Efficiency of Chicken Blastoderm Cell Transfection Combining two Nonviral Methods: Electroporation and Synthetic Carriers

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Blastoderm cells (BCs) are an efficient tool in the generation of chimeric and transgenic chickens. This study includes a comprehensive analysis of the transfection efficiency of BCs using a new method combining electroporation and lipofection. BCs derived from stage X fertilised eggs were subjected to transfection by electroporation and lipofection with the pOVAINT plasmid, which encoded the human interferon alpha 2a. Based on our previous results, the most optimal parameters for electroporation (iso-osmotic solution, two electrical pulses at a voltage of 25 V and a duration of 500 μ s) were used. For lipofection, 5 synthetic carriers were tested. Approx. 85.8% of the BCs were successfully transfected when electroporation with a cationic lipid DDAB / DOPE was combined. Injection of transfected cells *in vitro* into chicken BCs (stage X) of recipient embryos allowed for the introduction of a human interferon alpha-2a gene, the presence of which was detected by PCR, into their genome. Injection efficiency was 65% of the embryos, with a confirmed presence of introduced genes. The presence of the transgene in selected organs (gonads, femoral muscle, brain, pectoral muscle, torso, heart, blood of the embryo depending on the day of incubation) was also demonstrated.

Key words: Blastoderm cells, transfection, electroporation, lipofection, transgene.

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Apart from the benefits emerging from the intensive development of civilization, there are risks associated with increasing incidences of diseases such as diabetes, cancer, cardiovascular system malfunction, multiple sclerosis or AIDS. This alarming trend also refers to susceptibility to other diseases, including asthma, allergies and obesity (GANTEN *et al.* 2012). This has already imposed a demand for novel, effective drugs, including biopharmaceutical products based on human proteins (PAVLOU 2004; SCHELLEKENS *et al.* 2013). To prevent future economic problems and to ensure equal access to the latest medical achievements, a broader group of patients sought alternative sources of biopharmaceuticals.

Therapeutic proteins can be produced in prokaryotic bioreactors (NEUBAUER & WINTER 2001; BALBAS 2001; SWARTZ 2001; COLLET *et al.* 2002), yeast (NGUONG *et al.* 2011), mammalian tissue cell culture (HALDANKAR *et al.* 2006) or in mammalian bioreactors (DYCK *et al.* 2003; MAK-SIMENKO *et al.* 2013). A suitable solution seems to be the production of avian bioreactors. In this system, a potential place for secretion of foreign proteins is the oviduct, and a place to deposit proteins is eggs (BYUN *et al.* 2011).

There are many advantages of the avian bioreactor in comparison with other systems (ISHII *et al.* 2004). First of all, the interval between the generations of poultry is much shorter than in mammals (LI & LU 2010). In addition, it can be further minimised by stimulation with synthetic oestrogen receptors. This can result in a large number of individuals in a short time, especially in the case of artificial insemination (CHOJNACKA-PUCHTA *et al.* 2012).

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Transgenic birds have been produced by viral and nonviral methods (LI & LU 2010). The viral procedures are based on direct modification of embryos by using DNA or viral vectors (retro- and lentiviral vectors) (CYRIAK et al. 2012). This method exploits the natural abilities of retrovituses to combine their own genetic material with host DNA. The transfer of exogenes by viruses is considered the most efficient method of generation of transgenic chickens (PETITTE & MOZDZIAK 2007; NISCHIJIMA & IIJIMA 2013). However, viral methods have strong limitations e.g. danger of recombination with viruses that are widespread in the environment (ESMAEILZADEH & FARHADI 2011), limited transgene size to about 10 kb, requirement of tissue specific promoters with strong activity (BYUN et al. 2011), transgene silencing in next generations (MCGREW et al. 2004), etc. For technical and safety reasons, the use of viruses for agriculture is not recommended. Therefore, nonviral methods (cell-mediated) were developed for effective cell transfection and generation of transgenic chickens using germline chimeras. The nonviral procedures are based on the transfer of the genetically modified cells: embryonic stem cells, spermatogonial stem cells, primordial germ cells or blastoderm cells, directly into the recipient embryo (LI & LU 2010). The mains steps of this strategy involve: plasmid construction, isolation of donor embryonic cells, their transfection in vitro, injection into recipient embryos, identification of somatic chimeras, raising chimeras and identification of germ line chimeras (ROYCHOUDHURY et al. 2010). In our investigations, the production of chicken chimeras was conducted with a modified method of injection of BCs (PETITTE et al. 1990; PERRY et al. 1991; BEDNARCZYK et al. 2000, 2002; SIWEK et al. 2010; SAWICKA et al. 2015). Manipulation of BCs is very useful for producing transgenic chickens, as stage X BCs are transferred into recipient embryos and form somatic and germline chimeras (PETITTE et al. 1990; PERRY et al. 1991; SIWEK et al. 2010). BCs can be maintained in a culture for several days cryoproserved (SAWICKA et al. 2011; SAWICKA et al. 2015) and genetically modified in vitro (BEDNARCZYK et al. 2003; WAWRZYŃ SKA & BEDNARCZYK 2007; WAWRZYŃSKA et al. 2008).

One of the methods of cell transfection *in vitro* is electroporation. Electroporation relies on an electrical current that acts on the cell membrane, causing a momentary and reversible increase in the permeability of the cell membrane, resulting in the formation of nanopores that temporarily allow the transport of large and small molecules into the cytoplasm (PUC *et al.* 2001). The most important is determination of suitable parameters of the electric field (voltage, the number and duration of pulses) and the relevant conditions affecting the state of the cell (temperature, osmotic pressure, size, shape of the cells) (GOLZIO *et al.* 1998). If these parameters of transfection are relevant, the process is fully reversible. For chemical transfection, methods include the use of synthetic DNA carriers such as nanomolecules. Cationic lipids, liposomes and cationic polypeptides may be the carriers for DNA.

Different methodologies of transfection of chicken BCs are proposed in this study. We hypothesised that a combination of both methods: electroporation and lipofection, will be an effective nonviral manner for the introduction of an exogenous gene into avian cells.

Material and Methods

General scheme of the experiment is shown in Figure 1.

Isolation of blastoderm cells

Sixty fertilised eggs derived from Ross 308 chickens were used to obtain BCs. The eggs were stored at a temperature of 10-14°C for 4 days, afterwards the BCs were isolated. BCs were collected from stage X (HAMBURGER & HAMILTON 1951; EYAL-GILADI & KOCHAV 1976) using sterile filter paper rings and rinsed with Dulbecco's phosphate buffered saline (PBS) into sterile microtubes. In all experiments, one test constituted from 160 000 to 240 000 BCs isolated from five embryonic scutellum. Cells were centrifuged (3000 rpm; 5 min.) and washed 3 times with PBS to obtain a suspension of pure BCs. The cells were then transferred into a fresh OPTI-MEM medium supplemented with 10% foetal bovine serum (FBS) and Penicilin-Streptomicin antibiotic (PS) (1:100) and subjected to transfection procedures.

Transfection of blastoderm cells by electroporation

Electroporation was performed based on the results of previous studies (WAWRZYŃSKA et al. 2008), where we tested different conditions of electroporation in terms of the best viability of BCs (parameters at which the smallest number of cells were damaged), and the highest percentage of cells showed an expression of the introduced GFP marker gene. Electroporation was briefly performed in a Multiporator (Eppendorf). The electroporation solution was prepared in an iso-osmotic solution with interferon plasmid pOVAINT (IBA, Warsaw, Poland; described previously by BEDNARCZYK et al. 2003), which encoded the human gene, interferon alpha 2a. The BCs were mixed with a plasmid solution and then placed into a 2 mm electroporation slot in a polycarbonate cuvette (Eppendorf) and treated with an electric current of 2 pulses every 60 s, voltage = 25 V and a 500 μ s single pulse duration. After electroporation, the cells were centrifuged (3000 rpm; 5 min), suspended in a 20 μ l OPTI-MEM medium and subjected to lipofection.

Transfection of blastoderm cells by synthetic carriers

Six various synthetic carriers of DNA: DDAB/DOPE (Sigma), DDAP/DOPE, cationic polymers polyethyleneimine PEI 25 and PEI 600, CT/PEI-Chol (kindly obtained from Dr Aleksander Sochanik, Centre of Oncology, Maria Skłodowska-Curie Memorial Institute, Gliwice) and IFA (Freund's Incomplete adjuvant, Pierce Biotechnology) were examined in terms of the most suitable for avian BCs. The BCs were transfected with the GFP marker gene (plasmid pAcGFP1-C1) to compare the effectiveness of the applied carriers.

DDAP/DOPE was tested first: 230 μ l of DMEM medium (Gibco), 15 μ l of DDAP/DOPE (Escort TM Transfection Reagent - Sigma) and 10 μ l of pAcGFP1-C1 (0.5 μ g/ μ l) (Clontech) were mixed together and incubated for 15 minutes at RT. A suspension of BCs in 2 ml of a DMEM medium was added, and the mixture was incubated for 6 h.

The second synthetic carrier was DDAB/DOPE. An emulsion of liposomes of DDAB/DOPE was diluted 5 times with NaCl. 80 μ l of liposome suspension was then resuspended in a 320 μ l OPTI-MEM medium and incubated for 15 minutes at room temperature (RT). Simultaneously, 10 μ g of pOVAINT plasmid was added into 66.4 μ l of an OPTI-MEM medium and incubated for 15 minutes in RT. Both solutions (liposomes and plasmid) were then gently mixed together and incubated at RT prior to combining with the solution of BCs. After 15 minutes, the BCs were dispersed in a transfection solution and additionally incubated in a CO2 chamber (Heal Force) for 4 h at 37.7°C and 5% carbon dioxide.

Next, cationic the polymers polyethyleneimine PEI 25 and PEI 600 were tested. Emulsions of cationic polymers PEI 25 and PEI 600 were diluted with 150 mMNaCl to a final volume of 50 μ l. Simultaneously, 10 μ g of pAcGFP1-C1 (Clontech) was diluted with 150 mMNaCl to a final volume of 50 μ l. Both mixtures were incubated for 10 minutes at RT, followed by PEI combined with plasmid and incubated for a further 30 minutes. 3 ml of an OPTI-MEM medium with BCs was then added to the transfection mixture, and the suspension was incubated for 4 h in a CO2 chamber (Heal Force).

CT-PEI/Chol was tested as the fourth synthetic carrier. Cationic liposome was diluted 10 times. 10µg of CT-PEI/Chol was added to 90 µl of 150 mMNaCl. Simultaneously, 2 µg of pAcGFP1-C1 (Clontech) was diluted with 98 mMNaCl. The dilutions were then mixed with BCs and incubated for 30 minutes. After this, 850 µl of an OPTI-MEM medium was added, and 4 h of incu-

bation in a CO2 chamber (Heal Force) was performed.

We also tested the IFA carrier. 28.4 μ l of IFA (Freund's Incomplete adjuvant, Pierce Biotechnology) was mixed with 8 μ g of pAcGFP1-C1 (Clontech) and 14.2 μ l of DMSO and was incubated for 10 minutes. 500 μ l of DMSO was then added, and the mixture was incubated with BCs for 4 h in a CO2 chamber (Heal Force).

In each case, after transfection, the supernatant was removed (3000 rpm; 5 min), and the cells were resuspended in a fresh OPTI-MEM medium supplemented with 10% FBS and PS antibiotic (1:100). GFP expression was then analysed by a Cytomics FC 500 flow cytometer (Beckman Coulter, Miami, FL) and by intensity of fluorescence under a fluorescent microscope – in order to choose the most efficient carrier of DNA for BCs. Based on these results, the best synthetic carrier was chosen for implementation in the further step.

Injection of blastoderm cells transfected by electroporation and lipofection into stage X blastoderm

The BCs collected from 250 eggs were subjected to transfection as described previously by electroporation and lipofection with DDAB/DOPE together with the presence of a pOVAINT plasmid. After electroporation and lipofection, the BCs were centrifuged (5 min, 3000 rpm), and the supernatant was removed. The pellet from the BCs was mixed with 60 µl of an OPTI-MEM solution supplemented with 10% foetal bovine serum and Penicilin-Streptomicin antibiotic (1:100). A total of 50 newly laid eggs at stage X, stored pointed end down for 5 to 7 days prior to injection, were used for the host embryos (BEDNARCZYK et al. 2000). On the day of injection, the shell was swabbed with 70% (vol/vol) ethanol, and a window 1.0 to 1.2 cm in diameter was made in the blunt end of the egg shell (above the air chamber). A small piece (approximately 0.4×0.4 cm) of the membrane directly over the BCs was then carefully removed to expose the embryo. Approximately 1200 to 2000 donor BCs in 1 to 2 μ l of the medium was injected into the subgerminal cavity of the recipient embryo. All procedures for the injection and embryo manipulation were carried out under sterile conditions. Each window was sealed with adhesive tape, and the eggs were incubated at 37.8°C and a relative humidity of 55-60%.

DNA isolation of recipient embryos and organs

Embryos and their organs (gonads, femoral muscle, brain, pectoral muscle, torso, heart, blood) were collected on 2, 3, 4, 5, 6, 10 and 15 days of incubation. The obtained tissues were subjected to DNA isolation. DNA was extracted with the Master PureTM DNA Purification Kit (Epicentre Biotechnologies). After homogenisation, 5 mg of tissue was added to 300 µl of Tissue and Cell Lysis Solution, in which 1 µl of proteinase K was dissolved (50 mg/ml). The samples were incubated for 15 minutes at 65°C, mixing thoroughly every 5 minutes. The samples were then cooled in a refrigerator for 5 minutes, 175 µl of MPC Protein Precipitation Reagent was added and mixed thoroughly for 10 s to precipitate proteins. In order to separate the pellet, the samples were centrifuged for 10 minutes (10,000 rcf). The obtained supernatant was transferred to clean tubes so as not to stir the precipitate, and 500 ml of isopropanol was then added. To precipitate the DNA, the samples were rotated by hand approx. 40 times and then subjected to centrifugation for 10 minutes. After removal of isopropanol, the DNA was washed with 75% ethanol and dried for 30 minutes. Finally, 35 µl of a TE buffer was added to the DNA and stored for several hours at room temperature to dissolve the pellet. The DNA samples were stored at +4°C. The concentration was measured by a Perkin Elmer Lambda 25 UV/VIS spectrophotometer. In order to obtain appropriate concentrations, the DNA was dissolved to a 50 μ g/ μ l concentration using TE buffer.

PCR

PCR was performed by using the following primers: F-INOVOSP 5' CCA AGC AGC AGA TGA GTC C and R- INTOVASP 5' CCT CTG CTT TCT CAT ATA TCT GTC, generating a product with a length of 713 bp. The components of a single 22.5 μ l sample of PCR reaction were: 2.5 μ l of DNA solution; 2.5 μ l of 2 mM dNTP (Fermentas Life Science); 2.5 μ l of 25 mM MgCl2 (Fermentas Life Science); 2.5 μ l of PCR buffer (Fermentas Life Science); 1 μ l of 0.5 pmol/ μ l F-INOVOSP; 1 μ l of 0.5 pmol/ μ l INTOVASP; 0.2 μ l of Taq polymerase (Fermentas, Life Science) and 12.8 μ l of DNAse free water (Promega). PCR amplification was performed under the following thermal conditions: 30 cycles at 94°C-2 min, 94°C-30 s, 48°C -30 s, 72°C-30 s, $72^{\circ}C - 5$ min in a Mastercycler Personal (Eppendorf).

Electrophoresis

 $5 \ \mu$ l of the PCR product mixed with loading dye (Fermentas Life Science) was electrophoresed on a 1% agarose gel (Sigma). In order to better identify the desired bands, the O'Gene RulerTM 100 bp DNA Ladder (Fearmentas Life Sciences) was also run on each gel. After 1.5 hours of electrophoresis at a voltage of 126 V, the gel image was captured with a DNT Bioimaging System Ligot Bis camera.

Statistical analysis

For statistical analyses the SAS Enterprise Guide was used. All obtained results were evaluated using ANOVA and the Scheffe Test to determine whether there were any significant differences between groups.

Results

In order to obtain the best results for transfection, two nonviral methods were combined: electroporation and synthetic carriers. The experiment was carried out using a previously optimised electroporation programme (WAWRZYŃSKA *et al.* 2008), and the cells were then transfected using the most effective carrier in the presence of a pAcGFP1-C1 synthetic DNA plasmid (Clontech).

Transfection by electroporation and lipofection

Blastoderm cells were transfected by lipofection and electroporation or lipofection only. In this study, we demonstrated that the combination of electroporation and synthetic DNA carriers in all tested cases yielded a statistically higher percentage of BCs in which a foreign protein was expressed (Table 1). However, the most efficient proved to be the transfection of BCs using electro-

Table 1

Carrier	Electroporation+ lipofection (%) Mean value ± SD	Lipofection alone (%)
DDAP/DOPE	58.5 ± 1.11 bA	12.9 ± 2.34 b
DDAB/DOPE	$85.8 \pm 9.95 \text{ aA}$	$21.3 \pm 0.49 \text{ ab}$
PEI 25	76.2 ±8.49 abA	$17.9 \pm 3.37 \text{ ab}$
PEI 600	$82.0 \pm 4.32 \text{ aA}$	11.5 ± 1.11 b
CT-PEI/Chol	$74.5 \pm 18.05 \text{ ab}$	24.4 ± 8.01 a
IFA	$24.5 \pm 2.91 \text{ Bc}$	$18.0 \pm 2.18 \text{ ab}$

The percentage of the cells expressing the marker gene (GFP)

a, b - statistically different at P<0.05

A, B - statistically different at P<0.01



Fig. 1. General scheme of the experiment.



Electroporation + lipofection

poration with two pulses of current with a duration of 500 μs and a voltage of 25V carried out in an iso-osmotic environment, after which a synthetic DNA carrier - DDAB/DOPE (85.8%), was used. As a result of this combination, a four times higher effect was obtained than using the DNA synthetic carrier only. The second most effective carrier was PEI600, yielding slightly lower expression (82.0%). While the least efficient carrier was IFA, which resulted in the expression of the introduced gene at an average level of 24.5%. a value 6.5% higher than this carrier used without prior electroporation. When analysing transfection efficiency as a result of the utilisation of only synthetic carriers, the most advantageous are CT-PEI/Chol and DDAB/DOPE (24.4% and 21.3%, respectively). The smallest percentage of BCs expressing GFP wasobtained in groups where the transgene was introduced with PEI 600 (11.5%). Table 1 shows a significant (P < 0.05) difference in the efficiency of the transfection methods used (electroporation + synthetic carrier vs. carrier only).

In order to check the stability of the applied transfection method, the BCs were subjected to short-term *in vitro* cultivation, and then the fluorescence intensity was evaluated (Fig. 2). The most viable cells, proliferating normally and indicating proper morphology, were in a group where





Lipofection alone

GFP expression after electroporation and lipofection



GFP expression after lipofection

Fig. 2. Effect of transfection methods on expression of GFP after 61 hours of cultivation of blastoderm cells.

a combination of electroporation and lipofection was used. In turn, cells subjected to lipofection only were characterised by reduced viability, shrinkage of the cell membrane, dimming of cytoplasm and formation of apoptotic bodies. In this group, mitotic divisions were not observed, and the cells were grouped into aggregates.

Transgene detection

As a result of injection of transferred BCs into the bloodstream of chickens, we obtained embryos with the presence of introduced exogenes diagnosed by PCR (Fig. 3). The percentage of transgenic embryos was 65% and differs depending on the day of collection of the sample and analysed tissue. On days 4, 5 and 15, all embryos showed the presence of a transgene, regardless of the analysed tissue. In turn, on the third day, we obtained 44% of transgenic embryos, and this turned out to be the lowest (Fig. 4).

The analyses also showed that the amount of the transgene in embryos varies depending on the part of the body. However, differences in the percentage of embryos which revealed the presence of a band corresponding to the pOVAINF exogene and those analysed according to the type of collected tissue (Fig. 5) were not statistically significant and ranged from 40% (in the case of blood and heart) to 69% (for the entire embryo).



Fig. 3. Gel electrophoresis of PCR products amplified from DNA extracted from blastodem cells after transfection using electroporation+lipofection. S - ladder, P1-P9- embryos at 24 hours after transfection, K1, K2 - negative control, P0 - control without DNA, INT1 - plasmid containing the human gene - interferon-alpha 2a (10 ng/µl), INT2 - plasmid containing the human gene- interferon-alpha 2a (25 ng/µl).



Fig. 4. Presence of transgenes- human interferon alpha 2a- in embryos depending on the day of development.



Fig. 5. Presence of transgenes-human interferon alpha 2a- in embryos or in their tissues.

Discussion

Nonviral methods for introducing foreign genes into stem cells can be differentiated according to the method of administration of gene constructs (WAWRZYŃSKA et al. 2008; ROYCHOUDHURY et al. 2010; MAKSIMENKO et al. 2013). These include the injection of naked DNA into the developing embryo In vivo or a combination of the injection together with electroporation or with synthetic carriers (WAWRZYŃSKA 2008; ROYCHOUDHURY et al. 2010). DNA transfer can be carried out during stage X (in ovo injection). In addition, it is possible to conduct the transfection of stem cells outside the body in vitro; stem cells are then introduced into the embryo, forming a chimera. Selection of the transfection method depends on the type of transfected cells (RIBEIRO et al. 2001).

One method used to produce transgenic birds is microinjection, involving the injection of a naked DNA construct into an embryonic disc. The obtained chickens can transmit the introduced exogene into the next generation. Despite a successful transfection process, in most cases, the introduced DNA is not expressed, it integrates with the host genome in a random manner and becomes gradually silenced (PERRY *et al.* 1991; SANG 1994; INADA *et al.* 1997; ROYCHOUDHURY *et al.* 2010; MAKSIMENKO *et al.* 2013). Currently, this method is very rarely used and has been quickly replaced by alternative, more efficient ways of introducing human genes into the recipient cells (e.g. lipofection or electroporation).

Electroporation is another method of transfection that is used more often. This is a method which uses an electrical current that acts on the cell membrane, causing a momentary and reversible increase in the permeability of the cell membrane, with the result that the formed nanopores temporarily allow the transport of large and small molecules into the cytoplasm (NEUMANN et al. 1982; PUC et al. 2001). According to some researchers, this method may cause damage to the lipid groups in the cell membrane, which may lead to the creation of deep, hydrophilic pores (TIELEMAN 2004). It is most important to determine suitable method parameters that determine the optimal parameters of the electric field (voltage, the number and duration of pulses) and the relevant conditions affecting the state of the cell (temperature, osmotic pressure, size, shape of the cells) (reviewed in WAWRZYŃSKA & BEDNARCZYK 2007). In our previous work (WAWRZYŃSKA et al. 2008), the highest transfection efficiency (85.8% of cells) was obtained by combining electroporation (isoosmotic solution, two electric pulses at a voltage of 25 V and a duration of 500 µs) with a cationic lipid DOTAP/DOPE.

Chemical transfection methods include the use of synthetic DNA carriers such as cationic lipids and liposomes, cationic polypeptides and the latest generation dendrimer copolymers (WAWRZYŃSKA & BEDNARCZYK 2007). These methods of transfection are characterised by high efficiency. However, the effect of the expression of foreign genes disappeared with the passage of time. LUO *et al.* (2004) proposed nanoparticle silica as a carrier of DNA. A major advantage of this method is the ability to introduce foreign DNA into a cell covered with a protective layer. The coating provides protection for the DNA from digestion by endonucleases.

Presently, for the transfection of avian primordial germ cells and BCs, the most commonly used were lipids and cationic liposomes (WAWRZYŃSKA *et al.* 2008). These molecules are endowed with a positive charge, which allows them to attract the negatively charged DNA molecules. Thanks this feature, they connect to form a complex of DNA/lipid with a positive charge. This enables the penetration of the complex by a negatively charged membrane through endocytosis.

The success of synthetic carriers of DNA depends on the force of transport complexes target cells, DNA penetration into the cell, release into the cytoplasm, penetration of the nucleus, transcription and expression of a foreign gene. Transfection efficiency is influenced by the type of medium used and thus it should be selected to match the type of the transfected cells (LAMPELA 2004).

In previous research (WAWRZYŃSKA *et al.* 2008), high viability of cells was observed after application of the following electroporation programme: iso-osmotic solution, 4 pulses at a voltage of 25V and a duration of 50 μ s. After applying these parameters, 77.2% of the cells remained alive and undamaged. The highest transfection efficiency was obtained with synthetic carriers DDAB/DOPE and CT-PEI/Chol. After their use, we received average GFP expression levels at 21.3 % and 24.4% of the BC population, respectively. MURAMATSU *et al.* (1997) obtained similar results with bird cells, showing the poor performance of synthetic carriers.

In this study, as a result of electroporation after which incubation was carried out with the test BCs, synthetic DNA carriers in the presence of the GFP marker gene proved that most of the cells showed the presence of a foreign gene, using DOTAP/DOPE. Green illumination was observed in 85.8% of the cells. Our experiment demonstrated that the combination of electroporation with synthetic DNA carriers in all cases tested provided a statistically higher percentage of cells transfected efficiently. Moreover, the injection of chicken embryos with transfected BCs was successful and obtained 63.5% of embryos with introduced genes.

The differences between embryos at various days of incubation were observed, from 44% positive on the second day, to 100% positive on days 4, 5 and 15. The number of chimeric embryos (with an exogenous gene) depends on the day of incubation, and this conclusion is in accordance with the

observation of SIWEK *et al.* (2010). However, this percentage does not decrease along with the development of the embryo. The highest percentage of positive samples was observed in DNA isolated from the entire embryo, and the lowest samples from the blood and heart of embryos. In 60% of the analysed embryos, the presence of an exogenous gene in the gonads was detected. This arrangement would allow for the transmission of the transgene to subsequent generations. The analysis showed that the amount of transgenes in embryos varies depending on the organ.

Conclusions

Transgenic hens can produce specific therapeutic proteins. A combination of two nonviral transfection methods of chicken BCs: electroporation of a synthetic carrier, allowed for obtaining a statistically higher percentage of cells expressing the reporter gene GFP in comparison with the methods used separately. The highest transfection efficiency (85.8% of cells) was obtained by combining electroporation (iso-osmotic solution, two electric pulses at a voltage of 25 V and a duration of 500 μ s) with a cationic lipid DDAB / DOPE. The injection of the transfected cells in vitro into recipient embryos (stage X) allows for introducing a gene coding the human interferon alpha-2a, whose presence was detected by PCR, into their genome Therefore, the combination of electroporation and lipofection methods is an effective tool for the introduction of a human gene into chicken BCs. The presence of the transgene was observed in 63.5% of the embryos. In addition, the transgene was not supplanted from the DNA of the later stages of embryo development. In particular days of embryo development, there was no linear decrease in the number transformed chickens. The presented methods of production of transgenic chickens can be used successfully for further research into the production of biopharmaceuticals in avian bioreactors.

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