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Delivery of cloned offspring: experience in Zebu cattle (*Bos indicus*)

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Abstract. The production of a healthy cloned calf is dependent on a multitude of successful steps, including reprogramming mediated by the oocyte, the development of a functional placenta, adequate maternal–fetal interaction, the establishment of a physiological metabolic setting and the formation of a complete set of well-differentiated cells that will eventually result in well-characterised and fully competent tissues and organs. Although the efficiency of nuclear transfer has improved significantly since the first report of a somatic cell nuclear transfer-derived animal, there are many descriptions of anomalies concerning cloned calves leading to high perinatal morbidity and mortality. The present article discusses some our experience regarding perinatal and neonatal procedures for cloned Zebu cattle (*B. indicus*) that has led to improved survival rates in Nellore cloned calves following the application of such 'labour-intensive technology'.

Additional keywords: bovine, nuclear transfer, parturition.

Steps towards moving nuclear transfer to the field

There has been considerable discussion regarding advances in nuclear transfer (NT) in the past decade. Most of the discussion was triggered by the birth of Dolly, the first somatic cell nuclear transfer (SCNT)-derived mammal (Wilmut *et al*. 1997), which was quickly followed by many others (for a review, see Latham 2004). The possibility of producing identical animals was interesting enough to bring together researchers and technicians from most diverse fields (Wells *et al*. 1999; Wilmut *et al*. 2009). Since the early days, NT experiments have brought to light important issues in biology, leading to new discoveries and knowledge in many related areas, such as mechanisms controlling the cell cycle and nuclear reprogramming (Galli *et al*. 2003). In addition to its relevance to basic science, SCNT technology has received considerable interest for application in animal production (Bousquet and Blondin 2004; Heyman *et al*. 2004; Heyman 2005). This has enabled important improvements in the procedure, with the technology being gradually transferred to and applied in industry (Faber *et al*. 2003, 2004). Nowadays, many private companies are exploiting SCNT commercially in many species.

Cloned individuals are common in agriculture; for example, people often drive on roads bordered by huge forests of clones (e.g. eucalyptus). Why not apply the same principle to animal production? Cattle in particular could benefit from cloning because efforts to advance genetic merit are usually slow owing to large inter-generation intervals. Because of the large number of offspring, the poultry and swine industries already profit from such non-additive genetic applications. The possibility of producing cloned cattle on a large scale could also accelerate genetic gain, increasing production and introducing a paradigm shift in the beef and dairy industry.

Nonetheless, despite the potential benefits, pre- and postnatal abnormalities, including high rates of embryo and fetal losses, abortion, prolonged gestation, increased birthweight, placental anomalies and reduced neonatal survival are likely consequences of cloning by NT (Bertolini and Anderson 2002; Edwards *et al*. 2003; Miglino *et al*. 2007; Oback and Wells 2007; Oback 2008). The establishment and maintenance of SCNT-derived pregnancies have proven challenging, with the delivery and early care of these animals often being expensive, labour intensive and

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frequently frustrating (Hill and Chavatte-Palmer 2002; Wilmut 2006; Keefer 2008). Many factors contribute to the overall low efficiency of NT, with the number of births per number of embryos transferred still low, averaging 6% and usually varying between 0% and 12% (Wells *et al*. 1999, 2004; Panarace *et al*. 2007; Keefer 2008; Oback 2008).

The low success rate, combined with increased pregnancy and neonatal abnormalities and losses, seen after cloning has necessitated intensive care during the perinatal period, spawning considerable advances in neonatology in cattle. In the present review, we describe and discuss some management and clinical procedures and issues related to the peri- and postnatal care of cloned Nellore calves that, in our experience, have helped improve survival rates. Special attention is given to the most common risk-related clinical signs during the perinatal period. However, it is important to bear in mind that due to the considerable variations that exist between species, breeds, individuals, laboratories, procedures and personnel, cloning by SCNT may result in different phenotypes, thus requiring continuous adaptation to each specific condition or outcome that challenges us as scientists, practitioners, managers and producers.

Perinatal management

The perinatal period in cattle is defined as the interval that includes the 2–4 weeks before delivery and the first week of life. In our experience, as well as reported by others (Hill and Chavatte-Palmer 2002; Wells *et al*. 2004; Buczinski *et al*. 2009), this is the most critical period in clone production due to the high rate of unpredictable anomalies that may arise, leading to frustrating gestational outcomes. Indeed, approximately 50% of clone pregnancies do not end normally, and this figure ranges from 0 to 100% depending on the cell lineage. Hence, these pregnancies usually result in prolonged gestation of enlarged offspring with poorer neonatal (Heyman *et al*. 2002). The increased time *in utero* may be indicative of a need for further maturation, but is usually associated with increased birthweight, dystocia and higher morbidity and mortality (Hill and Chavatte-Palmer 2002).

In cattle and other domestic species, parturition is initiated by the activation and maturation of the fetal adrenal cortex, leading to an increase in fetal cortisol secretion (Challis *et al*. 2000). Previous studies have reported lower plasma cortisol concentrations in newborn cloned calves delivered by Caesarean section compared with vaginally delivered controls (Matsuzaki and Shiga 2002), but no differences in plasma adrenocorticotrophin (ACTH) levels, suggesting that the prepartum increase in plasma cortisol in clones may be insufficient to trigger parturition. Conversely, the responsiveness of the adrenocortical tissue to ACTH in cloned calves is normal and the difference in plasma cortisol levels in cloned calves seems to be due to the delivery method (Caesarean section *v.* natural delivery) rather than the cloning itself (Chavatte-Palmer *et al*. 2002; Hirayama *et al*. 2008). The prepartum increase in fetal cortisol leads to increased production of oestrogens by the placenta, which is accompanied by a reduction in maternal progesterone (P4) plasma concentrations (Conley and Ford 1987; Conley and Mason 1990; Challis *et al*. 2000). Under physiological conditions, these endocrine events stimulate myometrial oxytocin receptor expression, prostaglandin synthesis, cervical effacement, sacro-iliac relaxation and vulva distensibility (Huszar and Walsh 1991; Jenkin and Young 2004). Such events appear to be markedly decreased in females carrying SCNT-derived concepti. Hill and Chavatte-Palmer (2002) attempted to correlate maternal P4 profiles in the last 2 weeks of gestation of SCNT-derived pregnancies with the postnatal viability of cloned calves. The authors observed that atypical profiles (e.g. prepartum maintenance of elevated P4 levels) appeared to be associated with lower neonatal viability, whereas usual P4 profiles were associated with higher postnatal viability. Conclusive studies to determine the reason for the poor signs of parturition in cloned animals are still lacking. Hirayama *et al*. (2008) suggested that oestrogen sulfoconjugation, a mechanism that regulates oestrogen activity by impairing the binding of oestrogen to its nuclear receptors, may be responsible for the reduced parturition signalling because a lower prepartum oestrone : oestrone sulfate ratio was observed in cloned compared with control pregnancies.

When performed during the perinatal period preceding delivery, the induction of parturition is believed to improve fetal viability by stimulating the production of surfactant phospholipids by fetal Type II alveolar cells, enhancing the expression of surfactant-associated proteins and accelerating the overall structural maturation of the lungs (DeKruif and Benedictus 1993). Hence, the induction of parturition is desirable in recipient heifers and cows to reduce the interval to parturition, allowing a more assisted delivery; however, the incidence of retained fetal membranes usually increases (Lewing *et al*. 1985; Garcia *et al*. 1992). Parturition may be induced via different protocols (Lewing *et al*. 1985; Nasser *et al*. 1994), but is generally achieved by using a combination of corticoid treatment with a prostaglandin ($PGF_{2\alpha}$) analogue. The use of $PGF_{2\alpha}$ is highly conserved between different protocols, whereas the corticoid treatment is highly variable: treatments may consist of a single dose of 20 mg dexamethasone in the last 36 h before delivery or they may start 7 days before the expected date of delivery (EDD) using a slow-release, long-acting corticoid, such as opticortenol, betamethasone or triancinolone acetonide, followed by the standard protocol (Nasser *et al*. 1994, 2008). The aim of the latter treatment option is to accelerate final maturation of fetal lungs and organs, as well as the placenta.

Because the mean length of gestation in Zebu cattle is approximately 290 days, we generally induce parturition starting up to 5 days before the EDD (between Days 285 and 290 of gestation), as suggested by others (Hill *et al*. 1999). To evaluate the effect of the induction of parturition on perinatal survival of Nellore cloned calves, we tested two different protocols: (1) administration of 20 mg, i.m., dexamethasone 36 h before EDD, followed by administration of D-cloprostenol 24 h before EDD; and (2) administration of 8 mg, i.m., triancinolone acetonide 9 days before EDD, followed by drug administration as described for Protocol 1. Both protocols resulted in similar survival rates (13/20 (65%) and 11/15 (73.3%), respectively; $P = 0.72$). Although there were no advantages in terms of survival using the longer protocol, we routinely choose to use this second protocol because no negative effects on survival were observed and the protocol has potential benefits in that it may allow preparation of both the fetus and the recipient for delivery.

In fact, we have observed, by visual inspection, an improvement in udder development and vulvar distensibility in female recipients subjected to Protocol 2 compared with females subjected to Protocol 1, a phenotypic event readily detectable from the third day of triancinolone acetonide administration. In addition, the use of such protocol may be logistically useful; for example, if evaluation of fetal well-being in the last 4 days of pregnancy indicates signs of distress, an elective emergency Caesarean section is more likely to be successful than with no previous corticoid treatment. However, there is still a need for studies on non-invasive and risk-free methodologies to evaluate fetal well-being before birth that provide accurate results. Some studies using ultrasonography to assess fetal well-being have indicated that fetal inactivity or hyperactivity, as well as hyperechoic particles in fetal annexes, are possible signs of fetal distress (Buczinski *et al*. 2007, 2009; Kohan-Ghadr *et al*. 2008). Other strategies, such as the quantification of pregnancy associated glycoproteins in the first trimester, can also be used to predict the outcome of gestation (Heyman *et al*. 2002; Breukelman *et al*. 2005; Chavatte-Palmer *et al*. 2006). Nonetheless, we have been evaluating cardiotocography in animals in the last months of pregnancy. Thus far, the preliminary results suggest that hypoxia in the last trimester of gestation, especially from 90 to 30 days prepartum, is associated with fetal hypoactivity, the lack of a cardiac response after inter-digital stimulus and delivery of a meconium-stained calf. However, hypoactivity does not seem to invariably indicate hypoxic conditions near term, with some hypoactive calves showing being healthy and viable after delivery.

Delivery of SCNT-derived calves

Birthweight of calves produced by SCNT tends to be increased compared with the birthweight of non-cloned calves. It is important to note that nuclear donor cells are frequently taken from animals of high genetic merit that are born often heavier than the population average. However, deregulation of imprinted genes and/or mechanisms leading to large (or abnormal) offspring syndrome (LOS) also contribute to the increased birthweight. All these factors tend to reduce chances for survival due to a greater level of distress imposed upon the newborn. In our experience, aside from a group of oversized and heavier animals at birth (9/43; 20.9%), a large proportion of calves have been normal in both size and weight (30/43; 69.8%) at birth, with a smaller proportion being significantly smaller (4/43; 9.3%) (F. V. Meirelles, unpubl. data). The mean birthweight for Nellore cloned calves born in our laboratory is approximately 38 kg (range 14–62 kg) for a breed with an average birthweight of 34 kg. Figure 1 shows the distribution of birthweights for Nellore cloned calves in a real population of 9047 calves, along with a theoretical simulation using the Monte Carlo methodology for a similar number of clones based on the distribution we found in our study (F. D. P. Meirelles, unpubl. data).

The systematic evaluation of the absolute and relative (compared with the recipient) size of a fetus and the signs of parturition are important for planning the delivery procedure. Large animals are chosen to be delivered by Caesarean section, because these animals are less capable of enduring dystocia-induced hypoxia (Chavatte-Palmer *et al*. 2004). An experienced

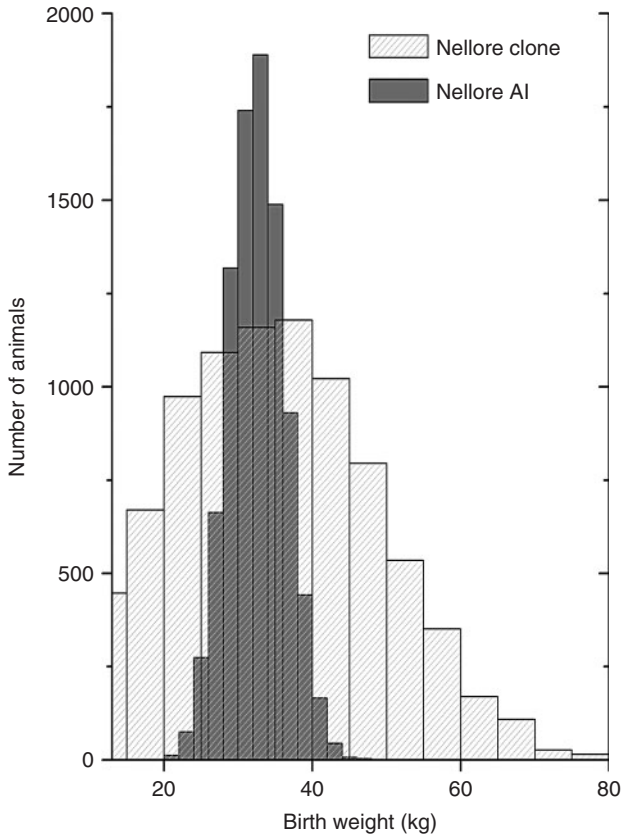

Fig. 1. Real and simulated data on calf weight at birth. Dark grey bars show the weight at birth of a real population of 9047 Nellore (Zebu) calves produced by AI. The light grey bars represent the distribution of weight at birth of a simulated population of 9200 Nellore calves produced by somatic cell nuclear transfer applying Monte Carlo methodology. The simulation applied the weight distribution we observed in our service in 43 animals delivered over a period of 4 years (2005 to 2009).

veterinarian with sound training in obstetric procedures may estimate the size of the animal by measuring the limbs or head by rectal palpation. In addition to identifying large fetuses, rectal palpation may help in the diagnosis of flexural deformities that may also complicate passage through the birth canal and require obstetric assistance during delivery (Fecteau *et al*. 2005).

A Caesarean section should be chosen if there is uncertainty about the size or other indications of potential suffering. This is the most appropriate method unless the neonate is known to be of normal size, thus having a high likelihood of passing quickly through the pelvic canal (Hill and Chavatte-Palmer 2002). Performing a Caesarean section provides an opportunity to better deal with the umbilical cord, which is frequently increased in diameter, allowing prompt draining of the umbilicus and clamping. We routinely clamp and cut large-diameter umbilici to avoid infections and haemorrhage, because an absence of blood vessel constriction is more likely in enlarged umbilici after rupture or cutting (Hill and Chavatte-Palmer 2002).

There are other factors in addition to the size of the calf that indicate the need for a Caesarean section. For example, visualisation of hyperechoic structures in the allantoic or amniotic fluids,

or within the placentomes, on ultrasound and/or the detection of a hyperactive fetus, placental oedema or excessive fetal fluid volume (hydramnio or hydroallantois) can be used as indicators for the need for a Caesarean section. The decision whether to perform a Caesarean section will depend on the time of gestation and on the time since starting the treatment to induce parturition. Two attempts to induce parturition 2 weeks before EDD in pregnancies in which hydrops was identified resulted in neonatal death due to atelectasis (F. V. Meirelles, unpubl. data).

Prior to surgery, we inject the recipient female with 50 mg, i.v., isoxsuprine chlorhydrate to relax the uterus and thus minimise the chances of fetal hypoxia during the procedure. There are no negative effects associated with the recipient's position (standing or in lateral recumbency) during surgery on survival outcome but, to avoid the risk of decreasing fetal viability, we discourage the use of drugs that may depress the central nervous system (CNS).

Initial care

It is important to note that most perinatal deaths generally occur within the first hour after birth (Nagy 2009). Consequently, special attention must be given to the calf during and immediately after delivery. After surgery, which usually takes 10–20 min, the calf is exposed to the atmosphere. It is essential to keep in mind that: (1) even apparently healthy animals require close monitoring; and (2) their condition can deteriorate rapidly (Fecteau *et al*. 2005). After delivery, fluid drainage from the respiratory system is facilitated with the calf being transported immediately to a warm room, in a clean space and laid in sternal recumbency. At this point, the first clinical evaluations are made with estimation of the Apgar score. The Apgar scoring index can be useful for assessment of neonatal viability and as a predictor of early signs of peripartum asphyxia (House 2002). A chart reporting the most important birth details is very important for keeping track of the parameters evaluated in newborns.

The most important parameters we take note are birth date and time, method of delivery (vaginal or Caesarean section), time to spontaneous respiration, the Apgar score at birth and after 5 min (Herfen and Bostedt 1999), initial respiratory and heart rate, initial rectal temperature, time of first meconium elimination, birthweight, haemorrhage in the umbilical cord, state of alertness, suckling reflex, time to standing, time to nursing, initial blood measurements (i.e. glucose, lactate, packed cell volume, total protein concentration, acid–base balance and arterial blood gas) and initial measurements of oxygen saturation in the haemoglobin. Other critical elements we make note of during the physical examination for the detection of respiratory disease include mucous membrane colour (nasal and vulvar mucous membranes), character and frequency of the respiratory effort and thoracic auscultation (Poulsen and McGuirk 2009). At the end of this initial period, the first clinical decisions are made and, depending on the neonate's condition, some initial precautions are prescribed and monitored over time.

Adaptation to extrauterine life

In utero, materno–fetal exchange within the placentomes provides oxygen and nutrient to the conceptus' blood. Oxygen and nutrients are then distributed through the fetal circulation by shunting blood away from the pulmonary circulation via the ductus arteriosus and foramen ovale, which is facilitated by hypoxia-induced pulmonary arterial constriction (Ardran *et al*. 1952). Under physiological conditions, the fetus survives in mild hypoxic conditions (P_aO_2 38 mmHg) compared with adult animals (P_aO_2 100 mmHg). The adaptation to this environment is due to efficient O_2 extraction from maternal placental blood by fetal haemoglobin (Poulsen and McGuirk 2009).

After delivery, the most important factor affecting the survival of a neonate is the respiratory system. Calves should make active respiratory movements within 30 s after being delivered. Initial hypercapnia is physiologically important, because it is detected as a stimulus for respiration by the chemosensitive area in the medulla. Primary apnoea is defined as the absence of spontaneous breathing for 1–5 min; if spontaneous breathing has not started in clones by 2 min, it is recommended that action be taken to help the calf. Rubbing calves with bedding or towels can help stimulate the phrenic nerve (Brunson 1981). The use of acupuncture points on the muzzle is also useful in early care (Rogers 1977). Pouring cold water over the calf's ear not only stimulates thermoreceptors in the skin, but has also been recommended for respiratory stimulation (Nagy 2009). In the case of insufficient fluid drainage from the airways, the induction of the sneezing reflex by inserting a clean straw into the nostrils or the use of suction devices may assist in expelling or removing the remaining fluids. Hanging the calf from the back legs for less than 90 s can also be used to clear the airways. It is also important to keep the calves in sternal recumbency in order to guarantee an adequate perfusion : ventilation rate. Both sternal recumbency and suspension by the hind legs improve respiratory adaptation to extrauterine life by newborn calves (Uystepruyst *et al*. 2002*a*).

Monitoring blood parameters (arterial blood gases, acid–base balance, lactate and glucose concentrations) over time is essential. For any animal diagnosed with any grade of asphyxia, hypoxia, hypercapnia or respiratory acidosis due to respiratory distress, we immediately place an intranasal catheter for O_2 supplementation at a rate of 5–7 L min^{-1} depending on the size of the calf. If the Apgar score does not improve significantly at the second evaluation, 5–7 mg kg^{-1} aminophylline every 12 h may be administered in the first days of life. Oxygen therapy should be maintained until P_aO_2 is near physiological conditions (i.e. 65 mmHg; Bleul 2009), when gradual withdrawal is initiated. If haemogasometer equipment is not available, we suggest maintaining O_2 supplementation for at least 24 h with gradual titration from 5–7 to 1 L min^{-1} depending on the clinical progress of the animal.

When hypoventilation or apnoea is present, drug intervention is indicated. Analeptic agents, such as doxapram hydrochloride, stimulate peripheral chemoreceptors and respiratory centres of the CNS. Doxapram hydrochloride has a wide margin of safety and has been used successfully to stimulate respiration (Nagy 2009). Many studies have demonstrated improved respiratory parameters and survival in calves and lambs following the intravenous or sublingual administration of doxapram (Szenci *et al*. 1980; Nagy 2009). Doxapram hydrochloride stimulates the medullary respiratory centres via aortic and carotid body

chemoreceptors and can be given at a dose of 0.5 mg kg^{-1}, i.v., or as an injection of 5–10 mg kg^{-1} at the base of the tongue for emergency resuscitation and anoxia (Sullivan *et al.* 2008). Several studies have demonstrated improvements only in mildly depressed newborns (Brown 1987), whereas severely depressed calves do not seem to respond to the agent (Nagy 2009).

Prior to birth, a fetus has a prompt supply of nutrients (e.g. oxygen, glucose), even if at relatively decreasing levels as parturition approaches. Moving from an intrauterine to extrauterine environment results in marked shifts in temperature and in discontinuation of the supply of nutrients, with all events being metabolically related to one another. Clinical changes are often seen in calves born following mild complications. Moderate to severe metabolic disturbances can mirror the changes in temperature (Nagy 2009). For example, rapid decreases in body temperature (the ambient temperature may be 20°C or more below that found *in utero*), failure in thermostatic regulatory mechanisms to restore normal rectal temperature and a delay in stimulation of the intermediate metabolism to maintain glycaemia and oxidative metabolism for efficient ATP production may occur in neonates, regardless of exposure to hypoxia, creating a vicious metabolic cycle that can result in the death of the calf. Not surprisingly, such conditions are often seen in cloned calves. In one study on the effects of infrared heaters on respiratory and metabolic parameters in newborns, calves exposed to an infrared heater for 24 h post partum had significantly improved rectal temperature, arterial haemoglobin oxygen saturation, tidal volume, dynamic lung compliance and respiratory rate compared with unheated calves (Uystepruyst *et al*. 2002*b*). This study highlights the need to ensure that adequate body temperature is maintained in cloned neonates, along with optimised gas exchange, acid–base balance and blood sugar monitoring. In our practice, neonates receive a cold water stimulus for breathing. This stimulus is followed by very fast cleaning of the calf with detergent and warm water to favour drying. Animals are then quickly moved to a 30°C room and dried immediately with towels and a heated blower. The animal is maintained under these conditions until it is capable of adequately regulating its own body temperature. Even using this protocol, 23.3% (10/43) of our animals developed hypothermia (body temperature < 36.5°C), which invariably resulted in death.

At this point (a few hours after birth), most of the initial clinical care has been applied. The calf is then closely monitored and colostrum offered. The colostrum not only provides passive immunity, but also reduces morbidity and mortality in young calves (Cortese 2009). When calves are born, the epithelial cells that line the digestive tract allow absorption of colostral proteins via pinocytosis. As soon as the digestive tract is stimulated by ingestion of any material, this population of cells begins to change to those that no longer allow such absorption. By 6 h after birth, only 50% of the absorptive capacity remains; by 8 h only 33% remains and by 24 h there is no absorption seen any longer (Cortese 2009), which is usually referred as 'gut closure'. This emphasises the importance of an early supply of good-quality colostrum as early as possible after birth. In our practice, we offer colostrum in small bottles until the animals receive approximately 10% of their bodyweight in the first day of life. If the animals do not have the suckling reflex we introduce the equivalent of 5% of the calf's weight in colostrum via an oesophageal catheter within the first 5 h of life. Maintaining a bank of frozen colostrum from healthy animals within a herd or environment is recommended. Only high-quality colostrum should be frozen or provided to calves after testing t with a colostrum densimeter. Whenever possible, we allow the calves to suckle the recipient, obviating the need for artificial feeding.

Glucose concentrations are evaluated every hour during the first 8 h of life or after 30 min of colostrum ingestion. Under our conditions, approximately half the calves are hypoglycaemic within the first 4–8 h of life. Some animals are capable of regulating glucose levels soon after colostrum ingestion, whereas others develop hypoglycaemia, especially after colostrum intake, requiring more intensive care for an additional 36–48 h. If an animal's plasma glucose level falls below 50 mg dL^{-1}, 0.2 g kg^{-1}, i.v., glucose supplementation is given starting with 2.5 g at 25% concentration, followed by 5% glucose solution at slow flow, along with the administration of 0.1 mg kg^{-1} dexamethasone. After treatment, animals are monitored frequently to ensure that glucose levels are maintained within normal limits (80–120 mg dL^{-1}).

Finally, special attention should be given to oxygen saturation, cardiac frequency and acid–base balance. These parameters provide important information regarding prognosis and should continue to be monitored closely to determine the extent of additional care the animal may need.

Additional care

Some of the issues discussed in the initial care section have consequences that must be addressed afterwards in more intensive care. The main problem, in our experience, is associated with cardiac and respiratory disorders likely due to insufficient placentation in late pregnancy and peripartum asphyxia. If not well monitored, these problems may progress into an acid–base unbalance, further evolving into respiratory and/or metabolic acidosis. Hypoxic neonates, likely affected by respiratory acidosis, have a weak or absent suckling reflex, difficulty in maintaining sternal recumbency and require more time to stand (Dufty 1977). A prolonged moderate hypoxic condition may promote the maintenance of the fetal circulation (patent foramen ovale and patent ductus arteriosus), intensifying the hypoxia. With time, a widespread chain of events may follow, such as depression and oedema of the CNS. Although respiratory acidosis can be controlled to a certain degree by offering O_2 and $NaHCO_3$, animals that are not capable of adequate gas exchange at the alveolar–capillary interface may develop a chronic, deteriorating condition of metabolic acidosis. This can progress even further due to less efficient energy production. Oxidative metabolism is stalled by the hypoxic conditions, leading to higher glucose consumption and lactate production, which metabolically deepens the acidosis. Often, over time the prognosis becomes poor, resulting in death (Fig. 2).

There are some rather effective therapies that can be used to improve an animal's respiratory condition when needed. To that end, distinct approaches can be used to tackle at least two sides of the same broader problem involving an inadequate ventilation : perfusion rate, depending on the diagnosis: (1) the

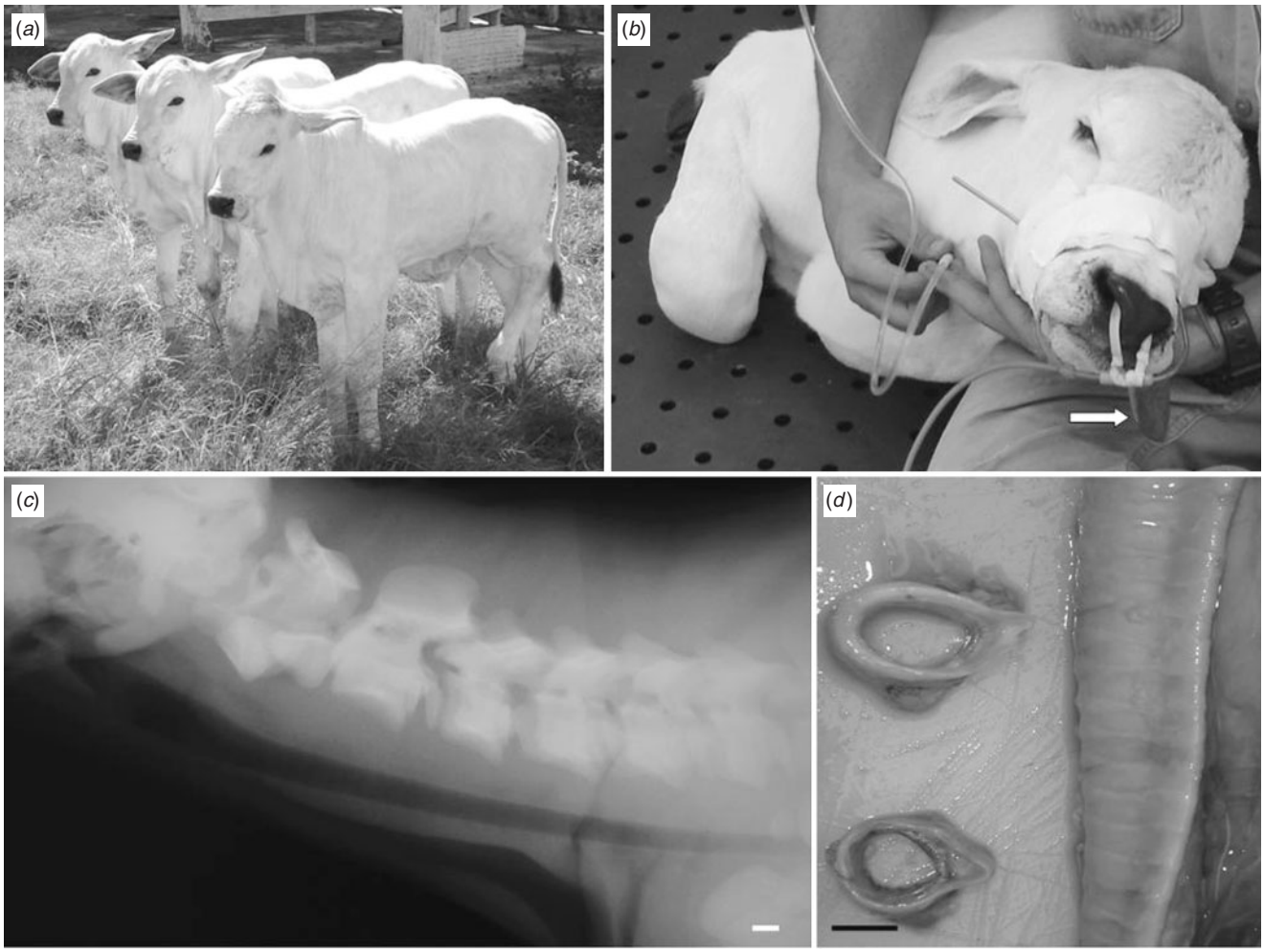

Fig. 2. Healthy Zebu male cloned calves (*a*) and a calf with a respiratory disterss (*b–d*). (*b*) Photograph of an animal with low P_aO_2 and clinical characteristics of respiratory distress and acidosis. The arrow indicates protrusion of the tongue (*b*), a very common finding in cloned calves with a macroglossia of the tongue base, and a stenosis in the distal third of the trachea (*c*, *d*). On post mortem examination (*d*), the diameter of the trachea in the caudal region (lower transversal cut) is notably smaller compared with the cranial region. Scale bars = 1 cm.

airway/alveolar compartment; and (2) the pulmonary circulatory system. Pathological conditions affecting one or both systems have often been reported in cloned animals and are one of the leading causes of death in the immediate postnatal period (Hill *et al*. 1999; Chavatte-Palmer *et al*. 2002; Wells *et al*. 2004).

If the origin of the respiratory problem in an animal is lung immaturity and there is little or no success after oxygen therapy and/or aminophylline administration, treatment with surfactants is a good option. Our experience suggests that surfactant treatment in animals suffering from meconium aspiration, for example, may improve blood oxygenation. There are many surfactants on the market and two main types may be available: (1) a crude surfactant extracted from bovine or swine lungs; and (2) a synthetic surfactant. A previous study showed that the use of crude surfactants is more effective (Karapinar and Dabak 2008). However, the cost of the treatment may hinder its use. In our practice, the surfactant is effective in rapidly increasing P_aO_2 in cloned calves when after administration of 300 mg–1 g in the first hour of life (P_aO_2 127.0 ± 11.1 mmHg after 6 h treatment *v.* 57.2 ± 5.8 mmHg in untreated animals; $P < 0.05$; Fig. 3). However, we did not observe any beneficial effect of treating animals 24 h after birth (88.7 ± 17.5 mmHg in animals treated before 1 h of life *v.* 64.7 ± 5.2 mmHg in animals treated at 24 h *v.* 62.1 ± 7.9 mmHg in untreated animals 48 h after birth). Other studies have also shown that administration of surfactant at an early stage of lung disease results in superior results compared with delayed treatment (Karapinar and Dabak 2008). Surfactant is generally administered by intubation, because it must be introduced intratracheally. In our facility, we do not perform intubations and have instead chosen to treat animals by injecting lower doses (300 mg) through the trachea with a 26-gauge needle. After injection, the animal is turned in three different positions to allow diffusion of the surfactant to the caudal positions and to each lung. Ideally, serial blood gas analyses must be done to monitor the calf's response to whichever therapy is implemented. Ultimately, calves with persistent hypercapnia,

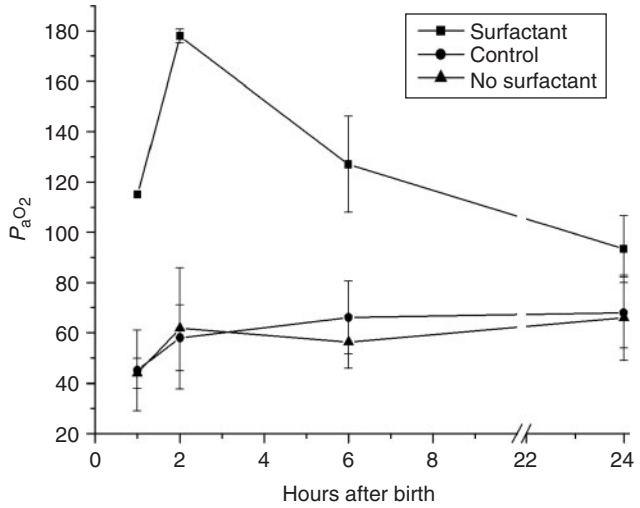

Fig. 3. The P_aO_2 of cloned and control calves 1, 2, 6 and 24 h after birth. (■), P_aO_2 of clones treated with 0.3–1 g surfactant during the first hour of life; (●), P_aO_2 of naturally delivered animals as reported in Bleul (2009); (▲), clones without surfactant treatment. The O_2 supplementation of cloned calves was stopped when the P_aO_2 was >80 mmHg or 24 h after birth. Data are the mean ± s.e.m.

despite therapeutic intervention, are candidates for mechanical ventilation, if available.

Additional care is frequently required to decrease the chance of pulmonary oedema, also favouring the elimination of alveolar mucus/meconium. As examples, mucolytics such as bromexin, along with 0.1 mg kg^{-1} dexamethasone, both given at the beginning and end of topical treatment using an inhalator, may help improve an animal's condition. Finally, if pulmonary oedema is diagnosed, using diuretics such as furosemide may help decrease the oedema and the related negative effects. Obviously, it is important to maintain an adequate hydration status during such treatment.

If the origin of the respiratory problem is related to the pulmonary circulation and/or lung perfusion, certain therapies may help ameliorate the hypoxic condition. Pulmonary hypertension, likely caused by an increased resistance to blood flow through the pulmonary arterioles, has been implicated previously in the early death of clones (Kishi *et al*. 2000; Cibelli *et al*. 2002). Even if not simple to diagnose in a non-invasive manner under field conditions, secondary signs can assist the clinician in suspecting the condition when respiratory insufficiency is present (e.g. positive jugular pulse and unresponsiveness to all treatments dealing with the alveolar/lung compartment, such as oxygen therapy). Oxygen therapy is the first line of treatment for improving lung perfusion, because oxygen can function as a vasodilator at the pulmonary level. However, vasoactive drugs can also be used depending on the intensity of the condition, including nitric oxide (NO) mixed with oxygen and given by inhalation or administration of drugs responsible for increasing vascular NO concentration. The validity of such treatments needs to be verified in broader clinical studies.

Despite the use of a range of drugs and therapies to improve survival rate, along with the time and effort provided by professional care at the intensive care unit, some animals will not respond to any treatment and will invariably die. In part, such an outcome is often related to congenital defects in vital tissues, organs or systems. In our experience, we have observed two such cases; in one case, almost the entire left ventricle was absent, whereas the other calf had dilated cardiomyopathy that resulted in a heart with 'two apexes'. However, post mortem findings are generally patent foramen ovale (13/22; 59%), myocardial hypertrophy (4/22; 18.2%), ventricle dilatation (2/22; 9.1%) and patent ductus arteriosus (2/22; 9.1%). Other post mortem findings include atelectasis, umbilicus haemorrhage and anasarca. Animals that survive the perinatal period have a good prognosis and are transferred to a facility where they receive care over the medium term.

Medium-term care

Medium-term care is important because calves are likely to show some symptoms at birth that require longer-term treatment or care, with some symptoms appearing only after a few days of life. Consequently, monitoring of the animal's health status may continue for weeks after birth.

One of the most important issues during the medium-term care is the umbilicus, which is more prone to turn into a patent urachus and ascending omphaloflebitis due its enlarged size at birth and the delivery procedure *per se*. The problem seems to be more frequent if the umbilicus is clamped during delivery, but can also occur in non-clamped animals. Patent urachus is also more frequent in the umbilici of cloned calves after clamping and rupture (Batchelder *et al*. 2007). Many descriptions of calves showing patent urachus appear in the literature, with surgical ablation the most indicated form of treatment, ranging from simple removal and ligation of the extra-abdominal portion of the vessels to a more invasive procedure for the intra-abdominal removal of all vessels and the urachus through laparotomy. Due to requirements imposed by Zebu breeders' associations in Brazil, surgical reduction in many genetically valuable cloned animals is not a choice. Therefore, we used iodine at an initial concentration of 10% followed by a later concentration of 2% for the treatment of all umbilical problems; this treatment has proven successful in preventing navel infections and in stopping urine drainage by the 3rd–4th day of life, but must be conducted frequently (four times a day).

Anaemia and other blood- and plasma-related conditions have also been examined carefully through sequential blood analyses by our group. Initially, SCNT-derived calves have normal erythrocyte counts (6.3×10^6 cells mm^{-3}). However, cell counts decrease 6–12 h after birth, reaching a significantly lower erythrocyte count (4.5×10^6 cells mm^{-3}) 24 h after delivery compared with counts in naturally mated or IVF control animals (6.0×10^6 and 6.1×10^6 cells mm^{-3} at 24 h, respectively). A similar decrease is observed for haemoglobin (10.9 g dL^{-1} at birth), which decreases to 7.2 g dL^{-1} in clones after 7 days, significantly lower than that in natural and IVF-derived calves (9.5 and 10.7 g dL^{-1} haemoglobin on Day 7, respectively). Perhaps part of this difference, particularly for cell counts, can

be explained by a higher haemodilution effect caused by the administration of colostrum, because cloned calves will likely receive more colostrum at an earlier time (first few hours; via oesophageal cannulation) than control animals. However, in accordance with what has been reported previously (Chavatte-Palmer *et al*. 2004), both blood parameters start increasing after 15 days, reaching normal values by 30 days after birth. Although these parameters are different between cloned and natural or IVF-derived animals, one must evaluate such information with caution, because our cloned calves have been exposed to oxygen therapy after birth, whereas IVF and natural calves are not. Regardless of the aetiology, if the animals are anaemic, we start administering iron ion supplementation 3 days after birth. In addition, a series of autologous blood transfusions (200–300 mL whole blood from a cell donor animal given daily in two or four transfusion sections or whenever deemed necessary) through the first few days of life is a good option to assist cloned calves in overcoming the unfavourable state caused by subclinical or even clinical anaemia. In addition, such transfusions help boost the transfer of passive immunity to the calf, along with cells from the white blood cell lineage (in fact, plasma can also be given to the calf for that same purpose). Nonetheless, the use of blood transfusions in tropical conditions requires further attention to potential infection with blood-borne diseases, such as babesiosis and/or anaplasmosis.

Many of our cloned animals develop diarrhoea days or weeks after birth. Cloned calves with diarrhoea are generally treated like any other calf, with special attention to hydration status. However, if needed, many other treatments are offered, including kaolin, activated charcoal, sulfonamides and antibiotics, depending on the severity of the condition (Constable 2009). Because many calves exhibit voracious appetites early in life, special care with the environment where the calf is located is essential because the ingestion of foreign bodies and bedding is rather common in such animals.

In general, hyperthermia should be viewed as a sign of sepsis and the origin and/or cause should be determined and treated. However, paradoxical hyperthermia in clones has been described in many studies (Chavatte-Palmer *et al*. 2002; Fecteau *et al*. 2005). Some of our animals had peak rectal temperatures of 43°C with no other associated clinical signs. This paradoxical hyperthermia typically does not respond to treatment with non-steroidal anti-inflammatory drugs and the cause of this hyperthermia remains unclear. In our experience, we have observed this phenomenon in 14.3% (4/28) of animals during the first week of life (Days 1–7). Treatment includes maintaining animals in a fresh and well-ventilated area, providing a 1 : 4 alcohol : water mix body bath and even i.v. or i.m. administration of drugs to relieve the fever, such as dipirone sodium, if the temperature reaches 40.5°C. Considering the disturbances seen in basic physiological functions in clones, such as altered appetite, satiety, temperature control and difficulties in regulating cardiorespiratory function on occasions, these clinical events may be linked to immaturity of the hypothalami regions. Studies designed to explore these possibilities are still needed in cloned animals.

Flexural and angular limb deformities are commonly seen in cloned calves to various degrees, affecting primarily the carpi and fetlocks (Fecteau *et al*. 2005). Our Zebu calves frequently show tendon laxity, most often affecting the rear limbs. This laxity results in difficulty standing in the first hours, which gradually disappears without any treatment. Although these animals have good prognoses, it is important to protect the animals' hooves and to avoid abrasive surfaces.

Another interesting, but less relevant, and common symptom in cloned calves in our experience is alopecia. This phenomenon is intense in approximately one-third of animals, with a mild manifestation generally occurring 30 days after birth or before the new hair coat is grown. Even though this is not relevant in terms of health and prognosis to adulthood, we observed that treating calves with vitamins A, D and E decreases the occurrence and severity of the alopecia.

Concluding remarks

As stated above, SCNT is a very promising biotechnology, especially for cattle. However, in order for it to achieve broader-scale application in the field, increased efficiency is required at many levels. One of the major areas for further development is the nuclear reprogramming that occurs during the cloning process, which ideally should enable normal placental and/or fetal development, resulting in normal pregnancies that reach term and end in the delivery of healthy offspring. Although advances in knowledge are most likely to increase SCNT efficiency, the progress in this direction is quite slow. Thus, in the mean time, there is a need for the development of obstetric and neonatal measures that can increase the chances of obtaining viable offspring. This will certainly contribute to our knowledge in managing high-risk pregnancies in cattle, as well as contributing to the implementation of SCNT after obtaining a greater knowledge of developmental reprogramming.

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