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Intensive Neonatal Care of the First Bitransgenic Bovine Clone for Human Lysozyme and Lactoferrin Production

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Abstract

Somatic cell nuclear transfer associated with transgenesis allows the production of animals with beneficial properties that cannot be obtained by conventional breeding programs. Unfortunately these biotechnologies are characterized by a very low efficiency being extremely important to optimize the results of each step from skin biopsy and cloning, until the birth of calf. Animals obtained by cloning have special requirements after birth that must be considered to ensure their survival capacity. Taken into account that there is not much information regarding neonatology of cloned animals, new cases represent a great challenge, and each clinical finding and treatment report should be considered of great value. In this work, we describe all facilities and medical procedures used to ensure the survival of the first bitransgenic bovine clone for human lysozyme and lactoferrin obtained by Somatic Cell Nuclear Transfer (SCNT), born under clinical patterns of Large Offspring Syndrome (LOS). We summarize all maneuvers performed from cesarean section and primary neonatal evaluation, to intensive cares such as sepsis management, internal medium evaluation and correction, and total parental nutrition over a total period of 80 days.

Keywords: Bovine; Clone; Transgenic; Neonatal; Care

Implications

It has been reported that only few cloned animals are capable to survive after birth. For this main reason, information related to the neonatal handling of such animals is limited compared to ordinary animals which turns each single case of great interest. This work summarizes every medical maneuver during a total period of 80 days from initial neonatal evaluation to total parental nutrition during 24 days. We made special focus on internal medium control, sepsis, nutrition and finally we tried to mention specific differences between ordinary animals and cloned ones which imply specific therapies to fix its requirements.

Introduction

Animals produced by cloning commonly have present high birth weight, placental abnormalities and low survival rates, among other conditions, which are grouped under the name of Large Offspring Syndrome (LOS) [1,2]. Due to the heterogeneity of the phenotypes of the animals defined under this syndrome, the name of Abnormal Offspring Syndrome (AOS) has been proposed to reflect the scope of anomalous developmental conditions seen after transference of embryos produced by cloning [3].

Somatic cell nuclear transfer associated with transgenesis allows the obtention of animals that produce valuable proteins for medical and/or nutritional purposes. However, despite the advance of knowledge of physiological processes operating in this biotechnology, there are still high rates of embryo loss both before and after transfer to recipient females, as well as high fetal mortality rates in the last third of gestation and during the peripartum period. Moreover, it has been reported that only 50-60% of calves born reach the age of 150 days, thereby increasing the costs of production of bovine clones [4,5]. Although general information regarding the management of these animals is available, each unique new case represents a great challenge and each every clinical finding and treatment of great value.

In this work, we summarize all medical procedures and intensive care used to ensure the survival of the first bitransgenic bovine clone for human lysozyme and lactoferrin production by Somatic Cell Nuclear Transfer (SCNT), born under clinical patterns of LOS/AOS.

Materials and Methods

Embryos and pregnancies

Cells for SCNT were obtained from an adult female Jersey cow skin biopsy and after primary culture, transfected with a bicistronic vector for human lysozyme and lactoferrin by using Lipofectamine 2000 (Invitrogen, CA, USA).

SCNT was performed as previously described by Ross et al. [6]. Briefly, after denuding *in vitro* matured oocytes, a single bitransgenic cell was inserted into the perivitelline space of an enucleated oocyte and fused in calcium-free sorbitol fusion medium by applying a single direct current pulse of 2.3 KV/cm. Fused Nuclear Transfer Units (NTU) were activated using 5 µ Mionomycin (Calbiochem, San Diego, CA, USA) for 4 min, followed by incubation in SOF medium containing 2 mM DMAP for 4 h. After activation, the NTU were cultured in 400 µl drops of SOF medium under mineral oil at 38.5°C, 5% O_2, 5% CO_2, 90% N_2 and maximum humidity for 7 days. Seven embryos were transferred to synchronized recipients. After 30 days,

two pregnancies were confirmed by ultrasonography. Both pregnancies were monthly checked until estimated time of birth.

Neonatological care facilities

Taking into account that embryos produced *in vitro* by nuclear transfer produce high-risk pregnancies, births and newborns, a Neonatology Unit (NU) was prepared at the EEA-INTA Balcarce (Buenos Aires, Argentina) in order to cover the requirements of these animals. The NU was designed considering our previous experience and data available [7,8]. The NU had an "outside dirty area" of pens and an "inside clean Neonatology Room (NR)", prepared for the intensive care of calves. Because the animal obtained was a genetically modified animal, infrastructure and animal management was performed under Resolution # 240 of the Argentine Ministry of Agriculture, Livestock and Fisheries, with the approval of the National Commission of Agricultural Biotechnology (CONABIA). Light sources were placed in the outside area to ensure the work at night, and the entire NU was fenced and equipped with motion sensors and alarm to prevent the entry and exit of animals and anyone other than related staff.

The NR was fitted with two main areas: the Primary Care Area (PCA) and the Intensive Care Boxes (ICB). Also, the NR had other dependencies as a room with beds, a kitchen, a restroom and shelves to store the materials and drugs necessary for animal handling. The PCA was equipped with a warm bath, a vacuum pump for upper airway hygiene, a refrigerator, a freezer (bank of bovine frozen plasma and colostrum), a pH meter, scales, an ultrasound scanner (Sonoscape A6 Vet, Fridimex S.A.), a glucometer (Bayer, Contour^{TM}) and oxygen tanks (2x10m^3). The ICB were equipped with an oxygen line, a heating lamp (infrared light), and several holders to hang parental solution bags. A full sheet was designed to record medical monitoring.

Results

Ten days before the estimated day of birth, fetal position and viability were examined by rectal palpation. On the same day, birth was induced by i.m. administration of 30 mg dexamethasone and 25 mg of prostaglandin. Due to the absence of parturition signs, thirty-six hours after induction, obstetric maneuvers were performed to evaluate both pregnancies but only one fetus responded to stimuli. A cesarean section in a standing position through a left flank approach was performed in both cows for the recovery of fetuses. One fetus was found dead and thus destined fornecropsy and histopathological studies, while the living one was transferred to the NR to assess its physiological status (Table 1) and receive its first veterinary care.

Body weight (kg)	Temperature °C	Heart rate (per minute)	Respiratory rate (per minute)	Glucose mg/ 100mL
45	38.7	142	40	79

Table 1: Physiological parameters recorded immediately after birth in the first bitransgenic female bovine clone.

Hygiene of the upper airways was performed by an intermittent suction performed with a vacuum pump and the animal in sternal recumbency, using a 50 mm diameter rubber tube. Clinical evaluation showed meconium staining, normal thoracic breathing pattern, and no signs of nervous depression. A therapy of 25 mg of doxapram (Viviram Holliday), 800 mg of dihydrostreptomycin, 400000 IU of procaine penicillin and 400000 IU of benzathinebenzylpenicillin (Dipenisol, Bayer), as Total Dose (TD), was administered as respiratory analeptic and preventive antimicrobial therapy, respectively. The umbilical cord, which was enlarged (7.5 cm diameter), was clamped immediately after birth and treated with hydrogen peroxide (10 v), iodized alcohol (10%) and healing powder every 8 hours.

Since all the clinical parameters evaluated were considered within normal ranges (Table 1), the calf was placed in the pen with the cow to wait for its stand up and colostrum suckling. After 3 h without incorporation attempts, we decided to transfer the calf back to the NR for further evaluations and try to administer colostrum from our frozen bank. Since blood glucose determination was 26 mg/dL and the suckling reflex was absent, a venous catheter (Arrow 24 Gax20 cm) was placed into the jugular vein. A 10% dextrose solution (45 drops/ min) was administered until blood glucose normal values were observed. A sample of 10 mL of blood was taken for blood culture and complete blood analysis (white cell blood count, electrolytes, hepatogram and values of renal function (urea and creatinine)) (results shown in Table 2). Oxygen was administered through a nasal tube (10 L/min) until oxygen saturation values from heparinized arterial blood (femoral artery) were reported from the laboratory. Once oxygen saturation result was obtained, oxygen administration was interrupted (Table 3).

Analysis	Calf values	Reference values
Erythrocytes (x10^6/L)	5.8	5-10
Hemoglobin (g/dL)	8.4	8-15
PCV (Packet Cell Volume %)	27	24-46
White blood cells (x10^3/µL)	10.8	4-12
Neutrophils (%)	69	22-30
Lymphocytes (%)	27	64-76
Monocytes (%)	4	2-4
Glucose (g/L)	0.7	1-1.2
Urea nitrogen (mg/dL)	22	19-25
Creatinine (mg/dL)	1.2	1-2
Protein: total serum (g/dL)	4.8	6.7-7.5
Albumin (g/dL)	3.2	3-3.6
Aspartate aminotransferase (IU/L)	78	43-127
Alkaline phosphatase (IU/L)	514	418-482
Total bilirrubine (mg/dL)	0.07	0.01-0.47
Direct reacting (mg/dL)	0.02	0.04-0.44
Indirect reacting (mg/dL)	0.05	0-0.3

Table 2: Clinical chemistry of the first sample. Reference values were adapted from Smith (2009) and Coppo (2008) [15,18].

Analysis	Calf values	Reference values
pH (arterial)	7.4	7.25-7.35

PCO_2 mmHg	48	45.1-55.6
PO_2 mmHg	149.8	48.8-70
HCO_3 mEq/L	31.9	20.7-26.3
O_2 saturation %	99.2	Up to 80%
Base excess mMol/L	+6.5	±3

Table 3: Acid-base (arterial blood) of the first sample. Reference values were adapted from Smith (2009) and Coppo (2008) [15,18].

Every time the calf urinated, samples were taken and tested using commercial urine strips (Bayer Multistix 10 SG).Urine samples showed normal values in all cases. Due to the lack of ingestion of frozen-thawed colostrum during its first 6 half life, we tried to administer fresh colostrum obtained from a nearby dairy farm. After 1 h of attempts, the calf ingested 0.5 L of colostrum, and 2 h later, 1 L of 50% colostrum/milk mixed. Meanwhile, frozen-thawed bovine plasma was intravenously administered (42 drops/min) as a partial parental nutrition providing an additional antibody source until the calf normalized its milk intake, 48 h later.

Thirty hours after birth, the calf reached a rectal temperature of 40.9°C, 184 heart beats/min and 96 breaths/min. A new blood sample was taken to perform a complete analysis and a new blood culture. Antimicrobial therapy was reinforced with i.m. administration of 250 mg TD of enrofloxacin (Baytril, Bayer) and 500 mg TD of gentamycin (Equi Systems Inc.)/24 h. To control hyperthermia, 1.5 g TD of dipyrone (Sanofi, Aventis) was i.v. administered every 8 hours. Laboratory results showed normal values for blood analysis (total white cells 10000; mature neutrophils 60%; immature neutrophils 0%; lymphocytes 36%) and four days later both blood culture results were negative. Since dipyrone did not modify hyperthermia, 50 mg TD of flunixin meglumine (Pharmavet) was i.v. administered every 12 h, together with cold water enemas, alcohol and water baths at room temperature. To depress the medulla and thereby decrease body temperature, 15 mg TD of xylazine (Alfasan) was i.m. administered. Echocardiographic images and pressure records were always within normal ranges. No positive responses were obtained.

Concomitantly with the high temperature, the calf developed seizures which were controlled with the i.v. administration of 5 mg TD of diazepam IV (Larjan) and 50 mg TD of phenobarbital (Cevallos). In all cases, when milk ingestion did not cover the fluid requirements, 60 mL 0.9% sodium chloride saline solution/kg/day+20 mL/kg/day extra for the hyperthermia was i.v. administered.

Failure to modify the deviations of the physiological parameters made us stop antimicrobial therapy in search of causality markers on day 7. Twenty-four hours later, new blood samples were taken for complete blood analysis, arterial blood gases, acid-base balance, and a new blood culture. Laboratory results showed leukocytosis (15300) and severe acidosis (pH 7.08), with low bicarbonate values (11.2 mEq/L) and base excess (-18.6 mEq/L). Sodium bicarbonate (8.4 g/100 mL hypertonic solution, 60 microdrops/min, Rivero) was i.v. administered to correct blood pH, until normal values were achieved. Antimicrobial treatment was changed to 1500 mg/24 h TD of metronidazole (FadaPharma) i.v. continuous drip, 1050 mg/24 h TD of florfenicol (Shield), and 1000 mg/12 h TD of amikacin (Richet). Twenty hours later, laboratory results indicated a decrease in white blood cell count (9600), arterial blood pH of 7.24, and a base excess of -9.7 mEq/L. Intravenous Metronidazol was replaced by a per rectum administered total dose of 750/12 (Flagyl, Sanofi). Blood culture results showed the growth of an anaerobic peptostreptococcus. The antimicrobial scheme was maintained for 13 days until all clinical signs and markers of sepsis from laboratory analysis were reversed.

Once the calf was considered healthy on day 22, it was placed in a nearby pen with the foster cow and was manually stimulated to teach it suckling behaviors. Three days later, the calf was able to suck enough milk to cover its requirements. On day 30, food overload and profuse mucous type diarrhea were observed. The general condition of the calf worsened and finally it turned lethargic with hyporeflexia. The calf was taken to the NR, where blood samples were taken for complete analysis. Whole milk fed was replaced with lactose-free milk to prevent diarrhea exacerbation. The same clinical picture was observed every three times we attempted to return the calf with the cow, becoming stable through bottle feeding with lactose-free milk. In each case, laboratory results showed increased white blood cell count (up to 15000) and decreased blood pH with negative base excess (below 3 mEq/L). pH and diarrhea were corrected by i.v. administration of sodium bicarbonate (previously described regimen) and oral administration of antidiarrheal syrup (Enteroplus Syrup, Zoovet). Fecal samples were taken for viral and bacteriological determinations, showing negative results. During the last episode (day 45), the calf turned lethargic with hyporeflexia, with a temperature of 36.9°C. Blood samples from the catheter and femoral artery showed leukocytosis, hypoglycemia and metabolic acidosis. Abdominal ultrasonography showed ruminal overload with gas and milk clots, confirming ruminal acidosis. Milk administration was interrupted and an esophageal tube (15 mm diameter) was placed. Ruminal content (5 L) was collected (pH 5.3, containing milk clots) and replaced with 2 L of a warm sodium bicarbonate solution (40 g/L water) to stabilize ruminal pH at 6.5. Saline (NaCl 9 g/L), sodium bicarbonate (8.4 g/100 mL) and 10% dextrose were i.v. administered until dehydration, blood pH and glucose were corrected. Ceftriaxone 1 g/12 h TD (Acantex, Roche) was i.m. administered to prevent ruminal bacterial translocation; 1.175 mg TD of flunixin meglumine (PharmaVet) was administered as an anti-endotoxic dose and 80 mg TD of ranitidine /12 h (Ranivet, Vetanco) was i.v. administered to prevent laminitis. After 24 h of treatment, the saline solution was replaced by bovine plasma as nutritional support, administered as described previously while teaching the calf to eat pelleted balanced food (Ruter). Although temperature records fluctuated, they did not exceed 39.5°C. Total parental nutrition was decided until the calf learned to eat enough solid food to cover its requirements. A Kabiven (Fresenius Kabi AV) Total Parental Nutrition (TPN) bag formulated for humans was used following veterinary Neonatal Intensive Care Unit Notes (J. Palmer, New Bolton Center notes) to reach calf requirements of 10 gm/Kg/day dextrose, 2 gm/Kg/day amino acids and 1 gm/Kg/day lipids. According to this protocol, dropping was modified by reducing it to 18 drops/min to prevent hyperlipidemia, becoming necessary to administer supplementary dextrose 25% 12.5 drops/min and, once acidosis was controlled, 504 ml amino acids (11.5%, Rivero) per TPN bag. A saline solution and a vitamin complex (Rivial Pediatrics, Rivero) were also administered to cover fluid and vitamin requirements. The TPN therapy lasted 24 days during which the animal regularized its metabolic functions, reversed signs of ruminal acidosis and learned to eat balanced food and hay. Table 4 shows the clinical and hematological responses after 24 day total parental nutrition.

Parameter assessed	Day 0 start of treatment	Day 24 end of treatment
White cellx10^9 cells/L	9.7 (granulocytosis)	3.8 (monocytosis)
Glicemia mg/dL	30	80
Arterial blood pH	7.18	7.35
Ruminal pH	5.23	6.5
Attitude	Dull, depressed	Bright, alert

Table 4: Clinical and haematological responses after 24 day total parental nutrition of a 37 day cloned female calf.

During this therapy, the calf began to develop abnormal patterns of ruminal pH, forcing corrections by oral administration of sodium bicarbonate. Perhaps for this reason, in an attempt to produce saliva and neutralize the low ruminal pH, the calf started to eat hay from the box bed, which produced ruminal impaction, as confirmed by ultrasonography. Ruminal content from adult steer was administered twice daily until ruminal fiber degradation and normal patterns of rumen movements were observed.

Once the calf was able to feed itself through water intake and balanced feed, diet was reformulated including soybean hulls and soybean expeller to provide rumen degradable protein. This allowed the production of rumen ammonia that prevented a decrease in pH. Once the rumen showed normal movement patterns and pH 6.5-7.2, the diet progressed to reach a formula that guaranteed a daily gain of 700 g, administered three times per day.

On day 80, the calf was discharged and resumed a normal life under an artificial breeding regime.

Discussion

Although hundreds of animals of several species have been cloned by different methods, the surviving individuals represent less than 5% of all embryos reconstructed and transferred into recipients [7]. For this reason, it has been concluded that SCNT is one of the few reproductive techniques that fails to achieve adequate efficiency to enable large-scale commercial uses [8]. Embryo losses take place at different stages during laboratory procedures, pregnancy, parturition and the neonatal period. Taking into account these data, neonatal care is one of the critical steps that appear necessary to be considered at the development of a SCNT or SCNT-transgenesis program.

Here, we presented valuable data regarding the first bitransgenic bovine clone for human lysozyme and lactoferrin production. We agree with Fecteau et al. [7] that early and aggressive monitoring and treatment of the calves immediately after birth is recommended over a conservative "wait and see approach". At first examination, large body size and weight, meconium staining, and enlarged umbilical cord vessels were considered as markers of fetal distress that could require extended neonatology care as has been described in LOS/AOS. For these reasons, the physiological status through complete blood analysis results is determinant after reception in the NR.

As described in the results section, thirty hours after birth, clinical signs led us to think about a picture of sepsis. However, laboratory results did not coincide with this preliminary diagnosis. Total leukocyte count was considered between normal values, although normal percentage of neutrophils and lymphocytes were inverted. Polymorphonuclear Leukocytes (PMN) are the primary mediators of neonatal innate cellular defense. In human neonates, PMN are activated and increased in number with the onset of sepsis [9]. Bone marrow PMN reserves are rapidly depleted during infection, which results in a release of immature "band" forms (described as a left shift). The proportion of these forms can be used to aid the assessment of sepsis probability [10,11]. Since none of these data were found in the first hemogram, we decided to search for other markers of causalities through arterial gas analysis. Acidosis is a common finding in critically ill patients during severe sepsis/septic shock, and a powerful predictor of mortality [12]. Moreover, negative base excess, below -3 meq/L under normal pH, is considered an indicator of acidosis associated with sepsis [13]. In our work, the attention to the base excess was very useful to conclude a case of sepsis although the first two blood cultures were negative. Blood pH correction was important to improve animal performance in terms of clinical parameters, as well as to enhance animal response to antimicrobial therapy.

The most likely source of pathogenic bacteria in neonate sepsis is the contaminated environment. However, in both human and veterinary medicine, it has been proposed that infection may also be acquired *in utero* or during parturition [14,15]. Many reports have shown that cloned animals present placental insufficiencies, including included degenerated inflammatory cells, edematous chorioallantoic membranes and decreased epithelial thickness,which may be associated with the poor clinical outcome of nuclear transfer pregnancies and offspring [16-19]. As mentioned, anaerobic peptostreptococcus was identified through the last blood culture. This bacterium is considered one of the predominant ruminal bacteria found in adult bovines [20], so it is possible to think that the sepsis diagnosed in our case could be established through *in utero* infection, generating at the same time the alteration in the white cell count recorded immediately after birth. Finally, we conclude that sepsis was favored by the lack of enough colostrum ingestion during the first hours of life.

The relative sizes of the four stomachs vary greatly during prenatal development from day 56 of gestation [21]. Initially, they have the same size and then increase remarkably in abomasal size, being larger than the rumen at birth [22]. During the first three weeks of life, the first three stomach compartments (rumen, reticulum and omasum) are not used by the calf, and their development takes variable time depending upon the ingestion of suitable dry feed. During this period, milk or milk replacer directly goes into the abomasum, where it coagulates and digestion continues as in monogastric animals. Results obtained from necropsies of LOS/AOS cloned calves have shown that these animals exhibit dilated stomachs, mainly the rumen (two necropsies, our own not published results). In veterinary practice, when normal or cloned calves present weak or absent sucking, reflex tube feeding is indicated, mainly during the first hours of life when proper absorption of colostrum has to be ensured [7]. In contrast, taking our own observations into account, we could suggest that there would be risks of development of ruminal acidosis in these animals, especially using tube feeding. For this reason, our approach was to administer nutrients orally in small doses together with studies in parallel to control or prevent the development of ruminal acidosis. As mentioned in the results section, on day 37, during the last phase of the intensive care, we diagnosed acidosis by milk ingestion, which triggered the need for a total parental nutrition therapy until the animal learned to eat solid food.

Nutritional support in the neonate has been extensively described by several authors in human and veterinary medicine, being fresh milk/colostrum of its same species the preferred source of nutrients. However, when fresh milk/colostrum is not available, frozen ones are the second best options to match the natural mother's milk. Although enteral nutrition is the best way to cover the neonate's requirements, under several conditions, Partial Parental Nutrition (PPN) or TPN is the only option to achieve it.

There are several reports about the use of TPN in foals, but not so many in calves [23-25]. These works proposed different ways to achieve nutritional requirements in calves by mixing several sources of carbohydrates, lipids, amino acids, micronutrients and vitamins, especially when TPN has to be used for more than 2 weeks. Moreover, several laboratories commercialize bags for TPN to be used in human medicine which can be used in veterinary medicine after making some modifications in the dropping and supplementing them with what is considered necessary. It is important to mention the need for constant monitoring of different blood parameters of the patient, such as those considered markers of the internal medium (pH, inonogram), leukocytes, glucose, and proteins, as well as urine samples to check at least, pH, glucose and proteins. To our knowledge, no previous data is available for such a long period of TPN in bovine neonates shown as in the present work.

As we mentioned, the ingestion of box straw caused ruminal impaction and due to the lack of cellulolytic flora the calf was not capable of degrading it. For this reason we started a therapy of flora transference from a ruminally cannulated steer fed with consociated pasture This treatment was not only indicated to favor fiber degradation but was also a way of accelerating the differentiation and maturation of absorptive and metabolic functions of the rumen [26-28].

This report presents the medical procedures necessary to enable the survival of a high risk animal while allowing an increase in efficiency of cloning and transgenesis. Practical and specific information regarding calf physiological parameters are summarized, representing a valuable guide to be consider for bovine clones neonatal care.

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