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Impact of cumulative gain in expertise on the efficiency of handmade cloning in cattle

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ABSTRACT

The aim of this study was to determine the effects of the cumulative gain in expertise in carrying out handmade cloning (HMC) procedures on embryo yield and pregnancy outcome in cattle. Results from *in vitro* and *in vivo* embryo development after HMC during three periods of 7 months, separated by 3-month intervals, were compiled and designated as P1, P2 and P3. Blastocyst yield, morphological quality and stage of development, and pregnancy per embryo transfer (ET) on Day 30 of gestation were compared. Zona-intact oocytes were activated chemically in each experiment replicate, and development of parthenogenetic blastocysts was used as a control measurement of oocyte quality and *in vitro* culture conditions. A total of 21,231 *cumulus*-oocyte complexes (COCs) were *in vitro*-matured, with 5,432, 10,721 and 5078 COCs used in 16, 18 and 10 replicates for P1, P2 and P3, respectively. Cloned blastocyst yields on Day 7 increased from 15.5% (124/798) in P1 to 21.6% (309/1428) and 36.6% (280/764) in P2 and P3, respectively. No differences were observed in blastocyst development of parthenogenetic embryos, which average 30.0, 37.6, and 36.4% in P1, P2, and P3, respectively. A 10-fold higher probability of obtaining cloned blastocysts at more advanced stages of development and of higher morphological grade was seen during P3 compared with P1. Pregnancy per ET on Day 30 also increased with gain in expertise, being 6.7% (2/30), 20.8% (10/48) and 40.0% (24/60) for P1, P2 and P3, respectively. The relative efficiency for the establishment of pregnancies (per total COC) increased from 0.04% (1:2716) in P1 to 0.22% (1:460) in P2, reaching 0.47% (1:212) in P3. Results demonstrated a gradual improvement in *in vitro* and *in vivo* embryo development over time after establishment of HMC procedures in the laboratory, highlighting the importance of gaining experience and technical skills on the overall cloning efficiency.

1. Introduction

Since the birth of Dolly the sheep, the first mammal produced by somatic cell nuclear transfer (SCNT) procedures [1], the number of animal species that have been cloned has expanded significantly. Applications range from the production of genetically identical animals for genetic preservation or multiplication of genomes of interest [2], to the generation of genetically modified (GM) animals, which stands out as one of the most notable applications of animal cloning to date [3]. Most cloned animals have been produced by classical cloning procedures, which require highly trained operators, and sophisticated and expensive pieces of equipment such as micromanipulators [4]. These technical demands constrain the

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number of structures that can be reconstructed per operator, and limit the extensive use of cloning by SCNT by a greater number of research groups [4]. The gain in knowledge in the field could increase with the widespread use of the technology for scientific and commercial applications, which can be attained by further simplification of the cloning procedures, and reducing the costs without compromising the efficiency [4].

The production of bovine cloned embryos by handmade cloning (HMC) proposed by Peura et al. [5] using blastomeres as a scientific strategy to study the effect of the cytoplasmic volume on cloning, and later adapted successfully to SCNT by Vajta et al. [6], entirely eliminated the need of micromanipulators for animal cloning. The association of HMC with the zona-free culture system in microwells, or the well-of-the-well (WOW) system [7], has encouraged the widespread use of cloning by SCNT on a large scale, potentially increasing the embryo yield and reducing the runtime by procedure [4,8]. Due to its simplicity and low cost, the HMC procedures made animal cloning more accessible to many laboratories in the world to implement its use. Also, the culture of zona-free embryos in the WOW system provides an excellent tool for studies in developmental biology, such as the effects of cytoplasmic volume changes [5], embryo aggregation and cell allocation [9,10], the effect of cytoplasmic complementation by the fusion of distinct cytoplasts and/or karyoplasts for the reconstruction of cloned embryos [11,12], or even the transfection of nucleic acids into zona-free pre-implantation stage embryos for multiple applications [13].

Results from the literature describing the rate of *in vitro* or *in vivo* developmental success by the application of any biotechnological reproductive procedure to livestock show a remarkable level of variation, even for different reports from the same laboratory [14]. For instance, research groups and private laboratories in the world have produced many bovine cloned calves in past years, mostly using conventional cloning procedures [15,16] but also applying the low-cost HMC technique [17,18]. Other studies have compared both methods [19,20] or even combined steps from each procedure within one [21], and all describe various degrees of overall efficiency of the cloning procedure. However, reports on the technical progress of HMC procedures from a single laboratory, over an extended period using a significant number of structures, are still lacking. Thus, the aim of this study was to evaluate the impact of gaining in expertise and technical ability in HMC procedures on embryo yield and pregnancy outcome in cattle during three distinct intervals of activities over a 27-month period. By providing a detailed description of the modified methodology used in our laboratory and its associated hurdles, we also aimed to assist in spreading the use of the HMC procedure by other research groups.

2. Material and methods

All reagents and embryo-tested water were from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), unless stated otherwise. Most reagents and media, as well as aliquots of medium components and reagents, were from the same batches in stock at the onset of the study. Also, most disposable materials and supplies were from the same brands or manufacturers throughout the study.

2.1. Personnel and data set compilation

At onset of the study, six technicians went through a period of training by an experienced instructor, focusing on all steps needed for the successful implementation of the HMC procedures in the laboratory (August to December, 2006). Initially, the technicians had advanced experience in *in vitro* embryo manipulation in cattle (*in vitro* fertilization procedures, well established in the laboratory), but no experience in manual cloning or in handling somatic cell primary cultures. When *in vitro* development of blastocysts was successfully and repeatedly attained (January 2007), procedures for the production of cloned bovine blastocysts by HMC were initiated aiming at the establishment of pregnancies for a study on placental metabolism on Day 225 of gestation [14]. Depending on availability of biological materials (slaughterhouse ovaries, female recipients), *in vitro* embryo production and subsequent *in vivo* embryo development after cloning by HMC were carried out in three periods of 7 months separated by 3-month intervals, within a total period of 27 months. Results were compiled and named as: Period 1 (P1), from January to August 2007; Period 2 (P2), from December 2007 to June 2008, and Period 3 (P3), from October 2008 to May 2009. All laboratory work was performed by trained technicians, under the close supervision of the instructor, having always at least three of the technicians working on each weekly scheduled set of activities throughout the study. The field or animal work (estrous synchronization, embryo transfers, ultrasonography, etc.) was carried out by experienced veterinarians.

2.2. Isolation and culture of bovine nucleus donor cells

Somatic cell primary cultures were established according to procedures by Gerger et al. [14,22]. Briefly, after collection of an aseptic ear skin biopsy from a 5-year-old donor Nellore female, explants were immersed sequentially for 20 s in 70% ethanol, culture medium, 100x antibiotic-antimycotic solution, and culture medium. Explants were diced into 3–4 mm fragments, and four to five fragments were placed in 35 mm tissue culture dishes (Corning Incorporated, NY, USA) containing 0.8 mL DMEM culture medium (Gibco-BRL, NY, USA) supplemented with 0.22 mM sodium pyruvate, 26.2 mM $NaHCO_3$, 100 IU/mL penicillin G, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B and 10% fetal calf serum (FCS, Gibco-BRL). Cell cultures were established, maintained, and expanded in an incubator at 38.5 °C, 5% CO_2 and saturated humidity up to the third passage, when cells were frozen or used for cloning. When cultured cells reached a confluence greater than 90%, cells were harvested using a 0.25% trypsin/0.5 mM EDTA solution for 5–7 min and centrifuged at 400 g for 3–5 min. Cells were re-suspended in DMEM culture medium with 10% ethylene glycol and packed in 0.25 mL sterile straws in a volume of 150 μL, at a concentration of 1×10^3 cells/μL. Straws were maintained at 2 to 4 °C for 15 min and then exposed to liquid nitrogen (N_2L) vapor at a temperature between −80 and −110 °C for 5 min, immersed in N_2L and stored for later use. Thawing was performed 72 h prior to use for cloning, by immersion of straws in a water bath at 36 °C for 30 s, followed by culture in four-well dishes (Nunclon®, Nunc, Roskilde, Denmark) by dilution of the volume of each straw in 500 μL of culture medium/well (one straw per well), to obtain a high confluence (>95%) 24 h ahead of its use [22]. Approximately 6 h after re-plating frozen-thawed cells, the culture medium was replaced by fresh medium to remove the remnants of the freezing medium and dead cells.

2.3. In vitro embryo production by somatic cell nuclear transfer and parthenogenesis

The *in vitro* production of bovine cloned embryos by HMC was established based on procedures by Ribeiro et al. [10] and Gerger et al. [14,22].

2.3.1. In vitro maturation (IVM)

Cumulus-oocyte complexes (COCs) aspirated from bovine ovaries collected at three regional slaughterhouses were selected by morphology (Grades 1, 2 and 3), based on Leibfried & First [23],