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Effect of Trichostatin-A on Embryons of Bovine Clones Modified Genetically with GFP

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Abstract

Objective: To evaluate the effect of treatment with trichostatin-A (TSA) on the production of bovine embryos, expressing the gene of the green fluorescent protein (GFP) generated by SCNT. **Materials**: 164 oocytes were distributed in three treatments, NT-GFP: newly reconstructed zygotes with genetically modified cells and not subject to TSA. NT-Trico-GFP: newly reconstructed zygotes with genetically modified cells and subjected to TSA. PART: Zygotes generated by parthenogenetic activation, used as a control for the process of oocyte activation and culture of embryos. The rates of cleavage, blastocysts, and embryos that expressed GFP were assessed by contingency tables and chi-square tests. **Results**: The percentage of cleavage in the zygotes in the NT-GFP treatment was greater but did not vary significantly from the NT-Trico-GFP treatment. However, this last treatment had a higher percentage of blastocyst formation ($p=0.077$). The percentage of blastocysts from cleaved zygotes, the produced embryos were significantly higher ($p<0.05$) for the NT-Trico-GFP treatment than for the NT-GFP. In both treatments, all the blastocysts generated expressed the GFP protein. **Conclusions**: TSA improves the embryonic development of clones of genetically modified cattle that express GFP.

Keywords: Embryonic Development, Epigenetic Modification, Nuclear Transfer

1. Introduction

Among the different techniques of genetic manipulation, there are several methods that can be used to generate transgenic animals. Among these we have, the transfer by means of viral vectors, DNA transfer mediated by spermatozoa, a nuclear transfer from transfected somatic cells (SCNT)[1] and CRISPR/Cas9[2] technology. The SCNT technique or animal cloning can be used as an effective alternative for the production of transgenic animals that express a protein of interest[3]. One of the major differences between the nuclear transfer of somatic cells and the other techniques is the ability to select and verify the expression of exogenous DNA when it is incorporated into the cellular genome before its use in nuclear transfer[3,4]. SCNT consists of transferring, by means of micromanipulation, the nuclei of a differentiated somatic cell, carrying a complete diploid genome, into enucleated oocytes, resulting in the production of genetically identical individuals to the nucleus donor animal[4,5].

The donor cell plays a fundamental role in cloning since it can determine the success or otherwise of the procedure. Somatic cells that can be used for the SCNT process include the mammary gland cells, fetal and adult fibroblasts, mural granulosa cells, uterine tuba cells, skin fibroblasts, cluster cells, embryonic stem cells and

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of Sertoli cells[6,7]. Some cell types seem to be more easily reprogrammed than others[6,7]. Additionally, adult somatic cells are usually harder to reprogram than fetal cells[8]. This fact is a consequence of the fact that the nucleus donor cells cultured for long periods have chromosomal instability and shortening of the telomeres, which favor the loss of the function of genes important for the development of the embryo[9,10].

In the phase of cellular reprogramming, cell division must begin and it has as limitation the chromosomal anomalies that can occur[11-14] and that lead to the death of cloned animals due to the epigenetic alterations that affect the structure, composition and remodeling of the chromatin that defines and maintains the accessibility and transcription of the genetic information contained in the DNA[10]. Thus, the regulation of embryonic development is dependent on the epigenetic modifications that occur in the nucleus donor cell after the SCNT, since the donor nucleus must undergo the processes of remodeling and reprogramming, so that it behaves like a zygotic genome. Reprogramming involves changes in nuclear material, such as the structure of chromatin and re-methylation of DNA. These events are necessary for the expression of the genome, silencing genes from the old differentiated cell and stimulating genes characteristic of embryonic cells[7].

Some epigenetic studies present a pharmacological approach and show that some compounds can alter the pattern of acetylation that would help in reprogramming the transferred nucleus with the purpose of improving the development of cloned embryos[15-16]. Acetylation of DNA histones is associated with the relaxation of chromatin structure, making transcription and replication more permissive, while deacetylation of histones has an opposite effect. While the chromatin of matured and newly fertilized oocytes has a low level of acetylation, the chromatin of embryos prior to implantation presents higher levels of acetylation[16].

Acetylation of histones is a major mechanism of genome epigenetic reprogramming of the gametes in order to establish a totipotent state to the normal development[17]. The deacetylation is catalyzed by histone deacetylases (HDAC) which remove acetyl groups and causes chromatin compaction and DNA segment silencing at this location[18]. The trichostatin-A (TSA) is an HDAC inhibitor that increases the number of acetylated histones and the demethylation of DNA[19]. TSA promotes hyperacetylation of histones through the inhibition of the histone deacetylase enzyme in newly constructed embryos[20,21]. In this sense, the drug has been used in an attempt to increase production efficiency of embryos by nuclear transfer (NT). Among the advantages of TSA, it can be highlighted by its low toxicity and the need for low concentrations for the induction of the desired effect[7,21].

Therefore, the objective of this research was to evaluate the effect of treatment with TSA on the production of bovine embryos, expressing the green fluorescent protein (GFP) gene generated by SCNT.

2. Materials and Methods

For this experiment, a total of 164 oocytes distributed in three treatments were used. NT-GFP: newly reconstructed zygotes with genetically modified cells not subject to TSA. NT-Trico-GFP: newly reconstructed zygotes with genetically modified cells subjected to TSA. PART: Zygotes generated by parthenogenetic activation, these were used as control for the process of oocyte activation and culture of the embryos.

2.1 Establishment of Genetically Modified Somatic Cell Lineages

The experiment was conducted in the Universidade Federal de Vicosa - Florestal Campus - Minas Gerais, at coordinates 19° 53' 12" South and 44° 25' 56". The project concerning to the present experiment was subjected to the Committee of Ethics of the Department of Veterinary Medicine, as being approved on March 05 2011. In the present experiment, 47 crossbred cows Bos taurus x indicus in nursing were used, as presenting cyclical luteal ovarian activity and with corporal condition score ≥ 3.0 based on the criteria used by Ferreira[6]. The animals were maintained under pasture (*Brachiaria decumbens*), with mineral supplementation during the rainy period and

with maize silage during the dry period, besides concentrate and water supplied *ad libitum* in both periods.

2.1.1 Donor cell nucleus

For the reconstruction of the cells of the embryo of adult females (fibroblasts) from the ear of a Gir breed cow were used, cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS) and 0,1% antibiotic (penicillin+streptomycin). The culture for the establishment of cell lineages was performed for 14 days, in an incubator maintained at 37°C, 5% CO_2 in the atmospheric air and 95% humidity. By means of an inverted phase microscope (ICM 405, Zeiss, Germany), cell growth was observed and upon reaching 80% confluence in the plate, the cells were frozen in DMEM solution with 20% FBS and 10% dimethyl sulfoxide (DMSO-SIGMA) in a concentration of one million cells/ml, in liquid nitrogen (N_2).

2.1.2 Transfection of the Fibroblasts with the Lentiviral Vector

For the gene transfection of the fibroblasts, we used the system of lentiviral vectors that contained only the green fluorescent protein (GFP) as gene report, without an antibiotic resistance gene, generated in the cell lineage HEK-293FT. The fibroblasts were thawed and cultured in the presence of the lentiviral vector for 48 hours and evaluated for the expression of GFP. Cells that expressed GFP were selected and replicated by 12 passages in order to establish lineages. After this process, the cells were frozen in DMEM with SFB and DMSO in N_2 for subsequent visual confirmation of the expression of the GFP protein and use in the SCNT[22].

2.1.3 Fluorescence Microscopy

The expression of the GFP gene in genetically modified nucleus donor cells was observed, by visualizing these in the white and ultraviolet light in a stereoscope (Nikon, SMZ800, 450-490 nm filter) and microscope (Motic, BA400, 465-495nm filter)[23] (Figure 1).

2.1.4 In Vitro Maturation (IVM)

The oocytes were classified with compact cumulus or more than three cell litters[24] and matured in medium TCM199 (Tissue Culture Medium 199) (Gibco/Invitrogen) supplemented with FSH (Follicle Stimulating Hormone) (20 mcg/mL) and cow serum in oestrus (10%). The maturation was carried out in groups of 15-20 structures deposited on Nunc plates of four wells, with 400µL of maturing medium previously equilibrated for a minimum of two hours in an incubator at 38.5°C with an atmosphere of 95% humidity and 5% CO_2. After maturation,

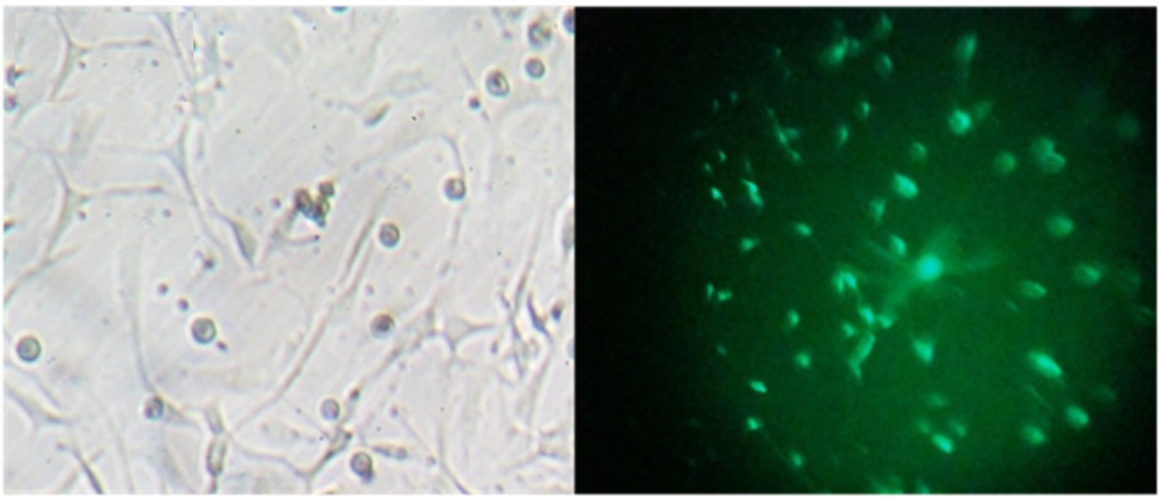

Figure 1. Bovine fibroblasts genetically modified with GFP.

they were randomly divided for SCNT and parthenogenetic activation[3].

2.1.5 Nuclear Transfer with Somatic Cells

For the production of embryos by SCNT, the fibroblasts genetically modified with GFP for NT-GFP and NT-Trico-GFP treatments were thawed and cultured in DMEM with 10% FBS for 1 day. The fibroblasts were recovered after trypsinization and used for zygote reconstruction[25].

2.1.6 Enucleation, Reconstruction, and Fusion

After 18 hours of IVM, the cumulus cells were removed from the oocytes by the addition of 1% hyaluronidase in mechanical agitation with vortex for 5 minutes, and then the oocytes presenting the first polar corpuscle were selected for enucleation. The aspirated material was subjected to ultraviolet light to confirm the removal of the genetic material, after coloring with Hoechst. The reconstruction was carried out by the transfer of the donor cells of the nucleus (fibroblasts) genetically modified expressing GFP, each cell (GFP+) was placed in the perivitelline space of the enucleated oocytes. Immediately after reconstruction, the receptor-donor nucleus-cytoplasm complexes were placed in an electric cell manipulator and the fusion was induced using two electric pulses (2.4 kV) in medium with mannitol[25].

2.1.7 In Vitro Culture of the Embryos

The assemblages of reconstructed embryos were cultured in CR2a medium for 2 hours and the sets that were presented fused were activated with ionomycin for 4 minutes followed by 4 hours in 6-DMAP. The newly constructed zygotes were cultivated in CR2aa medium with 2.5% FBS covered with sterile mineral oil, in 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C[25].

2.1.8 Treatment of the Embryos with TSA

During the culture with 6-DMAP and in the following seven hours, the NT-Trico-GFP treatment was cultured in medium containing TSA (50nM) dissolved in CR2aa medium without SFB or with 2.5% of SFB covered with sterile mineral oil, in 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C, totaling 11 hours of culture in the presence of TSA[20].

2.1.9 Parthenogenetic Activation (PA)

In order to evaluate the efficiency of the zygote activation process, the production of parthenogenetic embryos (control) was carried out. At the end of the maturation of the oocytes, the cells of the cluster were removed in 1% hyaluronidase and washed in TALP-Hepes medium. The oocytes were incubated at 5% CO_2 in atmospheric air with saturated humidity at 38.5°C, for 4 minutes in ionomycin solution (4.62µM) (Sigma St. Louis, USA) in CR2aa medium. Next, the oocytes were again washed in CR2aa medium and incubated for four hours in 6-DMAP solution (2mM in PBS) (Sigma St. Louis, USA) at 38°C in an incubator with 5% CO_2 and saturated humidity. After that incubation period, the oocytes were again washed in CR2aa medium and cultured *in vitro* in 4 well culture plates with 500µL of medium to 2.5% FBS, under mineral oil, in a greenhouse incubator at 5% CO_2, 5% O_2 and 90% N_2[20].

2.1.10 Evaluation of GFP Gene Expression

The evaluation of GFP gene expression was performed visually in the blastocysts, by exposing them to white and ultraviolet light in a stereoscope (Nikon, SMZ800, 450-490nm filter) and fluorescence microscope (Motic, BA400, filter 465-495nm). The blastocyst rates expressing the GFP for the treatments were calculated[23] (Figure 2).

2.2 Embryonic Development Evaluation

The cleavage rate was evaluated 72 hours after reconstruction, where embryos that had two or more cells were considered cleaved, without signs of fragmentation or cellular degeneration. The embryo production rates and the quality of the embryos were evaluated on day seven. The embryo quality was evaluated according to the criteria recommended by the International Society of Embryo Transfer[26].

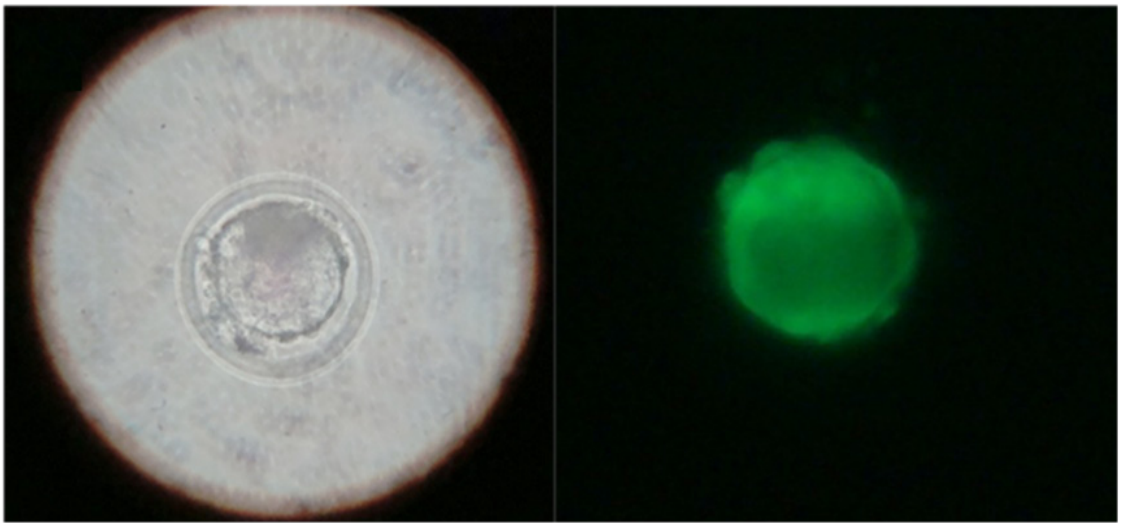

Figure 2. Bovine embryos clones treated with TSA expressing the GFP protein.

2.3 Statistical Analysis

The qualitative variables, rates of cleavage, blastocysts, and embryos that expressed GFP were compared in contingency tables and analyzed by the chi-square test, at 5% probability[27].

3. Results and Discussion

In this experiment, the effect of TSA on the development of bovine clone embryos, reconstructed with somatic cells genetically modified with GFP, was evaluated. The results of the viral translation in cells depend on several factors, such as initial cell concentration, type of cell; viral concentration added to the culture, among others, therefore, the positivity of the cells that express the transgene after translation is variable[12]. This characteristic allows quantification, genomic positioning and confirmation of transgene expression, making possible the establishment of cell lineages of interest, that is, when the transfected donor cells are properly selected, they eventually lead to the birth of exclusively genetic modification carriers[11,28-29].

The cleavage rate of the embryos subjected to a partial treatment did not vary significantly ($p>0.05$) from the embryos treated, which suggests an adequate management of the day. These results are different from those

Table 1. *In vitro* development of treated transgenic (NT-Trico-GFP) and untreated (NT-GFP) transgenic embryos with TSA in the first 11-12 hours after reconstruction

Treatment	Nº	Cleavage (%)	Blastocyst (%)	Blastocysts on cleavage (%)	Embryos that expressed GFP (%)
NT-GFP	55	72.9±11.3[a]	6.8±2.3[a]	10.3±3.6[a]	100 [a]
NT-Trico-GFP	49	66.1±14.4[a]	18.1±4.0[a]	26.7±3.8[b]	100 [a]

Different letters by columns indicate significant statistical differences ($p<0.05$), by the qui-square test.

reported by[29] who have incubated the embryos for 20 and 25 hours in culture media with and without 50% TSA, reporting a higher percentage of cleavage in the embryos.

Table 1 shows the results of cleavage, blastocyst production and embryo rate expressing the GFP protein. Although the percentage of cleavage in the newly reconstructed zygotes with genetically modified cells not subject to TSA (NT-GFP) was higher, it did not vary significantly from the NT-Trico-GFP treatment. However, this last treatment had a higher percentage of blastocyst formation (p=0.077).

When the blastocyst rate was calculated from cleaved zygotes, the embryo production was significantly higher (p<0.05) for the NT-Trico-GFP treatment than for the NT-GFP. In[29] reported a higher percentage of cleavage in embryos treated with TSA than in untreated embryos (36.22% Vs. 32.70%) (p>0.05), the cleavage rates reported by this author are greater than those presented here possibly due to a longer incubation time with the TSA (20 hours) than the time used in this investigation (11 hours).

In sheep[30] found that embryos cloned by SCNT treated with 50nM of TSA for 24 hours, significantly improved (p<0.05) the rate of blastocyst formation compared to the control. Furthermore, TSA treatment increased expression of the development-related genes-OCT4 and SOX2 in SCNT blastocysts. Whereas in buffaloes, the effect of TSA on embryos clones resulted in a greater in vitro development to the blastocyst stage, the reduction of the apoptotic index, in addition, transfer of cloned embryos produced with donor cells treated with TSA led to the birth of a calf that survived for 21 days[14]. Results similar to those presented here also report by[31] and[32] in pigs.

Histone methylation may be related to non-transcribed or transcribed regions, depending on which residue is methylated. For example, histone H3 lysine residue 9 methylation (H3K9me) and histone H3 lysine residue 27 methylation (H3K27me) are related to non transcribed regions, while histone H3 lysine residue 4 (H3K4me), 36 (H3K36me) and 79 (H3K79me) methylation are related to transcribed regions[7].

In both treatments, all blastocysts generated expressed the GFP protein (Figure 2). In[33] modified the fetal fibroblasts of bovines with the green fluorescent protein (GFP) using fibroblasts as core donor cells in the oocyte reconstruction and observed that GFP had been detected in the 8 to 16 cells phase and remained positive in all cases. Later phases. After birth the cloned calves presented a partial closure of GFP expression, suggesting an irreversible silencing of transgenes.

In a study[34] on the viability and genetic expression of cloned bovine pre-implant embryos in the presence and absence of TSA compared to embryos produced by in vitro fertilization or parthenogenetic activation. The treated embryos were found to be more viable with abated epigenetic errors.

In[35] proved the use of TSA as an inhibitor of histone deacetylase and 5-aza-2'-deoxycytidine (5-aza-dC), as an inhibitor of DNA methyltransferases at concentrations of 50nM TSA+5.5nM 5-za-dC in buffalo embryos cloned by SNTC, found that the blastocyst rate was higher in treated embryos than in controls (p<0.05).

Some studies have reported that embryo production is less efficient when genetically modified cells are used[36,37]. This may be due not only to genetic modification but also to the process of inducing modification and selection of modified cells. In this procedure the cells are cultured by various passages to confirm the success of the transgene and the establishment of the cell lineage. However, long-term culture can impair the viability of the cell, causing chromosomal alterations or even interfering with the reprogramming of the nucleus. The use of TSA in this type of cells may be more favorable for the development of the cloned embryo when compared to young cells, with few steps of culture. The above is corroborated by[38] found significant improvement in the in vitro and full-term development of nuclear transferred (NT) bovine embryos with the TSA.

Another point to be considered is that the genetically modified donor cells were not induced to quiescence as the cells without genetic modification. In most cases, TSA may favor reprogramming in transgenic cells without induction of quiescence, although studies have reported that quiescence is not necessary for the success of SCNT[35,36]. In this sense[21] used TSA, to observe the effects it had on the development and acetylation of the histones

of cloned embryos of pre-implanted cattle, they perceived that the treatment with TSA in SCNT embryos resulted in a development rate similar to embryos product of *in vitro* fertilization and significantly greater control treatment (clone embryos not treated with TSA) produced eight-celled embryos with levels of acetylation of histone H4 at lysine 5 (AcH4K5) similar to fertilized counterparts and was found to be significantly ($p<0.005$) greater than that of NT control embryos.

In murine embryos generated by SCNT and treated with TSA, it was observed that the increase in the levels of acetylation in the chromatin provided better rates of development and quality of the embryo, these authors suggest, in addition, that the hyperacetylation induced by the deacetylase inhibitor can stimulate a more effective chromatin remodeling, as well as DNA demethylation, commonly observed in fertilized embryos[18]. Other studies have shown that embryos clones of cattle and pigs treated with TSA improved the development and reduced levels of DNA methylation to values similar to those observed in embryos fertilized *in vitro*[39]. This fact seems to be associated with a new expression pattern to be formed in the fertilized embryo, different from that observed in the gametes. In this regard[40], indicates that TSA-modified acetylation status of reconstructed embryos enhanced the expression of anti-apoptosis and development-related genes and subsequently improved the developmental potential of porcine SCNT embryos *in vitro*. In addition, transgenic donor cells with DNA hypomethylation induced by TSA were more effective in preventing the decrease in EGFP expression in SCNT embryos. Therefore, both donor cells and reconstructed embryos treated with TSA not only prevented EGFP expression decrease but also improved development of porcine preimplantation SCNT embryos, which could provide an efficient means to produce transgenic cloned pigs.

The mechanism by which histone acetylation controls the expression of imprinted genes is not clear. Thus, two options are possible: one by directly leading the process of chromatin condensation-decondensation and genomic activation or by provoking the expression of translation factors such as promoters that regulate gene expression and imprinting control regions[41].

4. Conclusions

The TSA, promotes the increase of the production of clones embryos generated from genetically modified cells, without interfering in the expression of the transgene during the pre-implantation period.

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