is therefore prudent to routinely and methodically evaluate each body system for abnormalities at least for the first month after birth.

viii. Criteria and Methods for Euthanasia when Health Problems cannot be corrected.

Key skills required:

- Clinical assessment of viability of the lamb clones
- Humane euthanasia
- Pathology
- Detailed necropsies
- Diagnostic sample collection

Euthanasia is considered after indicated treatment options have been explored and response to treatment has not occurred. Severe respiratory distress and bacterial infections are the major problems occurring in the 2 weeks after birth that may lead to the decision to euthanize a lamb clone. Reported survival rates for neonatal lamb clones (non-transgenic) range from 17-87% [3, 6-9, 12] . Euthanasia of lamb clones or their dams is least traumatically performed by overdose of an injectable barbiturate (e.g., pentabarbitone) administered intravenously.

Table 1. Body systems in which abnormalities have occurred in neonate clones .

System	Abnormality
Respiratory	Surfactant deficiency.
	Meconium aspiration.
	Pneumonia
Cardiovascular	Pulmonary hypertension
	Ventricular hypertrophy
	Enlarged umbilicus
	Septicemia
Hemopoietic	Immunodeficiency
	Anemia
Metabolism	Hypoglycemia
	Diabetes
	Obesity
	Idiopathic hyperthermia
Gastrointestinal	Gastritis/enteritis
Musculoskeletal	Contracted tendons.
	Oversized
	Joint infection
Umblicus	Patent Umblicus
	Umblical infections (Navel Ill)
Reproductive	Placentation:
	Hydroallantois and/or edema.
	Reduced number of placentomes
	Enlarged placentomes
	Overweight placenta
Endocrine	Delayed or absent signs of parturition.
	Low postnatal milk production.
Urinary	Hydronephrosis

References

1. Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 1997; 278: 2130-2133.

2. Edwards L, Peura T, Hartwich K, Rudiger S, McMillen IC, Walker S. Postnatal growth and circulating ACTH and cortisol concentrations during the first month of life in cloned lambs. Endocrinology 2002; 143: 3699-3702.

3. Loi P, Clinton M, Barboni B, Fulka J, Jr., Cappai P, Feil R, Moor RM, Ptak G. Nuclei of nonviable ovine somatic cells develop into lambs after nuclear transplantation. Biol.Reprod. 2002; 67: 126-132.

4. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat.Genet 2001; 27: 153-154.

5. Peura TT, Vajta G. A comparison of established and new approaches in ovine and bovine nuclear transfer. Cloning Stem Cells 2003; 5: 257-277.
6. Wilmut I, Schnieke AE, McWhir J, Kind KL, Campbell KHS. Viable offspring derived from fetal and adult mammalian cells. Nature 1997; 385: 810-813.
7. Wells DN, Misica PM, Day TA, Tervit HR. Production of cloned lambs from an established embryonic cell line: a comparison between in vivo- and in vitro-matured cytoplasts. Biol Reprod 1997; 57: 385-393.
8. Peura TT, Hartwich KM, Hamilton HM, Walker SK. No differences in sheep somatic cell nuclear transfer outcomes using serum-starved or actively growing donor granulosa cells. Reprod Fertil Dev 2003; 15: 157-165.
9. Campbell KH, McWhir J, Ritchie WA, Wilmut I. Sheep cloned by nuclear transfer from a cultured cell line. Nature 1996; 380: 64-66.
10. McCreath KJ, Howcroft J, Campbell KH, Colman A, Schnieke AE, Kind AJ. Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 2000; 405: 1066-1069.
11. Denning C, Burl S, Ainslie A, Bracken J, Dinnyes A, Fletcher J, King T, Ritchie M, Ritchie WA, Rollo M, De Sousa P, Travers A, Wilmut I, Clark AJ. Deletion of the alpha(1,3)galactosyl transferase (GGTA1) gene and the prion protein (PrP) gene in sheep. Nat.Biotechnol 2001; 19: 559-562.
12. Peura TT, Kleemann DO, Rudiger SR, Nattrass GS, McLaughlan CJ, Walker SK. Effect of nutrition of oocyte donor on the outcomes of somatic cell nuclear transfer in the sheep. Biol.Reprod. 2003; 68: 45-50.
13. Ritchie WA, Taylor JE, Gardner JO, Wilmut I, Carlisle A, Neil C, King T, Whitelaw CB. Live lambs born from zona-pellucida denuded embryos. Cloning Stem Cells 2005; 7: 178-182.
14. Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, Thompson JA, Winger QA, Westhusin ME. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol.Reprod 2000; 63: 1787-1794.
15. De Sousa PA, King T, Harkness L, Young LE, Walker SK, Wilmut I. Evaluation of gestational deficiencies in cloned sheep fetuses and placentae. Biol Reprod 2001; 65: 23-30.
16. Wells DN, Misica PM, Tervit HR. Production of Cloned Calves Following Nuclear Transfer with Cultured Adult Mural Granulosa Cells. Biol.Reprod. 1999; 60: 996-1005.
17. Rhind SM, King TJ, Harkness LM, Bellamy C, Wallace W, DeSousa P, Wilmut I. Cloned lambs--lessons from pathology. Nat.Biotechnol 2003; 21: 744-745.
18. Dawson AJ, King TJ, Wilmut I, Harkness LM, Kelly BG, Rhind SM. Immunohistochemical characterization of cloned lamb nephropathy. J Histochem Cytochem 2004; 52: 1657-1664.
19. Hill JR, Chavatte-Palmer P, Cibelli JB, Lanza RP, Campbell KHS, West MD. Pregnancy and neonatal care of cloned animals. In: Principles of Cloning, vol. 1. San Diego: Academic Press; 2002: 247-266.
20. Hill JR, Roussel AJ, Cibelli JB, Edwards JF, Hooper NL, Miller MW, Thompson JA, Looney CR, Westhusin ME, Robl JM, Stice SL. Clinical and pathologic features of cloned transgenic calves and fetuses (13 case studies). Theriogenology 1999; 51: 1451-1465.
21. Wells DN. Cloning in livestock agriculture. Reprod Suppl 2003; 61:131-50.: 131-150.
22. Chavatte-Palmer P, Heyman Y, Renard JP. Cloning and associated physiopathology of gestation. Gynecol.Obstet.Fertil 2000; 28: 633-642.

23. Zaremba W, Grunert E, Aurich JE. Prophylaxis of respiratory distress syndrome in premature calves by administration of dexamethasone or a prostaglandin f-2-alpha analogue to their dams before parturition. American Journal of Veterinary Research 1997; 58: 404-407.
24. Liggins GC. Premature delivery of foetal lambs infused with glucocorticoids. J Endocrinol. 1969; 45: 515-523.
25. Ikegami M, Jobe AH, Newnham J, Polk DH, Willet KE, Sly P. - Repetitive prenatal glucocorticoids improve lung function and decrease growth in preterm lambs. Am J Respir Crit Care Med 1997; 156: 178-184.
26. Martens RJ. Neonatal respiratory distress: A review with particular emphasis on the horse. Compendium on Continuing Education 1982; 4: S23-S33.
27. Whitsett JA, Pryhuber GS, Rice WR, Warner BB, Wert SE, Avery GB, Fletcher MA, MacDonald MG. Acute Respiratory Disorders. In: Neonatology. Pathophysiology and Management of the Newborn, vol. Fifth Edition. Philadelphia: Lippincott Williams & Wilkins; 2000: 485-501.
28. Renard JP, Chastant S, Chesne P, Richard C, Marchal J, Cordonnier N, Chavette P, Vignon X. Lymphoid hypoplasia and somatic cloning. The Lancet 1999; 353: 1489-1491.
29. Chavatte-Palmer P, Heyman Y, Richard C, Monget P, LeBourhis D, Kann G, Chilliard Y, Vignon X, Renard JP. Clinical, hormonal, and hematologic characteristics of bovine calves derived from nuclei from somatic cells. Biol Reprod 2002; 66: 1596-1603.