SHORT-TERM CULTURE OF BOVINE BISECTED EMBRYOS. EFFECTS ON PREGNANCY RATES, SEX RATIO AND BIRTH WEIGHT OF CALVES[1]

RAFAEL HERRERA ALVAREZ[2], JACKSON BARROS DO AMARAL[3], RITA MARIA LADEIRA PIRES[3], ANTONIO CAMPANHA MARTINEZ[4], EUNICE OBA[5]

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[2]Pólo Regional de Desenvolvimento Tecnológico dos Agronegócios do Centro Sul (PRDTA), Agência Paulista de Tecnologia dos Agronegócios (APTA), Secretaria de Agricultura e Abastecimento do Estado de São Paulo (SAA), Rod. SP 127, Km 30, Caixa postal 28, CEP 13400-970, Piracicaba, SP, Brasil. E-mail: rherrera@apta.sp.gov.br
[3]Centro de Pesquisa e Desenvolvimento em Genética e Reprodução Animal (CPDGRA), Insituto de Zootencia (IZ), Agência Paulista de Tecnologia dos Agronegócios (APTA), Secretaria de Agricultura e Abastecimento do Estado de São Paulo (SAA), Rua Heitor Penteado, 56. 13460-000. Nova Odessa, SP, Brasil.
[4]Centro de Ciências Agrárias, Universidade Estadual de Maringá (UEM),Campus Avançado de Umuarama, Estrada da Paca, s/n, CEP 87507-190, Umuarama, PR, Brasil.
[5]Departamento de Reprodução Animal e Radiologia Veterinaria, Faculdade de Medicina Veterinária e Zootecnia (FMVZ), Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Distrito Rubião Jr, s/n, CEP 18618-000, Botucatu, SP, Brasil.

ABSTRACT: This study evaluated pregnancy rate, gestation length, calving rate, and also sex proportion and birth weight of calves derived from in vitro reconstructed bovine demi-embryos. Recovered embryos (grade I morulae) from superovulated cows were bisected with a steel blade and transferred to recipient heifers either immediately (group 1, n=38) or after a 24h period in culture medium (EARLE + 10% FCS) at 38.5 °C and 5% CO_2 (group 2, n=34) when showed a reconstituted appearance. Intact, uncultured embryos immediately transferred to recipients after collection (group 3, n=36) were used as control. Data were analyzed by ANOVA and Chi-square test. In vitro cultured demi-embryos that developed from morulae to blastocyst (78%, 44/56) were considered viable. Pregnancy rate at day 45 was 47.4%, 52.9%, and 52.8% for groups 1, 2, and 3 respectively (P> 0.05). Calving rate reached 26.3% in group 1. 29.4% in group 2, and 30.6% in group 3 (P> 0.05). No difference was found regarding pregnancy length (280.8 ± 1.8, 285.5 ± 1.7 and 285.3 ± 1.7 days), male proportion (60%, 50% and 54.5%) and birth weight of calves (38 ± 4.2, 32.4 ± 2.5 and 36.2 ± 2.6 kg) among groups 1, 2 and 3, respectively. It was concluded that even though *in vitro* reconstructed demi-embryos transfer can be of some utility to select the viability of demi-embryos, this procedure is not able to improve pregnancy rates since it does not differ from uncultured demi-embryos.

Key words: bovine, embryo splitting, , pregnancy rate, short-term culture.

CULTIVO IN VITRO TEMPORÁRIO DE EMBRIÕES BOVINOS BIPARTIDOS. EFEITO NA POSTERIOR TAXA DE PRENHEZ, SEXO E PESO DOS BEZERROS

RESUMO: O presente estudo objetivou avaliar a taxa de prenhez, a duração da prenhez e a taxa de parição após a transferência de embriões bipartidos e reconstituídos in vitro, bem como algumas características (proporção de sexo e peso ao nascimento) dos bezerros produzidos. Embriões (mórulas grau 1) recuperados de vacas superovuladas foram bipartidos com uma lâmina metálica e transferidos em novilhas receptoras logo em seguida (grupo 1, n=38) ou após 24 horas de cultivo (meio EARLE + 10% SFB) a 38,5°C e atmosfera de 5% de CO_2 (grupo 2, n=34). Como controle foram utilizados embriões intactos, não cultivados, transferidos logo após a coleta (grupo 3, n=36). Os dados foram analisados utilizando ANOVA e teste de χ^2. Os hemi-embriões culti-

vados que evoluíram ao estágio de blastocisto (78%, 44/56) foram considerados viáveis. A taxa de prenhez no dia 45 após a transferência foi de 47,4%, 52,9% e 52,8% para os grupos 1, 2 e 3, respectivamente (P> 0,05). A taxa de parição foi de 26,3% no grupo 1, 29,4% no grupo 2 e 30,6% no grupo 3 (P> 0,05). Não houve diferença (P>0,05) na duração da prenhez (280,8 ± 1,8, 285,5 ± 1,7 e 285,3 ± 1,7 dias), na proporção de machos (60,0%, 50,0% e 54,5%) e no peso dos bezerros ao nascimento (38,0 ± 4,2, 32,4 ± 2,5 e 36,2 ± 2,6kg) entre os grupos 1, 2 e 3, respectivamente. Foi concluído que o cultivo in vitro dos hemi-embriões durante 24 horas pode ser uma boa medida para eliminar os que não são capazes de continuar seu desenvolvimento, porém, a transferência dos que evoluem em cultivo não aumenta a taxa de prenhez quando comparada à transferência de hemi-embriões não cultivados.

Palavras chave: bipartição de embriões, cultivo in vitro, bovinos, taxa de prenhez

INTRODUCTION

The possibility of producing mammals throughout micro-surgically divided embryos was suggested many decades ago (Nicolas and Hall, 1942; Tarkowski, 1959). The first set of identical mice was produced in 1970 through mechanical isolation of blastomeres derived from two-cell embryos (Mullen *et al*., 1970). Later on, the use of bisection techniques applied to morulae and blastocyst stage embryos lead to the production of identical twins, triplets and quadruplets of several species such as ovine, bovine, swine, and equine (Willadsen, 1982).

The mechanical bisection process of embryos causes considerable loss and destruction of blastomeres and also leaves demi-embryos with 50% less cells than intact embryos. Although the remaining few cells of the demi-embryos have little effect in its further survival after transferred to recipients (Williams *et al*., 1984; Baker and Shea, 1985; Gray *et al*., 1991; Kippax *et al*., 1991; Nogueira *et al*., 1993), some authors (Reichenbach *et al*., 1998; Riedl *et al*., 1996) suggested that *in vitro* demi embryo culture can be an interesting alternative to raise pregnancy rate because this process permit to identify unviable embryos before transfer. *In vitro* culture during a certain period after bisection allows the exclusion of embryos unable to continue their development what enables a better utilization of recipients. In addition, the strategy of demi embryo culture has been successfully used to increase its resistance to the freezing process (Chesné *et al*., 1987; King *et al*., 1992).

In a recent study using demi-embryos cultured for 24h before transfer, Alvarez *et al.* (2004) did not observe differences neither in pregnancy rate nor in sex ratio evaluated by ultrasound imaging. However, there are evidences that specific culture systems, mainly those supplemented with bovine fetal serum, cause embryo modifications resulting in higher male proportions (King *et al*., 1992) and in larger and heavier newborn calves with health problems (McEvoy *et al*., 2001). The present study aimed the evaluation of pregnancy characteristics (rate and length) and calf traits at birth (sex and weight) after the transfer of bisected bovine embryos reconstructed *in vitro*.

MATERIAL AND METHODS

Experiment location

The experiment was carried out at the Laboratory of Biotechnology Applied to Animal Production from the Center of Research and Development of Genetics and Animal Reproduction of the Instituto de Zootecnia, located in Nova Odessa, São Paulo, Brazil (22°, 77′ S Lat and 47°, 29′ W Lon).

Animals and management conditions

Nelore and Caracu cows aged 8.4 ± 2.1 years (average ± SE) weighting 510.6 ± 52.8kg were used as donors, while crossbred heifers with average age of 2.9 ± 0.4 years and weighing 380.1 ± 50.3kg were used as recipients. Animals remained in guineagrass (*Panicum maximum*, Tanzania cultivar) pasture and received concentrate ration in the dry season, attempting to reach their nutritional requirements. Mineral salt and water were available *ad libitum*. Annual tests of the most important reproductive diseases (brucellosis, IBR and leptospirosis) were accomplished in laboratories registered by the Brazilian Agriculture Ministry to promote the animals' sanitary control. All procedures for using animals were approved by the Instituto de Zootecnia Animal Ethics Committee.

Superovulation protocol and embryo collection

Donor superovulation started four days after an intravaginal implant placement containing 1.9 g of progesterone (Eazi-Breed CIDR, Inter Ag, NZ) and an intramuscular injection of 2.5 mg estradiol benzoate (Estrogin, Lab. Farmavet, Brazil). Two FSH-LH injections (Pluset, Lab. Calier, Spain) were applied with a 48h-period interval between the first (320 UI, subcutaneous) and the second one (80 UI, intramuscular). Luteolysis was induced with a 150 mcg intramuscular injection of D (+) Cloprostenol (Veteglan, Lab. Calier, Spain) immediately after the second Pluset injection. Estrus expression was expected after the removal of the vaginal implant (12h after Veteglan injection). As soon as estrus started, donor cows were placed together with bulls of proven fertility for controlled mating. Embryo cervical collection was done through uterine wash with Dulbeco's Phosphate Buffer Saline (PBS, Lab. Nutricell, Brazil) seven days after mating. Recovered embryos (morulae and blastocyst) were morphologically evaluated according to International Embryo Transfer Society criteria (Robertson and Nelson, 1998) and only good quality morulae (grade I) were used.

Bisection, culture and embryo transfer

Embryos were randomly distributed into three groups after collection. Group 1 and 2 embryos were bisected as follows: embryos were placed in a PBS drop (0.5ml approximately) on a glass Petri dish without bovine fetal serum (FCS, Lab. Nutricell, Brazil). A microsurgical blade mounted on a micromanipulator (Narishige, Japan) fixed to an inverted phase-contrast microscope (Olympus, Japan) was used to bisect embryos. Each embryo half was removed from the dish (the zona pellucida was discarded) after the addition of 0.5ml PBS with 20% FCS, conditioned into 0.25ml straws and immediately transferred to the recipients by cervical transfer (group 1, n=38) or placed (both zona pellucida-free halves) into 1.5-mm x 1.5-mm culture dishes containing 1ml TCM 199 medium (EARLE, Lab. Nutricell, Brazil) and 10% FCS and cultured at 38.5°C in a 5% CO_2 atmosphere. Demi-embryos that developed from morulae to blastocyst after a 24 h culture period were transferred to recipients (group 2, n=34). Intact, uncultured embryos (group 3, n=36) were transferred to recipients at the same time as demi-embryos from group 1.

***Estrus synchronization*, pregnancy diagnosis and recipients parturition**

Recipients (heifers that expressed previous estrus within 7-14 days) received im 150mcg Veteglan 24h before donors, in order to have their estrus synchronized. Pregnancy was diagnosed 45 days after transfer by ultrasound imaging (Ultrasound Pie Medical, Nederland). After parturition calves were weighted and the sex was registered.

Statistical analysis

Data analysis was carried out using the program STATISTICA for windows (StatSoft, Inc., Tulsa, OK, USA). Categorical variables were analyzed by Chi-square test, while ANOVA was used to establish differences in gestation length and calve weight. The significance level considered was $P<0.05$.

RESULTS AND DISCUSSION

From 60 bisected morulae 114 (95%) halves were obtained. Six halves were lost during bisection process (adhered to microsurgical blade or were lacerated). Thirty-eight halves were immediately transferred to synchronized recipients, 20 were discarded because there were no available recipients and 56 halves were cultured. From the 56 cultured halves, 44 (78.6%) continued their development until the formation of a blastocoele cavity and inner cell mass region. These cultured blastocysts presented approximately half size of intact blastocysts. The *in vitro* development rate of demi-embryos (78.6%) was considered satisfactory and very close to results of McEvoy and Sreenan (1990) which found 83% of blastocists after 48h culture of good quality bisected morulae. These results showed that short-term culture of bisected embryos represent an interesting way to identify degenerated embryos, which are not appropriate for embryo transfer. Additionally, the present study showed that the bisection process of morphologically low quality embryos followed by *in vitro* culture allows the achievement of apparently good quality blastocysts able to continue their development when transferred to recipients.

Six from the 44 cultured demi-embryos that developed to blastocyst were lost when preparing the straw and other four were dismissed because there were no available recipients, thus only 34 were transferred. Results of pregnancy rates after transfer

of bisected and intact cultured embryos are showed in Table 1.

Table 1. Pregnancy rate of recipients after transfer of not cultivated and cultivated demi-embryos, and not cultivated intact embryos

Embryo	n	Pregnant at 45 days
Not cultivated bisected	38	18 (47.4%)a
Cultivated bisected	34	18 (52.9%)a
Not cultivated intact	36	19 (52.8%)a

[a]Difference was no significant (P>0.05).

An embryonic and/or fetal loss of 43% was observed in all groups, leading to calving rates of 30.6% (whole embryo), 26.3% (uncultured demi embryo) and 29.4% (cultured demi-embryos), respectively (P>0.05). Recipients that suffered fetal loss returned to estrus within 60 to 120 days after transfer. Individual transfer of cultured and uncultured demi embryos resulted in the birth of two identical calves in each group. In Table 2 is presented the pregnancy length, sex and birth weight of calves.

Table 2. Pregnancy lenght, sex (male proportion) and weight of calves after the transfer of not cultivated and cultivated demi-embryos, and not cultivated intact embryos

Embryo	Pregnancy length (mean ± SEM) (days)	Males (%)	Weight of calves (mean ± sem) (kg)
Not cultivated bisected	285.5 ± 1.7a	6/10 (60.0)a	32.4 ± 2.5a
Cultivated bisected	285.3 ± 1.7a	5/10 (50.0)a	36.2 ± 2.6a
Not cultivated intact	280.8 ± 1.8a	6/11(54.5)a	38.0 ± 4.2a

[a]Difference was no significant (P>0.05).

No difference (P>0.05) was found on average pregnancy length, nor in sex ratio and birth weight of calves. Therefore, the hypothesis that short-term culture of demi-embryos before transfer has a benefic effect was not confirmed in this experiment, since there was no difference among pregnancy rates obtained with cultured or uncultured demi-embryos and intact embryos. KING *et al*. (1992) obtained a pregnancy rate of 33% using demi-embryos that had been frozen after a 24-48h culture and 23% of pregnancy rate using demi-embryos that had been also frozen, but immediately transferred to recipients after bisection. In the same experiment there were more male than female fetuses suggesting that bisection and long period cultures can affect mostly female embryos. Even though these authors suggested that this sex difference could be associated to the culture system or to the bisection process, the present results make evident that this sex deviation would be associated to the freezing process and not to bisection or culture. According to Carvalho *et al*. (1996), male embryos develop faster *in vitro* than female embryos. The authors reported that both full-expanding blastocysts and hatched blastocysts had a higher (P<0.05) proportion of males (68 and 100%, respectively), while morulae had a significantly lower proportion of males (24%). Early blastocysts and blastocysts did not differ from a 1:1 sex ratio. Consequently, it is possible that our 24 hours culture system is suitable to obtain male and female blastocysts in similar proportion. Therefore it is necessary to increase the replicates in order to obtain more consistent results.

Fetal or embryonic loss (approximately 40%) registered in all studied groups makes evidence that this event has no relation to bisection process or culture. In this study it is hard to elucidate why recipients that received intact uncultured embryos had high gestation losses since the tests of the most important reproductive diseases (brucellosis, IBR and leptospirosis) were negative. Therefore, factors regarding embryos or recipients might be considered when evaluating the high embryonic loss. Such factors include the season, synchrony between the recipient and the embryo, parity, ovarian condition and recipient management, included forage supply (SPELL *et al*., 2001; STROUD AND HASLER, 2005).

Bisection and culture process did not affect gestation length neither calves' birth weight. THOMPSON *et al*. (1998) working with *in vitro* cultured embryo (FCS supplied or not) also did not find difference in gestation length neither in calves' birth weight. The same authors reported that one of the

bulls produced significantly heavier calves than the expected suggesting a bull-calf interaction. Therefore, the deleterious effect caused by embryo manipulation and culture system suggested by some authors (SANGILD *et al*., 2000; FARIN *et al*., 2006) was not confirmed in this study.

CONCLUSION

In conclusion, in spite of *in vitro* culture system being harmless for embryo later development, the present results do not recommend *in vitro* culture to improve pregnancy for practical purposes, since the pregnancy rate obtained was not higher than when using uncultured demi-embryos.

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