

Cattle offspring obtained after transfer of IVP, GFP positive and GFP positive-frozen/thawed embryos*

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The objective of the study was to compare the length of pregnancy, calving rate, birth weight and sex after transfer of IVP (group I), GFP positive (group II) and GFP positive-frozen/thawed (group III) cattle embryos. The calving rate was 80% after transfer of IVP embryos, 28.57% after transfer of GFP positive embryos and 16.66% after transfer of GFP positive-frozen/thawed embryos. The pregnancy length was normal and varied from 277 to 284 days, independent on group. After embryo transfer 73% bulls and 27% heifers were obtained. The normal cattle offspring was obtained both after transfer of IVP embryos (100%) and GFP positive embryos (83%), despite a heifer with lower birth weight (21.5 kg) and with some other abnormalities characteristic of LOS. After transfer of GFP positive-frozen/thawed embryos a heifer was obtained weighing 55.6 kg, which died in few hours.

KEY WORDS: cattle / embryo transfer / green fluorescence protein / *in vitro* embryo production / large offspring syndrome

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In vitro production (IVP) of cattle embryos is a biotechnique important both in cattle breeding (AI and MOET) and in biotechnology as assisted technique for cloning and obtaining transgenic animals. IVP of cattle embryos is established technique and many offspring has already been obtained in this way [Gordon 2003].

In this study, IVP of cattle embryos technique was used to create transgenic cattle by microinjection of gene construct into one of zygote pronuclei. Microinjection has been used to create many transgenic species including mice [Gordon *et al.* 1980], rabbits, sheep, pigs [Hammer *et al.* 1985] and cattle [Krimpenfort *et al.* 1991]. This technique is, however, inefficient and in most species less than 1% of microinjected zygotes lead to obtaining transgenic offspring [Eystone 1999].

In this research, as gene construct the plasmid (pbLGTNF-EGFP) was used containing the human tumor necrosis factor alpha (hTNF α) gene linked to the bovine beta-lactoglobulin promoter (β LG) in vector pCX-EGFP. Theoretically, using the *gfp* as reporter gene makes possible to detect the integration of gene construct with embryo genome. This allows to reduce the number of recipients required and thus decreases the cost of production of transgenic animals. [Duszewska *et al.* 2003, Rosochacki *et al.* 2001, Rosochacki *et al.* 2003]. GFP positive embryos were transferred to recipients or frozen/thawed and transferred later. Transferred were also several IVP embryos.

Many authors concentrate on the effect of different reproduction techniques on health and welfare of cattle offspring [Wagtendonk *et al.* 2000 – a review] and this is also the scope of the present study. The main problem with IVP of cattle embryos is large offspring syndrome (LOS), characterized, among other things, by longer gestation period, increased incidence of abortions, more problems during calving, increased birth weight, higher perinatal mortality, high rate of congenital abnormalities, more hydro-allantois, and relatively more bull calves born [Wagtendonk *et al.* 2000]. LOS has been associated with nuclear transfer and pronuclear injection [Eystone 1999, Renard *et al.* 2002]. The LOS problem has extensively been reviewed by Kruip *et al.* [2000], Wagtendonk *et al.* [2000], Young and Fairburn [2000] and Jaśkowski and Traczykowski [2002].

The objective of this study was to compare the length of pregnancy, calving rate, birth weight and sex after transfer of IVP (group I), GFP positive (group II) and GFP positive-frozen/thawed (group III) cattle embryos to recipients.

Materials and methods

Reagents

The reagents were obtained from SIGMA-ALDRICH (St. Louis, MO, USA), GiBco BRL (LIFE TECHNOLOGY, Paisley, Scotland), MERCK (Damstadt, Germany) and ART of CCD (Paris, France). The tubes, flasks and dishes were from NUNC (Roskilde, Denmark).

***In vitro* maturation (IVM) of cattle oocytes**

Bovine ovaries were obtained from a slaughterhouse and transported to the laboratory at 30°C in buffered saline. Cumulus-oocyte complexes (COCs) were collected by aspiration from follicles (2 to 6 mm in diameter) using a syringe with an 18-gauge needle. COCs were washed 3 times in manipulation medium (MM) composed of TCM199 buffered with 25 mM HEPES without NaHCO₃ (GIBCO) supplemented with 10% FBS (GIBCO), 50 µg/ml gentamicin sulfate (SIGMA), 100 IU penicillin (SIGMA), 50 µg/ml streptomycin (SIGMA) and adjusted to pH 7.4. A group of COCs (20 immature oocytes) was matured in 450 µl TCM199 buffered with 25 mM HEPES supplemented with 10% FBS, 0.02 IU/ml FSH (SIGMA), 1 µg/ml 17β-estradiol (SIGMA), 0.2 mM Na pyruvate (MERCK) and 50 µg/ml gentamicin sulfate [Marguant-Le Guienne *et al.* 1989]. The COCs were matured for 24 h at 38.5°C in 5% CO₂ in a humidified incubator.

***In vitro* fertilization (IVF) of cattle oocytes**

Frozen spermatozoa of a Jersey bull were thawed in a water bath (37°C), centrifuged for 10 min at 200g, and resuspended in 2 ml Sp-TALP medium containing 6 mg/ml BSA fraction V (SIGMA), adjusted to pH 7.4. Spermatozoa were prepared by the swim-up procedure. After maturation, COCs were washed 3 times in TL-TALP medium supplemented with 6 mg/ml BSA FAF (SIGMA), 0.2 mM Na pyruvate and 50 µg/ml gentamicin sulfate (pH 7.4). Groups of 10 matured oocytes (COCs) were placed in 4-well dishes (NUNC) in 450 µl Fert-TALP supplemented with 6 mg/ml BSA FAF (SIGMA), 0.2 mM Na pyruvate, 50 µg/ml gentamicin sulfate, 20 µM penicillamine (SIGMA), 10 µM hypotaurine (SIGMA), 1 µM epinephrine (SIGMA) and 2 µg/ml heparin (SIGMA), (pH 7.4) – Parrish *et al.* [1986]. Spermatozoa were used at a final concentration of 1×10⁶/ml. The COCs and spermatozoa were co-cultured for 17 h at 38.5°C in 5% CO₂ in a humidified incubator. Seventeen hours post-insemination, cumulus cells were removed by pipette stripping and the zygotes were transferred to manipulation medium (MM).

Microinjection of the pBLGTNF-EGFP construct into cattle zygotes

The pBLGTNF-EGFP construct contained a 0.5 kb fragment of the bovine *BLG* promoter, the 0.5 kb human TNFα cDNA and the 0.1 kb poly(A) signal from the *hGH* gene inserted in the pCX-EGFP plasmid vector containing the *gfp* reporter gene. The nucleotide sequence of the final construct pBLGTNF-EGFP was confirmed using a cycle sequencing kit and ALFExpress sequencer (PHARMACIA BIOTECH).

The part of zygotes were transferred to a microcentrifuge tube in 50 µl of MM and centrifuged for 6 min. at 12,000 g at room temperature. The zygotes were then transferred to a manipulation chamber (15µl drops of MM, covered with mineral oil). The manipulation chamber was placed under an Inverted Microscope with Nomarski contrast optics and micromanipulator (LEITZ). The pBLGTNF-EGFP construct was diluted to 3 ng/µl in TE buffer (pH 8.0) – Chan *et al.* [2002]. Successful injection into

one of the pronuclei was performed and confirmed by swelling of the pronucleus. The injections were made between 18-20 h post-insemination.

***In vitro* culture (IVC) of cattle embryos**

The IVP zygotes, at 17 h post-insemination and zygotes after microinjection were washed in Menezo B2 medium (ART of CCD) supplemented with 10% FBS and then placed in 40 μ l drops of the same medium under mineral oil (SIGMA). The zygotes from IVP group and the microinjected group were co-cultured on Vero cell monolayers until day 7 (168 h post-insemination) at 38.5°C in 5% CO₂ in a humidified incubator. During culture, the medium was renewed twice (at 48 h, 20 μ l of medium was removed and 20 μ l of Menezo B2 supplemented with 10% FBS was added; at 144 h, 20 μ l of medium was removed and 20 μ l of Menezo B2 without serum was added) – Duszewska *et al.* [2000].

Development of cattle embryos was evaluated after 48 and 168 h post-insemination.

Detection of green fluorescence protein (GFP) expression

After 168 h post-insemination, all embryos from the microinjected group and five embryos from IVP group were examined individually using a Fluovert FS microscope (LEITZ) with excitation of the chromophore at 488 nm and a standard GFP filter. The embryos were scored for GFP positive blastomeres within each of them. A GFP positive embryo was considered one in which at least one blastomere yielded a green fluorescence [Chan *et al.* 2002].

Embryo cryopreservation

The part of GFP positive embryos was frozen. These were washed in MM for 10 min. and exposed to 1.5M EG with BSA V and antibiotics in PB1 (AGTECH, Inc., USA) at 22 to 24°C for 10 min. Each embryo was loaded into 0.25 ml plastic straw (AGTECH, Inc., USA). The straws were placed into a programmable freezer (CRYCELL, Austria) at -6°C, and 1 min. later they were seeded at that temperature and kept for 10 min. The straws were cooled to -32°C at a constant rate of 0.5°C/min. before plunged into liquid nitrogen. After the storage, the straws were thawed in a 25°C water bath for 20 s and embryos were transferred directly to recipients.

Embryo transfer

IVP of cattle embryos, GFP positive embryos and GFP positive after freezing and thawing embryos were transferred to recipients. Heat synchronization of the recipients was induced by injecting 2 ml (0.5 mg) of the prostaglandin F_{2 α} -analogue Cloprostenol (BIOESTROVET-Vetoquinol, Gorzów, Poland) every 11 days. Seven to eight days after the standing heat, the fresh embryos were transferred into Embryo Transfer Medium (BioLife Transfer Medium, AGTECH, Inc. USA). The straws with GFP positive embryos were thawed at 25°C water bath for 20 s and transferred directly to recipients. After embryo transfer, heifers were monitored daily for heat behavior and examined ultrasonically after 5 weeks and then monthly to confirm the pregnancy.

Results and discussion

The results of transfer of IVP, GFP-positive and GFP-positive after freezing and thawing embryos are presented in Table 1. The integration of pbLGTNF-EGFP construct in the host genome will be described in the separate paper.

Table 1. The results of transfer of IVP, GFP positive and GFP positive-frozen/thawed embryos

Group	No of offspring/No of transferred embryos (calving rate %)	Offspring sex and name	Gestation length (days)	Birth weight (kg)	Weight (kg) at the age of (months)
I. IVP	4/5 (80.00%)	bull BOLEK	278	27.0	325 (10)
		bull CYPISEK	284	33.0	270 (10)
		bull COLARGOL	283	35.0	320 (10)
		bull LOLEK	278	35.4	318 (10)
II. GFP positive	6/21 (28.57%)	heifer GOPLANA	277	21.5	150 (11) ^a
		bull DYZIO	282	31.5	400 (13)
		heifer SAWA	277	34.5	170 (8)
		bull WARS	283	40.5	230 (8)
		bull WEDEL	278	35.7	340 (11)
		bull MARS	283	29.5	–
III. GFP positive-frozen/thawed	1/7 (16.66%)	heifer nn	284	55.6 ^b	–

^aSlaughtered.

^bDied in few hours after birth.

Generally, calving rates of IVP embryos after transfer are lower than of those obtained *in vivo*. Also, the calving rate of embryos injected with the gene construct produced *in vitro* is lower than of *in vivo* or *in vitro* produced embryos without injection [Han *et al* 2000].

In the present study the highest rate of calving was obtained after transfer of IVP embryos (80%) and it seems to be incidental event. Lower appeared the per cent of calving after transfer of GFP positive embryos (28.57%), probably due to the microinjection of the gene construct into one of pronuclei; this method includes a high magnitude forces (g) to visualize the pronuclei and making a small opening in the *zona pellucida* and in the plasma membrane [Ito *et al.* 1998]. This method is traumatic to the zygotes and reduces their early development rate [Eystone 1999]. The proportion of DNA-injected bovine zygotes developing into blastocysts is considerably lower than that of non-manipulated embryos [Ito *et al.* 1998], but our result indicates that the negative effect of microinjection may occur later, after transfer of these embryos to recipients. This supports the earlier observation on reduced pregnancy rates after transfer of gene-injected embryos by Eystone [1999] who obtained 17% of calving after transfer of microinjected zygotes. Similar result (21%) was obtained by Han *et al.* [2000].

After transfer of GFP positive-frozen/thawed embryos only one heifer (16.66%) was obtained. Taking into account that the calf died a few hours after birth, this result should be considered as failure. Generally, only *in vivo* obtained embryos as well as blastocysts produced *in vitro* may be cryopreserved. Morulae and early blastocysts produced *in vitro* are more sensitive to cryopreservation procedures. The first report on the generation of transgenic cattle from gene construct-injected embryos after freezing and thawing was published by Han [2000]; the pregnancy rate was 13.9% after transfer-expanded, mid- and early blastocysts and calving rate was 12.5%.

Independent on groups, the foetal losses occurred very early – during two first months of pregnancy. Although in several studies a longer gestation has been reported [Ito *et al.* 1998, Kruij and Den Daas 1997], in our study this period was normal and varied from 277 to 284 days, independent on group. After transfer of IVP and GFP positive embryos, recipients calved naturally. After transfer of GFP positive-frozen/thawed embryos, a heifer was obtained by Cesarean section due to the high body weight (55.6 kg). However, the gestation length in this case was normal. Therefore, higher birth weight was due to the faster prenatal growth rate what supports the earlier observation by Numabe *et al.* [2000].

In case of the calf obtained by transfer of GFP positive embryo, its weight at birth was markedly low (21.5 kg); after 11 months its weight (150 kg) appeared half-lower than that of its mate (340 kg). Additionally, this heifer demonstrated many symptoms which were characteristic of LOS (Duszewska *et al.*, in press).

Disregards these two abnormal cases, the birth weight of the other nine calves (Tab. 1) varied from 27 to 40 kg. Obtaining such calves may be explained by using for *in vitro* fertilization the semen from Jersey bull, *i.e.* of the breed characterized by the small body weight. Also, using for *in vitro* culture of embryos the Vero cells may affect obtaining healthy offspring by supporting the embryos development until blastocyst stage and also by metabolizing and absorbing inhibitory substances from the culture medium [Menck *et al.* 1997, Duszewska *et al.* 2000]. The weight of calves in the later period of life was satisfactory and the differences between specimens may result from the breed of the donors of oocytes. Since the oocytes were isolated from slaughter, the breed of donors is unknown.

After embryo transfer eight bulls (73%) and three heifers (27%) were obtained. This supports the observation that relatively more bulls are born after IVP than after AI or MOET [Wagtendonk *et al.* 2000].

In conclusion, the normal cattle offspring was obtained, both after transfer of IVP embryos (4 normal/4total – 100%) and GFP positive embryos (5/6-83%) despite the case of one abnormal calf. LOS should also include the offspring with lower body weight because it demonstrated many other abnormalities which are characteristic of LOS. The normal cattle offspring has not been obtained after transfer of GFP positive frozen-thawed embryos (0 normal/1 total – 0%).

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Uzyskiwanie potomstwa po transferze IVP, GFP-pozytywnych i GFP-pozytywnych zamrożonych/rozmrózonych zarodków bydłych

Streszczenie

Celem pracy było porównanie długości ciąży, odsetka oścień, masy ciała przy urodzeniu i płci noworodków uzyskanych po przeniesieniu do biorecyń zarodków IVP (grupa I), GFP-pozytywnych (grupa II) oraz GFP-pozytywnych zamrożonych/rozmrózonych (grupa III).

Najwyższy odsetek oścień uzyskano po transferze zarodków IVP (80%). Po transferze zarodków GFP-pozytywnych uzyskano 28,57%, a po transferze zarodków GFP-pozytywnych zamrożonych/rozmrózonych – 16,66% oścień. Długość ciąży była w normie i wahała się w granicach 277-284 dni niezależnie od grupy. Uzyskano 73% buhajków i 27% jałówek. Zdrowe cielęta uzyskano po transferze zarodków IVP (100%) oraz po transferze zarodków GFP-pozytywnych (83%) mimo, że u jednej jałówki, przy obniżonej masie ciała (21,5 kg) stwierdzono wiele zaburzeń charakterystycznych dla LOS. Po transferze zarodków GFP-pozytywnych zamrożonych/rozmrózonych uzyskano jałóvkę o masie 55,6 kg, która zaraz po urodzeniu padła.