Bovine Cloning Using Recipient Cytoplasts Recovered from Hormone-Stimulated Heifers

Clonación de Bovinos Utilizando Citoplasmas Recipientes Recuperados de Novillas Estimuladas con Hormonas

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ABSTRACT

This study aimed to investigate the efficiency of nuclear transfer (NT) using recipient oocytes collected by ultrasound-guided follicle aspiration (OPU) from heifers stimulated with follicle-stimulating hormone (FSH) and with synchronized follicular wave. Donor cells were isolated from ear skin of 9 years-old Junqueira cow (*Bos taurus*), specimen of endangered Brazilian Creole breed. For non-FSH stimulated group, sixteen cyclic crossbred heifers had the ovaries aspirated. And for FSH-stimulated group, 8 animals were selected from non-FSH group to be administered with 180 IU FSH (single dose, s.c.) 72h before OPU. For both groups seven sessions of OPU were performed every four days. The mean (±SE) number of follicles aspirated and oocytes collected per animal per OPU session was significantly lower (P<0.01) for non-FSH group (7.7±0.24 and 5.3±0.29) than for FSH group (11.2±0.52 and 8.1±0.50). No significant differences (P>0.05) were observed in fusion (84.4% vs. 89.03%) and blastocyst (46.36% vs. 37.66%) rates between NT using non-FSH or FSH stimulated oocytes, respectively. However, cleavage rate was higher (P=0.031) for non-FSH group (70.90%) than for FSH group (57.14%). Transfer of embryos showed that pregnancy rates between initial (40 days) to 180 days of gestation were not significantly different (P>0.05). These results suggest that FSH stimulation increased the number of oocytes retrieved by OPU. However, we did not observe improvement on in vitro and in vivo NT efficiency administering FSH in donor oocyte heifers with synchronized follicular wave.

Key words: Bovine, animal cloning, oocyte competence, FSH, follicular wave

RESUMEN

Este trabajo objetivó evaluar la eficiencia de la Transferencia Nuclear (TN) utilizándose ovocitos receptores colectados por aspiración folicular guiada por ecografía (OPU) en novillas estimuladas con hormona folículo estimulante (FSH). Células donadoras de núcleos fueron aisladas de la piel de una vaca Junqueira (*Bos taurus*), raza en vías de extinción, a los 9 años de edad. En el tratamiento1 (T1) 16 novillas tuvieron ovarios aspirados. En el T2, se separaron 8 novillas del T1 para administración 180 UI de FSH (Pluset®), s.c., 72h antes de cada punción, en aplicación única. En ambos grupos se realizaron 7 sesiones de OPU con intervalos de 4 días entre ellas. El promedio (±SE) del número de folículos aspirados y ovocitos colectados por animal en cada sesión fue significativamente menor (P<0,01) en el T1 (7,7±0,24 y 5,3±0,29) que en el T2 (11,2±0,52 y 8,1±0,50). Se recuperaron 470 y 411 ovocitos de los T1 y T2, respectivamente. No se observó diferencia significativa en las tasas de fusión (84,40% vs. 89,08%) y blastocisto (46,36% vs. 37,66%), mientras que la tasa de clivaje fue significativamente mayor (P=0,031) en el T1 (70,90%) que en el T2 (57,14%). En el T1, se realizó la inovulación de 46 embriones en 34 receptoras y, en el T2, de 36 embriones en 29 receptoras. Evaluaciones ecográficas revelaron que no hubo diferencia significativa (P>0,05) entre el T1 y el T2 en los índices de preñez, desde inicial (61,76% vs. 41,38%) hasta 180 días (5,88% vs. 6,9%) de gestación. Los resultados de este experimento sugieren que la administración del FSH en donadoras de ovocitos no influenció en los parámetros evaluados.

Palabras clave: Bovino, clonación animal, competencia ovocitaria, FSH, ondas foliculares.

1. INTRODUCTION

The somatic cell nuclear transfer (SCNT) encloses several processes and most of the molecular events are still poorly understood. In the last years, many works have demonstrated the feasibility of using a variety of somatic donor cells types and genotypes for NT (Inoue et al., 2003). However, up to now, the source of recipient cytoplasm remains restrict to matured oocyte (Jouneau and Renard, 2003), due to its particular cellular and molecular content. It has been largely emphasized that maternal lineage, the nuclear-cytoplasmic interactions and the nutritional status of oocyte donors (Peura et al., 2003) significantly affect nuclear transfer outcomes.

The aim of this study was to investigate whether the FSH administration in oocyte donor heifers influences the OPU rates, as well as in vitro and in vivo developmental competence of somatic cell nuclear transfer. Emphasizing that the oocytes were recovered during the growth phase of the first follicular wave, that is, from animals with synchronized follicular waves, since OPUs were performed every four days in both groups. To our knowledge, this is the first work that reported the effects of FSH stimulation in recipient cytoplasts, recovered by OPU technique, for NT purposes in bovine.

2. MATERIALS AND METHODS

Non-FSH group

Sixteen cyclic crossbred (*Bos indicus* x *Bos taurus*) heifers were used as oocyte donor for nuclear transfer. Oocytes were retrieved by transvaginal ovum pick-up (OPU) using an ultrasound equipped with an 18 gauge needle connected to a vacuum pump. Prior to oocyte collection, the large follicles (5mm) were aspirated for dominant follicle ablation (considered as day 0–D0). On D4, oocytes were aspirated by OPU from >2mm follicles. For this group, the OPUs were performed every 4 days (7 sessions). Each 2 OPU sessions the donor heifers received a dose of prostaglandin (Ciosin).

FSH group

Eight animals were selected from non-FSH group according to ovary status and response to epidural anesthesia. There were 7 sessions of OPU for this group. As proceeded in non-FSH group, before starting the experiment, all follicles ^{3} >5mm were removed (D0). On D1, the FSH was administered subcutaneously in a single dose of 180IU (Pluset). The OPU session was performed 72h after FSH injection (D4), following the same non-FSH procedures. All experimental procedures were carried out according to the relevant guidelines for the care and use of animals.

2.1 In vitro maturation

All oocytes were, in groups of 20 to 30, transferred to 400µL of maturation medium (TCM 199 supplemented with 10%FCS, 24IU/mL of LH, 10g/mL of FSH, 100IU/mL of penicillin and 100g/mL of streptomycin), covered with 400µL of silicone oil and kept at 39°C in 5%CO_2 air atmosphere with high humidity.

2.2 Isolation and culture of adult ear skin fibroblasts

Donor cells used as karyoplast donors were isolated as described previously (Iguma et al., 2005). Briefly, a small ear skin biopsy of a 9 years-old Junqueira cow (*Bos taurus*), a specimen of endangered Brazilian Creole breed, was minced into small pieces and these fragments were placed on the bottom of 25cm^2 tissue culture flasks filled with 2mL of cell culture medium (Dulbecco Modified Eagle Medium [D-MEM]) supplemented with 10%v/v fetal calf serum 100IU/mL penicillin G and 100g/mL streptomycin. The flasks were put into an incubator at 39°C, 5%CO_2 in air with saturated humidity. When cells reached confluence they were trypsinized for 10min at 39°C and then centrifuged to recover the cells.

2.3 Nuclear transfer

Cumulus-oocyte complexes (COCs) were striped from cumulus cells at 19-21h of IVM by pipetting them for 5min in 0.15%w/v hyaluronidase. Only oocytes that extruded the first polar body and with homogenous or slight heterogeneous cytoplasm were used. The polar body and metaphase chromosomes were removed in manipulation medium drops, using a beveled glass pipette measuring 27-30µm outside-diameter.

Reconstruction and fusion

Following enucleation and reconstruction, karyoplast-cytoplast complexes (KCCs) were subjected to electrofusion applying double DC pulses of 1.8kV/cm each for 30 msec.

From 3 to 5 hours postfusion, KCCs were exposed to 2μM ionomycin for 5min, and then incubated for 5h in 2mM 6-DMAP. After activation, the reconstructed embryos and oocytes were co-culture with granulosa cells monolayer in 200μL drops of culture medium consisting of SOFaaci supplemented with 5%FCS covered with silicone oil at 39°C and 5%CO_2 in humidified air. The cleavage rate and blastocyst rate were evaluated on day 2 and day 7 after nuclear transfer, respectively. The blastocysts were transfered to surrogate cows.

2.4 Embryo transfer and pregnancy evaluation

On day 7 after nuclear transfer, 1-3 blastocysts from both experimental groups were non-surgically transfered to synchronous recipient heifers.

Pregnancy was assessed by transvaginal ultrasonography between 37 and 42 days of gestation, and monitored every two weeks for pregnancy status, fetal and placental development.

The effects of FSH administration on OPU data were analyzed using a non-parametric Kruskal-Wallis test followed by Mann-Whitney test for two independent samples. The results were presented as mean±SEM values provided by descriptive statistics. For comparison of fusion, cleavage and blastocyst rates as well as initial and 60days pregnancies rates qui-square test was used to test the results between FSH and non-FSH treatments, and $P<0,05$ was considered significantly different. Fisher exact test was performed to analyze pregnancy rates from Day 90 to Day 180 of gestation.

3. RESULTS

Parthenogenotes were used as control of activation procedures and culture system. Then, a sample of oocytes from both groups (FSH and non-FSH) was parthenogenetically activated and cultured in vitro, and the overall rate of cleavage (78.31%vs70.33%) and blastocyst (54.24%vs53.38%) did not show significant differences between FSH and non-FSH groups, respectively (Table 2).

The OPU observations and results for non-FSH and FSH groups are shown in Table 1.

Table 1. Effects of treatment on numbers of follicles aspirated and oocytes retrieved (7 OPUs per treatment)

Experimental group	Mean* n°of follicles aspirated/heifer/session	Mean* n°of oocytes retrieved/heifer/session	Retrieval rate %**	Total n°° of oocytes retrieved	Total n°° of viable oocytes (%)
Non-FSH (n=16)	7.7 ±0.24[a]	5.3±0.29[a]	66.0 ±2.64	470	367 (78.08)
FSH (n=8)	11.2 ±0.52[b]	8.1±0.50[b]	71.5 ±2.93	411	319 (77.61)

Values within column with different superscripts are significantly different

[a,b] $P < 0.001$; Kruskall-Wallis and Mann-Whitney tests

*Mean ±SEM

**Number of oocytes retrieved / Number of follicles aspirated; $P = 0,297$

Table 3. Parthenogenetic control of NT cytoplast recipient

Parthenogenetic groups	Replicates	Cleavage rate (%)	Blastocyst rate (%)
Non-FSH	8	70.33	53.38
FSH	7	78.31	54.24

3.2 In vitro developmental capacity of NT embryos

The in vitro development of cloned embryos derived either from non-stimulated or FSH-stimulated oocytes is summarized in Table 3.

Table 3. Effects *in vitro* of FSH administration in oocyte donor cows for nuclear transfer using Junqueira breed ear skin fibroblasts

Cytoplast type	KCCs*	Fusion rate**	Cleavage rate ***	Blastocyst rate ***
Non-FSH	173	84.40%	70.90%[a]	46.36%
FSH	236	89.08%	57.14%[b]	37.66%

Values within column with different superscripts are significantly different
[a,b] $P < 0.05$
*Karyoplast-Cytoplast Complexes
**Based on the number of KCCs
***Based on the number of fused KCCs

3.3 Pregnancy rates of NT embryos

The data of NT embryo transfer and pregnancies results analyzed statistically are shown in Table 4.

Table 4. Effects *in vivo* of FSH administration in oocyte donor cows for nuclear transfer using Junqueira breed ear skin fibroblasts

Cytoplast type	Transferred embryos	Recipients	Initial pregnancy (%)*	60 DP (%)**	90 DP (%)**	120 DP (%)**	150 DP (%)**	180 to 210 DP (%)**
Non-FSH	46	34	21 (61.76)	9 (26.47)	4 (11.77)	3 (8.82)	3 (8.82)	2 (5.88)
FSH	36	29	12 (41.38)	5 (17.24)	3 (10.35)	3 (10.35)	3 (10.35)	2 (6.90)
TOTAL	82	63	33 (52.38)	14 (22.23)	7 (11.12)	6 (9.52)	6 (9.52)	4 (6.35)

DP = Days of Pregnancy. Based on the number of recipients.
*Ultrasonographic assessment 30 to 35 days after embryo transfer. Based on the number of recipients.
**Ultrasonographic assessments

4. DISCUSSION

The present study may suggest that FSH stimulation of oocyte-collected heifers increases the average number of follicles observed and, consequently, the average number of oocytes recovered, as previously reported (Goodhand et al., 1999). Actually, the effect of exogenous gonadotrophins administration prior to OPU on the oocytes used as a source of recipient cytoplasm in bovine nuclear transfer has not been described so far.

We observed a lower cleavage rate in FSH group comparing with non-FSH group (57.14% vs. 70.90%, respectively). This result may be attributed to uncoupled follicle and oocyte maturation induced by rapid follicular growth, leading to an asynchrony between nuclear and cytoplasmic factors of oocyte maturation (Izadyar et al., 1998). Likewise, the observations of Combelles and Albertini (2003) also may explain our data in terms of lower cleavage rate in FSH group than in non-FSH group, since these authors reported that oocytes subjected to repeated gonadotrophin stimulation have reduced ATP content in mouse. Considering that ATP storage is the main source of energy to support cellular processes during oocyte maturation and early cleavage stages it seems plausible that up to embryonic genome activation the zygote development may be affected in individuals derived from stimulated oocytes.

The majority of superovulatory protocols includes multiple and declining doses of FSH. Our reasons for choosing a single FSH injection scheme were based on previous studies carried out in our laboratory (Pivato, 2001), which evaluated different hormonal treatments in IVP process.

Gonadotrophins stimulation reduces the number of animals necessary to provide a source of recipient cytoplast. Therefore, this work would support the decision of at what extent is interesting to keep a batch of heifers and/or cows, as a source of oocytes, to reach a more efficient NT system.

Another research is in process, in view of comparing the competence of oocytes collected from animals during the growth phase of the first follicular wave and COCs recovered from slaughterhouse ovaries, which would be at different stages of estrous cycle. We also intend to evaluate the effects on OPU

rates, as well as in vitro and in vivo developmental capacity of oocytes recovered from heifers and/or cows treated with FSH associated to LH and/or bovine somatotropin (bST).

CONCLUSION

These results suggest that FSH stimulation increased the number of oocytes retrieved by OPU. However, we did not observe improvement on in vitro and in vivo NT efficiency administering FSH in donor oocyte heifers with synchronized follicular wave.

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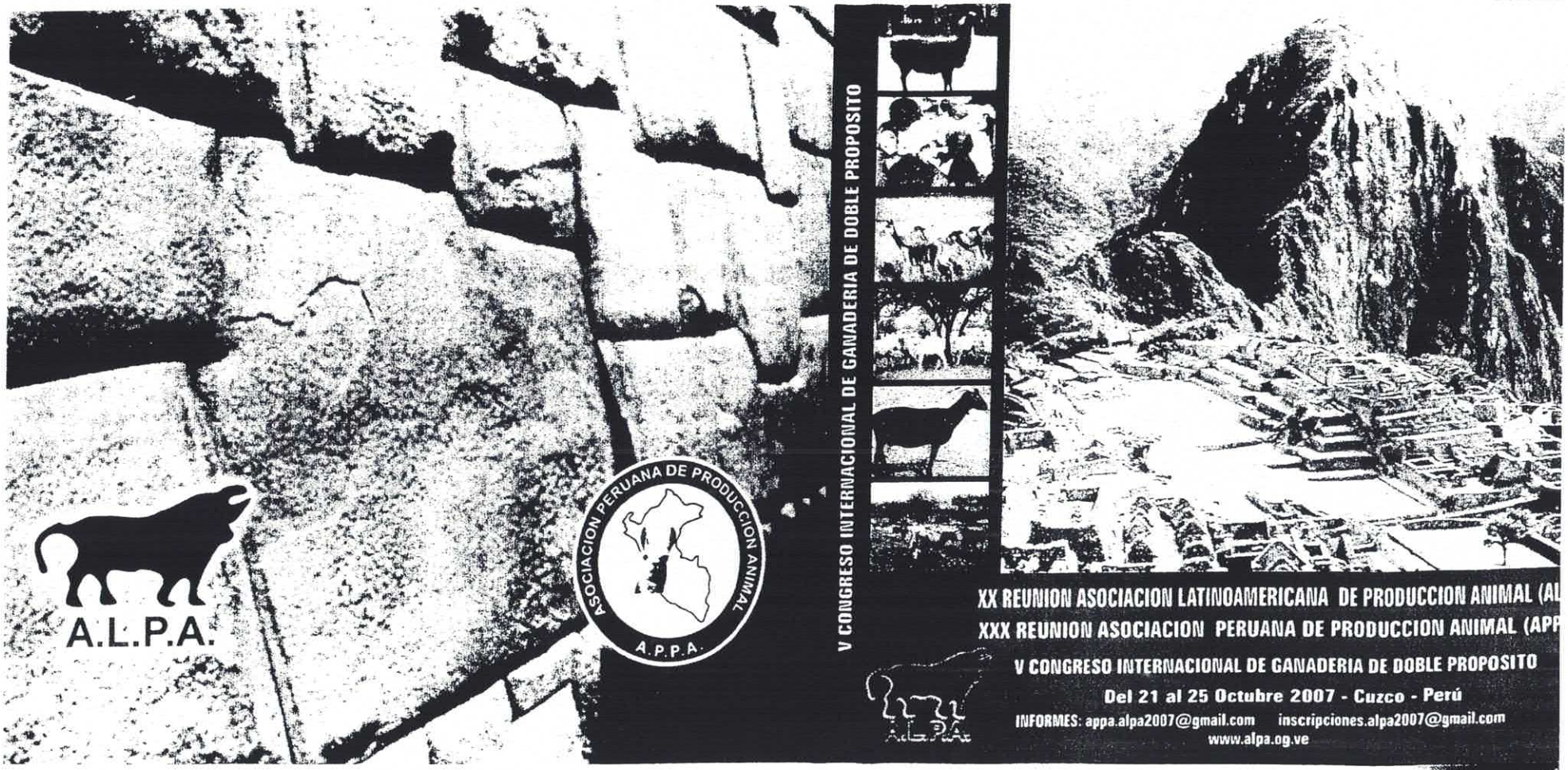
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