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

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(Received October 14, 2004; accepted December 2, 2004)

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

*Supported by Polish Academy of Sciences Institute of Genetics and Animal Breeding, Project S.III-1.5.
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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 (hTNFα) gene linked to the bovine beta-lactoglobulin promoter (βLG) 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 [Wagtgendonk 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 [Wagtgendonk 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], Wagtgendonk et al. [2000], Young and Fairburn [2000] and Jaœkowski 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 regents were obtained from SIGMA-ALDRICH (St. Louis, MO, USA), Gibco BRL (LIFE TECHNOLOGY, Paisley, Scotland), MERCK (Damstad, 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 (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), 1μg/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 TNFa 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 μl 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 μl of medium was removed and 20 μl
of Menezo B2 supplemented with 10% FBS was added; at 144 h, 20 μl of medium was
removed and 20 μl 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 posi-
tive 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 recipi-
ents was induced by injecting 2 ml (0.5 mg) of the prostaglandin F₂α-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 di-
rectly 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

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

<sup>a</sup>Slaughtered.  
<sup>b</sup>Died 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, Kruip 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 [Wagtgendonk 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%).
REFERENCES


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Uzyskiwanie potomstwa po transferze IVP, GFP-pozytywnych i GFP-pozytywnych zamrożonych/rozmrożonych zarodków bydłęcych

**Streszczenie**

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

Najwyższy odsetek ocieleń uzyskano po transferze zarodków IVP (80%). Po transferze zarodków GFP-pozytywnych uzyskano 28,57%, a po transferze zarodków GFP-pozytywnych zamrożonych/rozmrożonych – 16,66% ocieleń. Długość ciąży była w normie i wahala 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 jałowki, przy obniżonej masie ciała (21,5 kg) stwierdzono wiele zaburzeń charakterystycznych dla LOS. Po transferze zarodków GFP-pozytywnych zamrożonych/rozmrożonych uzyskano jałówkę o masie 55,6 kg, która zaraz po urodzeniu padła.