Folia biologica (Kraków), vol. **56** (2008), No 3-4
doi:10.3409/fb.56_3-4.197-201

Influence of Electroporation on Chicken Blastoderm Cell Viability *in vitro**

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Accepted April 22, 2008

WAWRZYNSKA M., BEDNARCZYK M., LAKOTA P., LUBISZEWSKA M. 2008. Influence of electroporation on chicken blastoderm cell viability *in vitro*. Folia biol. (Kraków) **56**: 197-201.

The aim of this study was to compare two types of devices used for blastoderm cell (BC) transfection: the Nucleofector (Amaxa, Biosystems) and the Multiporator (Eppendorf). To assess the influence of electric current on BCs, different conditions of both nucleofection and electroporation were used. Next, the viability of cells was assessed. The highest number of cells (90.8%) was viable after nucleofection in the G10 program,. After transfection in the presence of pmaxGFP, the A23 program was found to be most advantageous. The elecroporation experiment with the Multiporator (Eppendorf) showed a significant influence of osmotic pressure and voltage on BC viability. Namely, in the isoosmolar buffer BC viability was statistically higher ($P \leq 0.05$) in comparison to the hypoosmolar buffer. The, viability of cells was statistically higher ($P \leq 0.05$) after application of 25V as compared to 50V. The efficiency of transfection in the presence of EGFP-C1 after electroporation in 2 pulses, 25V, 500 μs in the isoosmolar buffer was better than in the recommended conditions in the Amaxa Biosystems A23 program.

Key words: Blastoderm cells, electroporation, nucleofection, efficiency.

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Production of transgenic birds is a more complicated process than creating transgenic mammals because of the specific avian reproductive system. Microinjection, the most often applied technique in mammals, is impractical in birds because polispermic fertilization in hen occurs in the infundibulum of the reproductive tract. Therefore, identification of the male pronucleus among the supernumerary spermatozoa is difficult, just as the return of the ovum to the oviduct of a fistulated hen.

Although viral transfection systems enable efficient introduction and expression of transgenes in chicken cells (SCOTT & LOIS 2006), it is difficult and hazardous to use these in transgenic bird production. Another methodological disadvantage is the limited capacity of transgene size. Besides methodological problems, this method is not accepted by society because viral vectors, including replication-defective vectors, are able to recombine with wild-type viruses (CRITTENDEN & SALTER 1992).

A strategy developed in recent years is the use of transgenic chimeric intermediates. The main steps of this strategy include the isolation of donor embryonic cells, their transfection *in vitro*, injection into recipient embryos, identification of somatic chimeras, raising of chimeras and identification of germline chimeras, and their inter se mating in order to obtain the transgenic birds (BEDNARCZYK 2003).

In general, chimeric chickens have been produced by two different procedures. The first is based on blastoderm cell (BCs) injection into the subgerminal cavity of the recipient embryo, and the second uses primordial germ cells (PGCs) transferred directly into the bloodstream of the recipient embryos. In our investigations, the production of chicken chimeras was conducted with a modified method of BC injection (BEDNARCZYK *et al*. 2000b). We did not only achieve a high rate of hatchability of manipulated embryos (41 %) and high percentage of somatic chimeras among hatched chickens (87 %, BEDNARCZYK *et al*. 2000a; ŁAKOTA 2001), but also a satisfactory rate of chicken chimeras raised until maturity (87 %), as well as a high percent of germline chimeric chickens (30%, BEDNARCZYK *et al*. 2002).

*Supported by the Ministry of Scientific Research and Information Technology, project No. 6 P06D 029 26.

By investigating the use of cationic liposome/DNA complexes (lipofection), we achieved reporter gene expression (GFP and luciferase) in cultured BCs *in vitro* (BEDNARCZYK *et al*. 2003). Moreover, it was demonstrated that under analogous conditions of lipofection the introduction and expression of human interferon 2a and growth hormone genes under the control of the ovalbumin promoter into BCs was also possible. However, the transfection efficiency of lipofection-assisted DNA delivery is still low and accompanied with relatively high toxicity (BEDNARCZYK *et al*. 2003).

Another transfection method, electroporation (EP), has been used successfully for the transfection of particular cells/tissues during embryonic development (KRULL 2004). EP appears to be one of the most efficient methods for chicken embryo transfection as well (MURPHY & MESSER 2001). The main parameters that affect EP effectiveness are: pulse amplitude, pulse duration, number of delivered pulses, osmotic pressure etc. (KOTNIK *et al*. 2003). If these parameters exceeds certain high values of the electric field, irreversible membrane permeabilization and cell death will occur (VALIČ *et al*. 2004). On the other hand, survival of successfully transfected cells has a great impact on efficiency of EP (BRIELMEIR *et al*. 1998). Thus, the electroporation parameters must be determined for each type of cells used. Lately, a new method of transfection, nucleofection (NF), introduced by Amaxa, Biosystems was developed.

In this study, we determined the effects of different electroporation parameters on the survivability of chicken blastoderm cells *in vitro*.

Material and Methods

Stage X blastoderms from freshly laid donor eggs were prepared with the use of filter paper rings. Blastoderms were isolated from the yolk and washed several times with PBS without Ca^{2+} and Mg^{2+} ions (Gibco) in order to prepare one probe. Then cells were dispersed in PBS by repeated aspirations into a Pasteur pipette. After dispersion, the cells were centrifuged for 5 min at a relative centrifugal force (RCF) of 300 x *g* and then washed three times with PBS. Each supernatant was discarded, and the cells were nucleofected or electroporated.

Nucleofection

In Amaxa Nucleofector™ technology the NF was performed with 10 different programs: A17, C16, G10, S18, T14, T20, T23, U09, U14, U30 in 10 mm gap cuvettes. Coded programs differed in intensity of the electric pulse (field strength) and pulse length (further details are proprietary information of Amaxa). As a result two programs were chosen as the least harmful (G10, C16) and one as the most destructive (U09) for cells. Next the influence of the different electroporation buffers (Cell Line Nucleofector Kit V, Human T Cell Nucleofector Kit and Mouse ES Cell Nucleofector Kit) on the survival rate of BCs was investigated in G10, C16, and U09 programs. Experiments in all tested programs were repeated three times. Viability of cells was assessed after 24 hours of culture using a dye-exclusion (Trypan Blue) assay.

Subsequently, cells were nucleofected with 20 µg pmaxGFP under CMV promoter (Amaxa Biosystems) and Mouse ES Cell Nucleofector Kit solution in a 2 mm gap cuvette according to the Amaxa Biosystems protocol using G10 or C16 programs selected by us and the programs A23 and A24, suggested by Amaxa for embryonic cells. The green luminescence of cells was detected using a fluorescence microscope.

Electroporation

EP under different voltage conditions (25 V, 50 V), duration of pulse (15 µs, 50 µs, 500 µs) and pulse numbers (1 pulse, 2, 4, 6, 8 and 10 pulses) in 10 mm gap cuvettes was performed in hypoosmolar and isoosmolar buffers. Experiments were repeated three times. Cell viability was assessed by cell counting after 24 hours of culture using a dye-exclusion (Trypan Blue) assay.

The best conditions (cell viability) were chosen and approximately 600 000 BCs were electroporated in the presence of EGFP-C1 (Clontech). The cells were harvested after transfection, and the expression of the marker gene was examined by fluorescence microscopy (Axiovert 200) using appropriate filters (excitation 558 nm and emission 583 nm).

All obtained results were evaluated using the SAS 9.1 program. ANOVA and Tukey tests were employed to determine whether there were any significant differences between treatments.

Results and Discussion

An advantage of electroporation as a nonviral transfection method is the possibility of using cells which have been isolated from the blastoderm disk several minutes before transfection. After a procedure lasting a few seconds, cells can be injected into recipient embryos. Therefore, it is an alternative to methods that need long cultures. EP bypasses the unresolved problem of proper culture medium composition maintaining PGCs *in vitro*. Although this is an undeniable merit, a serious

drawback of EP is its low efficiency, which is due to an inappropriate choice of process parameters. Some experiments with EP on BCs with application of different devices have been performed (MURAMUTSU *at al*. 1997; ATKINS *et al*. 2000; WANG *et al*. 2006), however, it is difficult to compare the results because of different parameters of the electric field. In this study, in order to systematize these data, two types of devices were used: the Nucleofector ™ (Amaxa, Biosystems) and the Multipolator (Eppendorf).

In the Nucleofector technology DNA was introduced directly into the cell nucleus. This process was called nucleofection by the producer and was tested as a very efficient nonviral transfection method. The efficiency of NF was confirmed in different lines of cells, including stem cells (HAMM *et al*. 2002; GARTNER *et al*. 2006; ZEITEHOFER *et al*. 2007). However, experiments on BCs and PGCs have not been reported to date. A drawback of NF are the coded programs which make it difficult to assess the influence of parameters of the electric current on cells.

In our experiment ten different programs were chosen and their influence on cell viability was assessed. As shown in Figure 1, the best results were achieved in program G10. After NF with G10, almost the entire population of cells (over 90%) was viable and theoretically capable of expressing the introduced gene construct. We assessed program C16 as safe for cells as well because more than 50 % of BCs were undamaged. In contrast, programs U09 and T20 were completely useless for avian embryonic cells because only 18.2 % and 28.7 % of cells remained viable, respectively.

In order to exclude the negative influence of osmotic pressure, NF was performed in the presence of a solution recommended by producer: the Cell Line Nucleofector Kit, the Human T Cell Nucleofector Kit and the Mouse ES Cell Nucleofector Kit (Table 1). A statistically higher ($P \leq 0.05$) viability was observed in programs G10 and C16 in comparison to U09, regardless of solution type. However, in the most harmful program U09, viability increased from 18.2 % in the previous experiment to 51.4 % on average in all tested solutions; in program G10 the viability decreased from 90.8 % in first experiment to 78.6 % on average. It is likely that disadvantageous results of the electrical current in the C16 program are partly eliminated if NF is performed in the presence of the Human T Cell Nucleofector Kit solution. Analogously, in the program U09 necrosis was minimized in the presence of Mouse ES Cell Nucleofector Kit.

In contrast to the Nucleofectior™ technology, the application of the Multiporator (Eppendorf) allows controlling current parameters through regulation of voltage, pulse duration and number of

Table 1

Influence of the electroporation buffer on the survival rate (%) of BCs; a, b – values significantly different ($P \leq 0.05$)

Programs	Amaxa's buffers			
	Cell Line Nucleofactor Kit V	Human T Cell Nucleofactor Kit	Mouse ES Cell Nucleofactor Kit	Mean
G10	80.0	80.8	75.0	78.6 a
C16	77.7	81.7	75.6	78.4 a
U09	43.3	47.7	63.6	51.4 b
Mean	66.7	69.6	71.5	

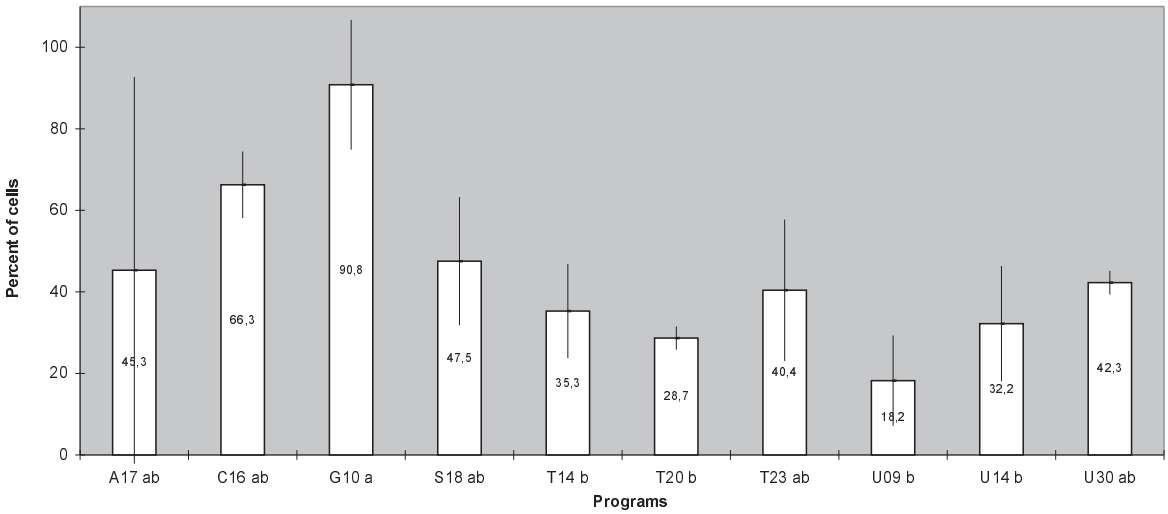

Fig. 1. Influence of different programs in nucleofection (Nucleofector™, Amaxa) on survival rate (%) of BCs.

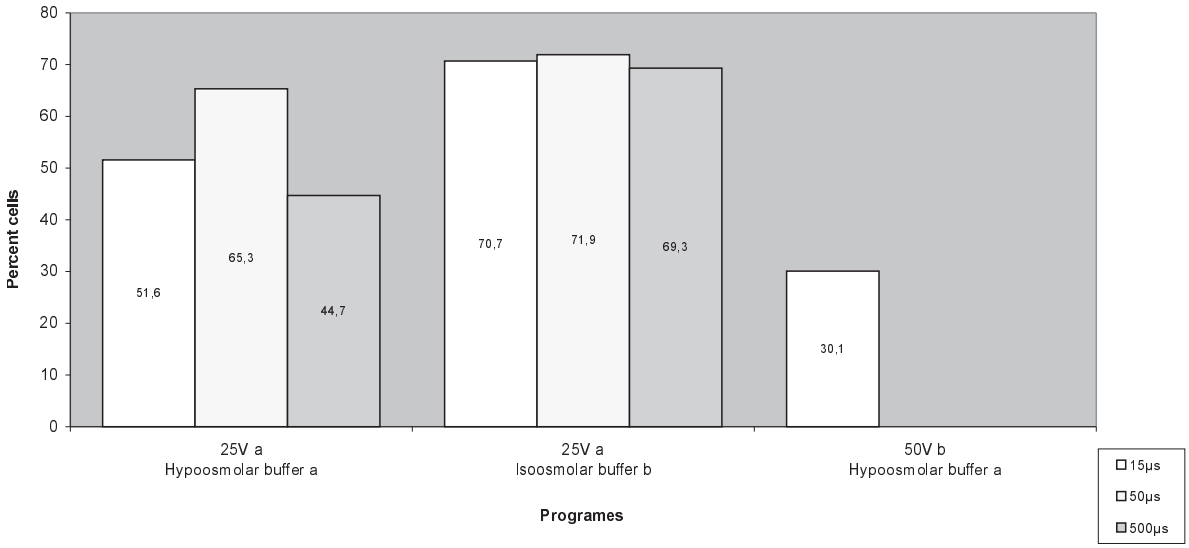

Fig. 2. Influence of electroporation conditions (Multiporator, Eppendorf) on survival rate (%) of BCs.

delivered pulses. Similarly, application of two different electroporation buffers (iso and hypoosmolar), the composition of which is known, allows for the assessment of the influence of osmotic pressure on cell viability. In this study several combinations of voltage and duration of pulse at different osmotic pressures were tested (see Fig. 2). The highest viability of cells (almost 72 %) was detected under the following conditions: 25V, 50 μs and isoosmolar buffer. This result is comparable to the best result achieved in the Nucleofecor ^{TM} technology. The most detrimental for BCs was a hyposomolar solution accompanied by the 50V pulses lasting for 15μs. However, BC viability in this program was still approximately 10 % higher than in program U09, the most harmful for cells. Moreover, in the isoosmolar buffer BC viability was statistically higher ($P \leq 0.05$) in comparison to the hypoosmolar buffer. Similarly, if a lower voltage was applied (25 V), BC viability was statistically higher ($P \leq 0.05$) compared to 50 V, revealing the sensitivity of the examined cells to this factor.

Gene transfer involves two distinctly separate processes. The first step must provide a mechanism in which the genetic information can be transported from extra cellular space, across biological membranes, into the nucleus. There the incoming genetic information co-mingles with the genome of the target organism. The second step of the process affords a means for the new genetic information to become a part of the target genome (WALL 2002). In order to assess the conditions and efficiency of the process, NF and EP in the presence of reporter plasmids were performed.

NF with pmaxGFP in the Mouse ES Cell Nucleofector Kit solution was performed according to the Amaxa Biosystems protocol using G10 and C16 programs and also the most advantageous programs A23 and A24, recommended by Amaxa. Illumination was observed in the 37th hour of incubation in all employed programs except for G10.

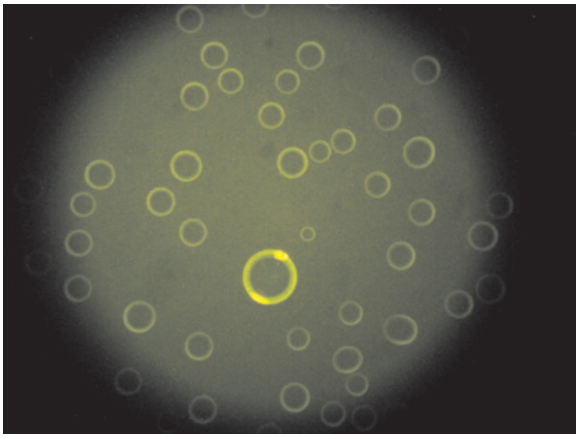

Fig. 3. Nucleofection – pmax GFP expression (program A23, 37th hour of incubation).

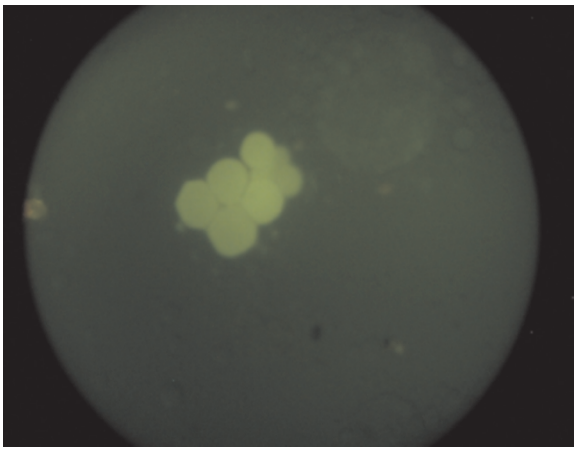

Fig. 4. Electroporation – EGFP-C1 expression (isoosmolar buffer, 2p, 25V, 500 μs, 26th hour of incubation).

In the G10 program weak illumination was observed after the 61st hour, which lasted only for 6 hours. In programs C16, A23 and A24 cells were illuminated for 30 hours. The best fluorescence was achieved in the A23 program (Fig. 3).

In a parallel experiment, the Multiporator (Eppendorf) reporter gene GFP in plasmid EGFP-C1 was used. For transfection, 1 and 2 pulses, 25 V, 500 μs and an isoosmolar buffer were chosen as the best conditions of electroporation. After application of 1 pulse, 25 V and 500 μs, illumination was first observed in the 46th hour of incubation. It lasted for only 10h. In the second program (2 pulses, 25 V, 500 μs). the result was better (Fig. 4). The first green fluorescent cells were observed after 32 hours. The duration of the phenomenon was 38 hours. This result was better than in A23, the best program recommended by Amaxa Biosystems. This proved that using double pulses has an impact on the efficiency of electroporation.

Our experiment indicates that both types of tested electroporators are suitable for chicken BCs. Small changes in electroporation conditions can have a significant influence on transfection efficiency. Osmotic pressure and voltage have the largest influence on BC viability. Because in the Nucleofector ™ electroporation technology it is impossible to regulate the conditions of the process, this device is more suitable for popular cell lines. However, for BCs the use of Multipolator (Eppendorf) gave better results.

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