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Production of Transgenic Bovine Embryos by Microinjection Method of a Lentiviral Vector in Zygotes

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Abstract

Objective: To produce bovine transgenic embryos by microinjection of a lentiviral vector that carries the eGFP gene as a marker in zygotes six hours after fertilization. **Methods:** 834 oocytes were matured and subjected to one of four treatments designed as follows: CC: Control: IVF with Cumulus-oocyte with (COCs), cultivated in CR2 medium supplemented with 10% FBS and incubated at 38.5°C in atmosphere of 95% humidity and 5% CO_2.CCM: Control culture medium: fertilized in vitro for six hours, cultured in medium SOF aluminum in pouches under the same conditions of CC. MC: Microinjection control: Fertilized under the same treatment conditions CCM. After six hours they were microinjected with TALP medium and cultured in sachets with the same conditions of CCM treatment. ML: Microinjected with the lentivirus: Fertilized in the same conditions of the CCM treatment. After six hours they were microinjected with the lentiviral vector carrying the eGFP transgene and cultured in sachets with the same treatment conditions CCM and MC. **Findings:** The cleavage rate found in CC was higher ($p < 0.05$) than that observed in the other treatments. The rates of blastocysts found between CC, CCM and MC did not differ significantly ($p > 0.05$) in them, but yes, with ML ($p < 0.05$). On average, 76.4% of the zygotes obtained in ML expressed the green fluorescent protein. **Application/Improvements:** The cult ure conditions used were suitable for CC, CCM and MC, microinjection with lentiviral vector has some influence on embryo development, it succeeded in obtaining transgenic zygotes.

Keywords: Green Fluorescent Protein (eGFP), Genetic Modification, Micromanipulation of Zygotes

1. Introduction

Genetically Modified Organisms (GMOs) or transgenic organisms are organisms that have DNA sequences from another species inserted in their genome[1,2]. The gene that is introduced or transgene is a construct that contains the coding and promoter region of the protein of interest[2], which can be from another animal of the same species of a bacterium or plant[3,4].

Transgenic Animals (TA) have many applications, such as in vivo study of gene function in organogenesis, development and aging, as experimental models for the knowledge of mechanisms involved in the development of diseases and in animals Farm. This technique helps in breeding programs with the multiplication of animals with desirable characteristics of economic interest[5–7].

There are several methodologies for the generation of TA, among them: Pronuclear microinjection[8], DNA transfer mediated by sperm, nuclear transfer from transfected somatic cells (SCNT)[9] pronuclear DNA microinjection[10], CRISPR/Cas9[11], microinjection of transposons[12], retroviral vectors[5] and effector nucleases, activators of transcription[13]. These techniques vary in efficiency[1].

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When retroviral vectors are used, lentiviruses are the most used[5], retroviral vectors are obtained by replacing viral genes gag, in and env by one or more genes, whereas, the set of regulatory genes necessary for encapsulation (sequence ψ), reverse transcription (PBS, R, PPT) and expression of the genes (LTR) are conserved[5, 14]. The most used transgene, because it shows stable expression in mammalian cells and can be tracked in situ quantitatively or qualitatively and non-invasively[15] is the protein eGFP described in Aequorea victoria[16].

In the first investigation where transgenic animals generated using lentiviral vector concluded that the microinjection of lentivirus in the perivitelline space of fertilized oocytes could increase the production efficiency of TA[17]. Then, the comparison of the classical DNA microinjection technique with the transfer of genes in a lentiviral vector resulted in a four to eight time's higher rate[18]. Today the technique has become more widespread in different animals[12], although, there are still some limitations to the use of lentiviruses, especially when the use of high viral titers is necessary, since the lentivirus must surpass the Zona Pellucida (ZP) of the embryo and the extracellular glycoprotein matrix that confers external protection to the embryo[19] even against infectious agents[20]. However, in this case the ZP ends up acting as a physical barrier that prevents the penetration of the lentivirus[19]. Therefore, the preferred method for the microinjection of viral particles is within the perivitelline space (subzonal injection) allowing the virus to overcome the membrane of the oocyte or the zygote. What makes sophisticated equipment necessary for subzonal injection[19]? The aim of this investigation was producing bovine transgenic embryos by microinjection of a lentiviral vector that carries the eGFP gene as a marker in zygotes six hours after fertilization.

2. Materials and Methods

2.1 Collection of the Ovaries and Manipulation of the Oocytes

We used 360 ovaries, without defining the breed in a different phase of the estral cycle, slaughtered in a cold storage facility located in the city of Juiz de Fora, Minas Gerais, Brazil. Immediately after sacrifice and evisceration, the ovaries were removed and immersed in a thermal carafe with physiological solution (0.9% NaCl) increased with streptomycin sulfate (0.05 g/L), at a temperature between 35-38°C. The ovaries were transported to the animal reproduction laboratory in a maximum time of thirty minutes. The ovaries were washed with a physiological solution, previously placed in a water bath at 37°C; the ovarian follicles (≤ 10 mm) were suctioned with a syringe. The follicular fluid was deposited in a conical calyx, at a temperature of 37°C. Once decanting of the oocytes occurred, they were re-suspended in Talp-Hepes medium, after removal of the supernatant fluid and transferred to Petri dish, on the heating plate at 37°C and then classified morphologically, according to[20, 21]. Only immature oocytes classified as cells of compact cumulus and with at least three cell layers were transferred to a third Petri dish containing Talp Hepes medium.

2.2 Maturation (IVM) and Fertilization In vitro (IVF)

We used 834 immature oocytes, which were matured in TCM 199 medium (Tissue Culture Medium 199) (Gibco/Invitrogen) with FSH Hormone (20 mcg/ml) and cow serum in heat (10%). The maturation was carried out in groups of 50-60 structures, deposited in plates Nunc (Thermo Scientific, Cat.176740) of four wells, containing 400 µL of maturing medium previously balanced for at least two hours in cell culture incubator at 38.5°C with an atmosphere of 95% humidity and 5% CO_2. The oocytes were cultured under these conditions of temperature and atmosphere for 22 to 24 hours[21].

Semen from a single Gir bull was used, previously evaluated microscopically. Only doses with motility equal to or greater than 50% and vigor at minimum 3 were used. The semen was thawed at 37°C for 30 seconds; the spermatozoa were processed by the Percoll gradient method (Nutricell) with the concentrations of 90% and 45% for separation of mobile sperm in centrifugation. The Percoll gradient was previously prepared and stabilized in the incubator for 15 minutes before use. The semen was diluted, with a concentration adjusted to obtain a fertilizing dose of $4x10^6$ sperm/mL and transferred to the fertilization drops (100 µl of FERT-TALP medium) (2). The gametes remained co-incubated under the same conditions cited for IVM for a period of six hours for the experimental groups CCM, MC and ML and for 20 hours for the CC treatment. The concentration 4 x 10^6 SPTZ/mL, was used due to the fact that the oocytes of groups CCM, MC and ML, only remained for six hours in the

FERT-TALP medium and consequently, a higher sperm penetration rate was sought[22].

2.3 Treatments

Four treatments were designed, as follows: **CC = Control**: IVF with Cumulus-Oocyte Complexes (CCOs) with a concentration of 1 × 10^6 sperm (SPTZ)/ l, cultured in CR2 medium enriched with 10% FBS and incubated at 38.5°C in an atmosphere of 95% humidity and 5% CO_2. **CCM = Control of Culture Medium:** IVF with a concentration of 4 × 10^6 SPTZ/ml for six hours, grown in SOF medium in aluminum sachets with a gas mixture of 5% CO_2, 5% of O_2 and 90% of N_2 and saturated humidity at a temperature of 38.5°C. **MC = Microinjection Control**: IVF with a concentration of 4 × 10^6 SPTZ/ml and cultured under the same treatment conditions CCM. After six hours of fertilization time, they were microinjected with TALP medium and cultured in sachets with the same treatment conditions CCM. **ML = Microinjected with the Lentivirus**: IVF with a concentration of 4x10^6 SPTZ/ml and cultured under the same conditions of the CCM treatment. After six hours of fertilization time, they were microinjected with the lentiviral vector and cultured in sachets with the same treatment conditions CCM and MC.

2.4 Lentiviral Vector

The lentiviral vectors were produced by transient transfection, using four plasmids: The packaging plasmid (pMDLg/pRRE), which encodes the envelope protein (pMD2.G), the plasmid that encodes the Rev protein (pRSV-Rev) (Addgene, USA) and the plasmid containing the transgene (pLGW). Cell lineage HEK-293F (ATCC CRL 1573) cultured in DMEM medium was used more than 10% FBS until reaching the 80% confluency state. Transfection with the lentiviral vectors was performed with a mixture containing the four plasmids, in concentrations of 6µg of DNA from each of the structural plasmids (pMDLg/pRRE, pMD2.G, and pRSV-Rev) and 12µg of the plasmid of interest (pLGW).

Two mixtures have been prepared separately: A mixture of 18 mM polyethyleneimine (PEI, Sigma) plus 5% glucose and another mixture of plasmid DNA plus 5% glucose. The ratio of 1 µL of PEI (with pH adjusted to 7) per 1 µg of DNA was used. The two mixtures were vortexed for one minute and left to rest for 5 minutes, then both mixed and put into vigorous vortexing, all the contents were kept at rest for 10 minutes, during which time a new one was made, medium exchange without SFB. 1 mL of DMEM without SFB was added. After 6 hours, SFB was added to the medium to obtain the concentration of 10%. 48 hours later the medium was centrifuged for an hour and a half at 70 thousand gravities at a temperature of 4°C. The lentivirus was re-suspended in 100 µL of DMEM without SFB and frozen at -80°C until used[2].

2.5 Microinjection of Zygotes six hours Post-Fertilization with Lentiviral Vector (MIZL)

After sixty hours the onset of IVF, the putative zygotes were removed from the drop of fertilization and subjected to complete removal of the cumulus cells by mechanical vortexing for five minutes and then transferred Petri dish. The putative zygotes were washed with TALP medium and kept in drops of 20 µl of the medium covered with mineral oil until the moment of microinjection[2]. The MC zygotes were microinjected with the TALP medium and the ML with the lentiviral vector in the perivithelial space using an inverted microscope (Axiovert 135M, Carl Zeiss) equipped with a micro-injection and microinjection system (Nikon Narishige NT-88V3)[2]. The microinjection was considered successful when the zona pellucida grew visibly[2]. After the injection of the TALP or subzonal lentivirus, the putative zygotes were washed three times in TALP medium and again transferred to the corresponding medium. During this period, the oocytes from the control treatments (not microinjected) remained in the FERT-TALP medium until completing 20 hours of fertilization.

2.6 In Vitro Culture of the Embryos (IVC)

After fertilization, the presumed zygotes were removed from the drop of fertilization, washed in TALP-HEPES medium and divided into two culture plates, where one was the control and the other the treatments. In the CC treatment, the supposed zygotes were subjected to the nakedness with the help of a pipettor and washed in TALP medium. The culture was carried out in drops of 50 µL of CR2 medium plus 10% SFB under mineral oil, distributed in Petri dishes of 10 x 35mm. The culture condition was 5% CO , 95% humidity and 38.5°C[23].

The IVC of the CCM, MC and ML treatments was performed in Nunc-type four-well plates containing

500 µL of SOF medium supplemented with 2.5% FBS, under mineral oil, in groups of 30-40 structures in each well. During the whole culture, the plates containing the embryos were placed airtight bag (Aluminum Sachet) containing a gaseous mixture of 5% CO_2, 5% O_2 and 90% N_2 and saturated humidity, maintained at a temperature of 38.5°C.

2.7 Evaluation of Cleavage and Blastocyst Rates

The cleavage rate was evaluated 72 hours by IVF. The blastocyst production rate and the morphological classification of the embryos were evaluated on the seventh (D7) and eighth (D8) days of culture, using a stereoscope (Nikon SMZ 645), following the parameters established by[21]. A randomized complete block model with five repetitions per treatment was used. The averages of each treatment were compared using the 5% Ducan test[22].

2.8 Expression of the eGFP Protein

eGFP was visually, in blastocysts, by exposure to white and ultraviolet light in stereomicroscope (Nikon, SMZ800, 450-490 nm filter) or the fluorescence microscope (Motic, BA400, 465 filter-495 nm). In the blastocysts microinjected with the lentiviral vector, the percentage of the expression of the eGFP gene was estimated and the position of the fluorescence presentation was noted[22].

3. Results and Discussion

Cleavage rates at 72 hours, production of blastocysts at days D7 and D8 and the rate of zygotes expressing the eGFP gene are presented in Table 1.

For CC, the cleavage rate obtained was higher ($p < 0.05$) than that observed in treatments CCM, MC and ML. This fact can be explained by the manipulation to which the possible zygotes of the treatments CCM, MC and ML were subjected, since, six hours after the start of the IVF, these were removed from the drop of fertilization and subjected to the complete removal of cumulus cells. This procedure is necessary to allow the micromanipulation process (Table 1). On the other hand, it was possible to notice that the highest cleavage values for CC with respect to the other treatments were not reflected in higher blastocyst rates at D7. In Figure 1, eGFP gene expression can be observed in embryos cleaved from the ML treatment.

The blastocyst rates found between CC (14.5%) and CCM (10.3%) ($p > 0.05$) at D7, similar results are reported by[2], who suggest that the atmospheric conditions and the culture medium used do not interfere with the production of blastocysts, thus, the variations found can be attributed to the development of embryos.

The rate of blastocysts at day 8 for the fertilized oocytes of CC (19.0%) and MC (16.3%) was higher ($p < 0.05$) than that observed in the other treatments. This rate in CC is a reflection of the higher rates of cleavage and blastocysts presented in previous days and can be explained by the fact that this control group was not subjected to the manipulation of cells removed from the clusters and also remained by[20] hours in incubation with the sperm, while, the other treatments, only remained for six hours in fertilization.

The similarity between BL to D8 rates between CCM and MC ($p > 0.05$) indicates that the microinjection procedure with TALP medium (MC) was not able to cause damage to the embryos, to the point of not interfering with their development, even when your ZP was drilled. Additionally, when CCM and MC are compared with the group microinjected with lentivirus (ML), cultured in the same atmosphere and using the same culture media, significantly lower values can be observed in ML ($p < 0.05$), which are not related to the microinjection mechanism but to the presence of the lentivirus particles. This fact suggests that microinjection of this particle into zygotes is detrimental to the growth of the embryos until the initial blastocyst stage.

The first studies using microinjected lentiviral vectors in zygotes reported a higher efficiency to generate transgenic rodents[17, 23] and swine[24, 25]. The blastocyst rate at days D7 and D8 were significantly lower ($p < 0.05$) for ML than for the other treatments. In contrast, when injecting vectors into the perivitelline space, 22% of the zygotes developed until the blastocyst stage, resulting in the birth of four calves, none of them transgenic[18]. The microinjecting a retroviral into the perivitelline space resulted in a rate of 21%[26]. In other species, rates of blastocyst formation are 25% in pigs[24] and 76% in macaque[26] is reported. A possible cause of the low efficiency may have been the age of the zygotes since the MIZL was carried out at 18 h post-in vitro fertilization. It has been argued that one of the possible reasons for the inefficient transduction of lentiviruses in bovine zygotes could be that the lentiviral genome is not able to penetrate efficiently in the bovine pronucleus[27].

It is necessary to consider the phase of the cell cycle and the state of chromatin at the moment on the integration of retroviruses, in this regard, sperm penetration during in vitro fertilization begins with four hours of culture, the formation of pronucleus starts with four hours and lasts up to 11 hours, while DNA synthesis (S phase) starts with 14 to 15 hours after fertilization with a duration of 8 to 10 hours. Some retroviruses, such as HIV, can be integrated into cells that are not in the cell division phase, but decondensed or open chromatin can facilitate access and correct integration of the viral genome[28, 29]. This may be one of the reasons whereby the use of vectors in matured oocytes, followed by fertilization is more efficient[15, 30]. Additionally, it is likely that in bovine zygotes, lentiviral integration could be significantly delayed in the presence of a nuclear membrane, also, that retroviral infection can delay embryo cleavage, thus decreasing the integration capacity of the pre-integration complex[18].

Other possible reasons for the low rate of blasts may be the negative effect of microinjection on embryonic development due to a detrimental effect on the expression of genes involved in these stages of embryogenesis[2, 31], the concentration of MIZL and the incubation time that affect the eGFP gene may reduce the viability of transfected embryos[26, 32] and the processes of DNA methylation, which has been identified as a critical factor in the regulation of gene expression[33].

On days seven and eight, the expression of eGFP was 75% and 77.8% respectively (Figure 2). In the embryonic regions of the trofectoderm and cell mass was where the highest fluorescence was found (32). This confirms that the microinjection of the lentivirus is efficient for the incorporation of a DNA fragment in the oocyte genome[2].

By microinjecting lentiviral vectors in pig zygotes, the expression of the interest protein was similar to that found here 65%[24].

The expression this gene was only 45% blastocysts of bovine embryos[24]. Another report indicates a 90% yield of GFP expression in at least 80% of the generated transgenic mice[17]. Using lentiviral vectors developed from the genome of equine infectious anemia (AIE), transgenic pigs were obtained, obtaining 31% of transgenic animals from zygotes injected with the virus in the perivitelline space and 95% of the founding animals exhibiting fluorescence. Green[25]. In other species, transgene expression is reported, 95.5% in mouse[23], 97.4% in sheep[15], 70-90% in pigs[34].

eGFP expression can be an interesting tool to solve an infinity of questions in biotechnology and in the biology of embryonic development. It can be used to study epigenetic modifications, such as DNA methylation, study of biological processes, since it allows an accurate visualization of the anatomical structure of transgenic animals expressing gene (eGFP), in order to obtain a real visualization of organs and tissues in vivo, also to study maternal-embryonic interactions[13– 35].

Transgenic embryos produced could be implanted in recipient bovine females and generate TA. This methodology is an alternative of genetic modification in animals in a safe, fast and profitable way[15, 36] when compared for example with pronuclear DNA microinjection[19, 23, 26, 27], partly because, it has been shown that unlike simple retroviruses,

Table 1. Production of transgenic embryos through the microinjection method of a lentiviral vector in zygotes six hours post-fertilization

Treatment	Culture conditions	N	Clivage Rate	Blastocyst Rate		eGFP+*	
				D7	D8	D7	D8
CC	CR2aa	269	72.1%a (194/269)	14.5%a (39/269)	19.0%a (51/269)		
CCM	SOF	232	45.0%b (76/169)	10.3%a (24/232)	12.5%b (29/232)		
MC	SOF	209	49.0%b (75/153)	12.4%a (26/209)	16.3%a,b (34/209)		
ML	SOF	233	40.8%b (69/169)	3.4%b (8/233)	3.8%c (9/233)	75.0% (6/8)	77.8% (7/9)

* Rate of zygotes that expressed the eGFP gene. Values with different overwritten letters in the same column indicate significant statistical differences ($p < 0.05$)

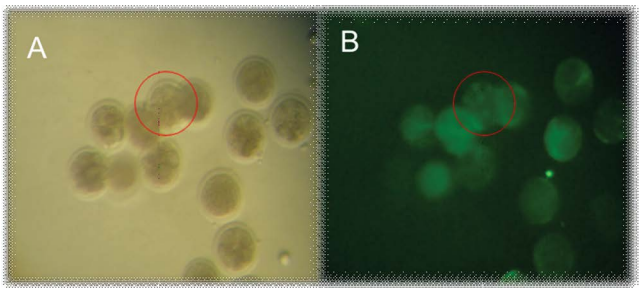

Figure 1. eGFP gene expression in bovine embryos microinjected with the vector. Dark field (A) micrograph (B) exposed to ultraviolet radiation, stereo microscope (Nikon, SMZ800, 450-490 nm filter).

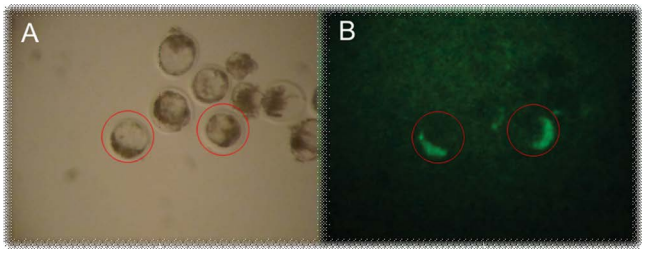

Figure 2. eGFP gene expression in bovine embryos day seven (D7). Light-field (A) and dark (B) micrograph in ultraviolet radiation (Nikon, SMZ800, 450-490 nm filter).

lentiviruses cannot activate proto-oncogenes by their insertion, also, because the use of high titers of lentiviral vectors does not need high biosafety confinements due to the low volume used during microinjection[15,19].

4. Conclusions

The culture conditions used were adequate, since no significant statistical differences were found in the production of blastocysts between the control treatments and the microinjections with TALP medium. The lentiviral vector influenced in some way the embryonic development, deduced from the fact that the percentage of blastocysts found in ML significantly lower than in the other treatments. The expression of the transgene in the zygotes was high, indicating that the technique used here is highly efficient for obtaining transgenic animals.

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