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ISOLATION, CULTURE AND CRYOPRESERVATION OF UMBILICAL CORD CELLS, ADIPOCYTES CELLS AND FIBROBLASTS FROM BOVINE FETUSES

Oliveira, V.V.G.[1,2]; Silva, C.G.[2];Pires, N.L.[2]; Cunha, E.R.[2]; Guimarães, C.O.[2]; Sereno, J.R.B.[2]; Martins, C.F.[2]

[1]Universidade Federal Rural de Pernambuco, Recife, PE; [2]Laboratório de Reprodução Animal - Embrapa Cerrados, Planaltina, DF - Brasil

E-mail: carlos.frederico@cpac.embrapa.br

In the nuclear transfer technique, the ability of the oocyte remodel the cell's nucleus and result in embryo development is influenced by the cell types. The formation of a somatic cells bank is an important strategy, and involves a cryopreservation process. Considering the importance of bovine cloning, this work had the following objectives: a-evaluate the possibility of isolation and culture of umbilical cord cells, adipocytes cells and fibroblasts from bovine fetuses b-test the effect of two cryopreservation solutions containing DMSO or DMF to protect the cells, c-determine if the trypan blue dye is better than the fluorescent probes (carboxyfluorescein acetate and propidium iodide) for identify the viability of frozen cells. Bovine tissues were collected at the slaughterhouse, cut into small pieces, placed in petri dishes, covered with 3 mL of Dulbecco's Modified Eagle Medium (DMEM) and incubated in an incubator at 38.5 ° C and with $CO_2$5%. After 7 days, biopsies were retrieved and the medium changed. Passed over seven days, the cells were treated with trypsin and placed in culture bottles until reach confluence.For cryopreservation, cells were distributed in two cryoprotectant solutions: T1-DMEM with Fetal Bovine Serum (FBS) 10% and DMSO 10%, T2- DMEM with FBS 10% and DMF 5%. Then, the cell solutions were placed in 0.25 ml straws, kept at -80°C for 24 hours and stored in liquid nitrogen. The rate of cell viability after thawing was assessed with trypan blue dye and carboxyfluorescein acetate- propidium iodide. In all tissues studied was possible to isolate cells with the same growth pattern in DMEM. The medium with cryoprotectant DMSO preserved 85.50 ± 4.95%, 37.5 ± 10.6% and 82 ± 8.48, of fetal fibroblasts, umbilical cord cells and adipocytes cells, respectively. Already the cryoprotectant medium with DMF preserved 54 ± 5.65%, 28.5 ± 21.92% and 56± 1.41%, these same cells, respectively.The medium with DMSO was significantly (P=0.0096) more efficient than the medium with DMF to preserve the viability of different cell types. There was no statistical difference between the trypan blue and carboxyfluorescein acetate-propidium iodide to detect cell integrity. Thus, we conclude that is possible to isolate and cryopreserve fetal fibroblasts, umbilical cord cells and adipocytes cells to compose a cryobank with potential use in nuclear transfer technique. The cryoprotection medium with DMSO is indicated to cryopreserve the cell types studied and cell viability can be efficiently evaluated with trypan blue or carboxyfluorescein acetate and propidium iodide.

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EFFECT OF TECHNICAL PROGRESS ON THE EFFICIENCY OF HANDMADE CLONING IN CATTLE

Ortigari Jr., I.[1,2,3]; Gerger, R.P.C.[1,3,4]; Ribeiro, E.S.[1]; Mezzalira, J.C.[1]; Zago, F.[1,5]; Gaudencio Neto, S.[1]; Ohlweiler, L.U.[1]; Martins, L.T[1].; Bertolini, L.R.[1]; Forell, F.[1]; Costa, U.M.[1]; Lopes, R.F.F.[6]; Rodrigues, J.L.[6]; Ambrósio, C.E.[7]; Miglino, M.A.[4]; Mezzalira, A.[1]; Bertolini, M.[1,8]

[1]CAV, UDESC, Lages, SC; [2]FURB, Blumenau, SC; [3]Central Santa Rita de Embriões, Itajaí, SC; [4]FMVZ, USP, São Paulo, SP; [5]EPAGRI, Lages, SC; [6]FAVET, UFRGS, Porto Alegre, RS; [7]FZEA, USP, Pirassununga, SP; [8]UNIFOR, Fortaleza, CE - Brasil

E-mail: centralsrita@brturbo.com.br

Research groups and private labs in Brazil have produced many bovine clones in the past years using conventional cloning methods. However, reports on technical development or even on births of clones using the handmade cloning (HMC) procedures are still lacking in the country. The aim of this study was to determine the effects of the gain in expertise in handmade cloning on embryo yield and pregnancy outcome in cattle during three periods of activities (28 months). Results from *in vitro* embryo production and subsequent *in vivo* embryo development after cloning by HMC during three periods of seven months, separated by three-month intervals, were compiled as Periods 1 (P1), 2 (P2) and 3 (P3). Cloned bovine embryos were produced and transferred to recipient females according to our previous procedures (Gerger *et al.*, Genet Mol Res, 2010, 9:295-302). Data regarding blastocyst rates, morphological quality (Grades 1-3) and stage of development (Stages 5-9), based on the IETS Manual (Stringfellow & Seidel, IETS Manual, 1998, 173-176), and pregnancy rates on Day 30 of gestation were compared by the ÷2 test, for $p<0.05$. A total of 21,231 COCs were *in vitro*-matured, with 5,432, 10,721 and 5,078 COCs used in 16, 18 and 10 replications per period (P1, P2 and P3), respectively. Blastocyst yield on Day 7 of development increased from 15.5% (124/798) in P1 to 21.6% (309/1428) and 36.6% (280/764) in P2 and P3, respectively. A 10-fold higher probability of obtaining blastocysts at more advanced stages of development (Stages 7 and 8/9) and of embryos of higher morphological grade (Grade 1) was seen during P3 when compared with P1, improving chances from 5.0% in P1 and 26.7% in P2 to 49.7% in P3. Pregnancy rates also increased as a function of technical progress over time, 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 on Day 30, based on the total number of COCs used in each period (pregnancies per COC), increased significantly from 0.04% (1:2,716) in P1 to 0.22% (1:460) in P2, finally reaching 0.47% (1:212) in P3. Our results demonstrated a significant and gradual improvement in the rates of *in vitro* and *in vivo* embryo development over time after cloning, highlighting the importance of the gain of experience and technical skills on the overall HMC efficiency. This work also demonstrated the feasibility of manual cloning procedures under our conditions, which was translated by the birth of a Nellore clone calf in Brazil by handmade cloning in 2008. Financiamento CAPES/PROCAD