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COMPARISON OF TRANSFECTION METHODS FOR PORCINE ZYGOTES

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Abstract

Pronuclear microinjection of DNA into a zygote is the standard procedure used for production of transgenic pigs. This method is characterized by low efficiency, with up to 2% of microinjected zygotes resulting in transgenic animals. In addition, DNA microinjection is a complex and labour-intensive method. Lipofection is the method of choice for transfection of somatic cells. Lipofection is a simple and effective procedure, but its direct use in zygotes is limited by the zona pellucida of the zygote, which prevents the penetration of lipofectant into the cytoplasm. Introduction of the lipofectant/DNA mixture into the perivitelline space enables it to penetrate the cytoplasm and transfer exogenous genetic information into the cell nucleus. The aim of the study was to compare the efficiency of standard DNA microinjection procedure and a new DNA lipomicroinjection method, used for transfection of porcine zygotes.

Key words: pig, transgenesis, DNA microinjection, DNA lipomicroinjection, efficiency

The production of transgenic animals is a complex procedure. The process as a whole is affected by several factors, which result in the production of a transgenic animal with modified genotype. Despite increasingly sophisticated technologies, the efficiency of the process remains disproportionately low compared to the labour inputs and costs. In pigs, transgenesis efficiency using standard DNA microinjection, which continues to be the leading technique in this species, does not exceed 2% in terms of the number of zygotes subjected to microinjection (Jura et al., 2007).

Cloning using transfected somatic cells or their nuclei to produce transgenic animals is even less efficient. This is due to the inefficiency of the cloning process, the complexity and limitations of which have been well documented in the literature (Kruip and Den Daas, 1997).

In the near future, application of the new-generation lentiviral vectors, whose transfection efficiency is increasingly promising, will likely improve the efficiency of the production of transgenic animals, including transgenic pigs. However, this will take place only when researchers have developed commercially available vectors using safer types of lentiviruses, which will enable their use in laboratories with lower security levels (Jura et al., 2007).

Likewise, technologies that use transfected stem cells to produce transgenic mammals are still undergoing improvement and standardization in the case of farm animal species. The main constraint to full use of stem cell technology in transgenesis of pigs and other farm animal species is the availability of pure stem cell germ lines suitable for transfection.

As already mentioned, transgenesis is a complex process and factors related to reproduction physiology as well as embryological and molecular factors have a significant effect on its efficiency (Williams and Jurkiewicz, 1992; Jura et al., 2006; Jura et al., 2007). A key role is played by method of introducing exogenous genetic information. The efficiency of the process is low when standard DNA microinjection technique is used. Therefore, there is an urgent need to develop a less complex transfection method, whose efficiency will surpass or match that of DNA microinjection (Jura and Jurkiewicz, 2006; Melo et al., 2007).

The objective of this study was to determine the efficiency of lipomicroinjection, a new zygote transfection method, in comparison with standard DNA microinjection. The comparative tests also accounted for the "molecular factor", or gene constructs (vectors) used to carry genetic information to induce certain changes in the genotype, which plays a key role in the transgenesis process. The gene constructs were equipped with reporter genes, which enable the procedure efficiency to be determined under *in vitro* conditions and transgenic embryos to be selected before transfer to synchronized recipients.

The paper presents the results of transfection of porcine zygotes obtained using standard DNA microinjection in comparison with a new, alternative method of transfection named lipomicroinjection.

Material and methods

Polish Landrace, Duroc and line 998 donor and recipient gilts aged 6–8 months and their crossbreds (90–120 kg body weight) were used.

Collection of zygotes

Donor gilts were superovulated by intramuscular injection of 1500 IU of PMSG (Serogonadotropin, Biowet, Poland), followed after 72 h by intramuscular injection of 1000 IU of HCG (Biogonadyl, Biomed, Poland). After detection of oestrous signs (24 h after HCG administration), donor gilts were inseminated twice at 12-h intervals (Smorąg et al., 1999; Jura et al., 2007). Zygotes were recovered surgically or postmortem by flushing oviducts with PBS containing bovine albumin (Sigma, USA). The eggs obtained were subjected to morphological evaluation

under a stereoscopic microscope. Morphologically normal eggs were used for microinjection.

Lipomicroinjection

Morphologically normal eggs were placed in a micromanipulation chamber filled with PBS containing 20% fetal calf serum (FCS) (Sigma, USA). The procedure was carried out under an inverted microscope equipped with Nomarski optics and two micromanipulator units. The liposome/gene construct complex was inserted under the zona pellucida into the perivitelline space. After lipomicroinjection, transfected zygotes were transferred to a small vessel containing PBS and albumin and again subjected to morphological evaluation. Only zygotes with an intact cytoplasmic structure were selected for culture.

DNA microinjection

Prior to DNA microinjection, morphologically normal eggs were centrifuged to reveal the pronuclei (15000×g, 5 min) and placed in a micromanipulation chamber filled with PBS containing 20% fetal calf serum (FCS) (Sigma, USA). The procedure was carried out under an inverted microscope equipped with Nomarski optics and two micromanipulator units. The gene construct was inserted into one of the exposed pronuclei. After DNA microinjection, transfected zygotes were transferred to a small vessel containing PBS and albumin and again subjected to morphological evaluation. Only zygotes with an intact cytoplasmic structure were selected for culture.

The following 2 gene constructs were used for microinjection and lipomicroinjection:

1. WAP-*INFeGFP* – expression gene construct – containing a fragment encoding the *human interferon* gene with the eGFP reporter gene.

2. CMV *RFP* – reporter gene construct – containing the *Red Fluorescent Protein* reporter gene.

***In vitro* culture**

Transfected zygotes were transferred to four-well dishes filled with culture medium. Cultures were performed in NCSU 23 medium (North Carolina Univ., USA), in a laminar flow incubator (38.5°C, 5.0% CO_2, maximum humidity) (Peters et al., 1993). After 5 days of culture, embryos were assessed morphologically by choosing blastocyst-stage embryos for *in vivo* assessment (Gajda et al., 2004). Blastocysts were transferred to PBS (Sigma, Germany) supplemented with 20% FCS (Sigma, Germany) and mounted in groups of 5 on a depression slide.

***In vivo* assessment of transfection efficiency**

After 5 days of *in vitro* culture, embryos were evaluated under a fluorescence microscope (Nikon, Japan) using the following UV filters: 450–490nm for embryos transfected with WAP-*INFeGFP* vector and 510–560 nm for embryos transfected with CMV-*RFP* vector. Embryos that reached the morula/blastocyst stage and showed marked luminescence whose colour corresponded to a reporter gene (green for *eGFP* or red for *RFP*), were classified as positive (transgenic).

Results

Thirty pigs were superovulated to obtain zygotes for lipomicroinjection and standard microinjection. A total of 435 zygotes were obtained. Lipomicroinjection with the WAP-*INFeGFP* gene construct was performed on 122 zygotes and lipomicroinjection with the CMV *RFP* vector on 60 zygotes. The control group were 70 zygotes subjected to standard microinjection with the WAP-*INFeGFP* construct and 85 zygotes subjected to microinjection with the CMV *RFP* vector. Treated zygotes were subjected to *in vitro* culture. As a result of culture, zygotes transfected using lipomicroinjection produced 44 (36.1%) blastocysts after introduction of the WAP-*INFeGFP* gene construct and 23 (38.3%) blastocysts after the CMV-*RFP* vector was used. The blastocysts obtained were evaluated under a fluorescence microscope. Expression of the WAP-*INFeGFP* insert was found in 5 (11.5%) blastocysts. Of the 23 blastocysts obtained from zygotes subjected to lipomicroinjection with the CMV-*RFP* vector, expression was found in 4 (17.4%) blastocysts. Standard microinjection was performed on 70 zygotes using the WAP-*INFeGFP* vector and on 85 zygotes using the CMV-*RFP* gene construct. Zygotes transfected with the standard DNA microinjection method were the control group in relation to zygotes subjected to lipomicroinjection. As a result of transfecting 70 zygotes with the WAP-*INFeGFP* vector, culture to the blastocyst stage resulted in 19 (27.1%) blastocysts. A total of 25 (29.4%) blatocysts were obtained after the CMV-*RFP* vector was introduced. *In vivo* evaluation under a fluorescence microscope showed expression of the WAP-*INFeGFP* insert in 4 (21.1%) blastocysts. Expression of the CMV-*RFP* insert was observed in 6 (24.0%) blastocysts (Table 1, Figure 1).

Table 1. Comparison of porcine zygote transfection results obtained using lipomicroinjection and standard microinjection methods

Transfection method	No. of transfected zygotes	No. of blastocysts obtained (%)	No. of transgenic blastocysts (%)
Lipomicroinjection			
WAP-*INFeGFP*	122	44 (36.1)	5 (11.4)
CMV-*RFP*	60	23 (38.3)	4 (17.4)
Standard microinjection			
WAP-*INFeGFP*	70	19 (27.1)	4 (21.1)
CMV-*RFP*	85	25 (29.4)	6 (24.0)

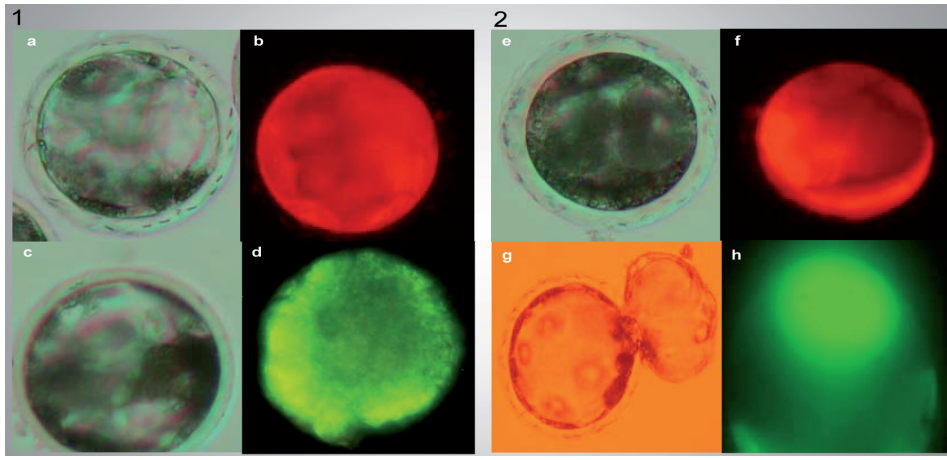

Figure 1. Transgenic pig blastocysts
1) Blastocysts obtained from zygotes after lipomicroinjection: a-blastocyst transfected with CMV-*RFP* vector; b-blastocyst under UV light; c-blastocyst transfected with WAP-*INFeGFP*; d-blastocyst under UV light
2) Blastocysts obtained from zygotes after standard microinjection: e-blastocyst transfected with CMV-*RFP* vector; f-blastocyst under UV light; g-blastocyst transfected with WAP-*INFeGFP*; h-blastocyst under UV light

Discussion

Efficiency of DNA microinjection, the most common method used to date to produce transgenic pigs, is low. Other popular transfection techniques used to produce transgenic mice are either unavailable for pigs or highly complex, which makes them unsuitable for routine use under farm conditions because of the extensive equipment required. In a search for an alternative zygote transfection method for DNA microinjection, which would result in comparable or better efficiency of transfection (and thus transgenesis) in pigs, an attempt was made to use modified lipofection for transfection of porcine zygotes. Lipofection is a popular method for transfection of somatic cells. It is a highly efficient procedure of DNA transfection using liposomes. In this method, exogenous DNA (gene construct) is carried by monolayer or multilayer liposomes, made of cationic lipids and spontaneously interacting with DNA. In addition, they are capable of penetrating cell membranes and this capacity is used to transport exogenous DNA into the nucleus. Zygote transfection by conventional lipofection, which is used to transfect somatic cells, where the carrier (liposomes) linked to exogenous DNA comes into direct contact with cell membranes surrounding the cytoplasm, is not feasible for zygotes, which are surrounded by the zona pellucida. To enable contact between liposomes linked to exogenous DNA and cell membranes surrounding the zygote, a method for microinjection of the liposome/DNA complex into the perivitelline space, named lipomicroinjection, was used. The present study showed that injection of the complex under the zona pellucida of the zygote is a straightforward procedure. What is more, a much greater volume of the DNA mix-

ture can be deposited in the perivitelline space than is possible with direct injection of DNA mixture into the pronucleus (about 300pl into perivitelline space, 5pl into pronucleus). In theory, a greater number of insert copies should offer a greater chance for integration of exogenous DNA with the host genome. Furthermore, unlike standard DNA microinjection, lipomicroinjection causes no damage to intracytoplasmic or pronuclear structures. Insertion of the injection pipette into the cytoplasm itself causes about 10% of the transfected zygotes to die. Compared to standard DNA microinjection, lipomicroinjection turned out to be a less invasive method, which clearly translates into the number of blastocysts obtained from transfected zygotes cultured *in vitro* (Table 1). In our study, the type of gene construct used for transfection had no substantial effect. In addition, the type of the reporter gene used did not influence the clarity and quality of the *in vivo* evaluation of insert expression (Figure 1). Both reporter genes enable transfection efficiency to be assessed rapidly (about 48 h after *in vitro* culture) and effectively. Comparison of transfection (transgenesis) efficiency leads us to conclude that it was much better when the standard DNA microinjection technique was used. The proportion of "transgenic" blastocysts obtained from zygotes transfected using standard DNA microinjection was almost twice that of "transgenic" blastocysts obtained using lipomicroinjection (Table 1).

Because the study was conducted under *in vitro* conditions, we cannot state conclusively which method will produce a lower percentage of mosaic individuals. It is known that this proportion is relatively high for standard DNA microinjection.

The DNA lipomicroinjection method is much simpler to perform and causes lower loss of transfected material. The more complex DNA microinjection technique makes it possible, at least at this stage, to obtain a greater percentage of "transgenic" embryos. Standard DNA microinjection has reached its limits as it cannot be modified to improve method efficiency. Lipomicroinjection is an alternative method and a number of parameters have to be fine-tuned (e.g. optimum parameters for the liposome/DNA complex have to be determined). Another advantage is that new and increasingly efficient and capacious generations of carriers (liposomes) are being developed.

References

Gajda B., Smorąg Z. (2004). Cell number in pig blastocysts cultured in different media. Ann. Anim. Sci., 4: 315–320.

Jura J., Jurkiewicz. J. (2006). Methods for the production of transgenic animals. Ann. Anim. Sci. Suppl., 1: 29–38.

Jura J., Smorąg Z., Słomski R., Lipiński D., Gajda B. (2007). Factors affecting the production of potential transgenic pigs by DNA microinjection; a six-year retrospective study. J. Anim. Feed Sci., 16: 641–650.

Kruip Th.A.M., Den Daas J.H.G. (1997). *In vitro* produced and cloned embryos: effects on pregnancy, parturition and offspring. Theriogenology, 47: 43–52.

Melo E.O., Canavessi A.M.O., Franco M.M., Rumpf R. (2007). Animal transgenesis: state of the art and applications. J. Appl. Genet., 48 (1): 47–61.

Peters R.M., Wells K.D. (1993). Culture of pig embryos. Reprod. Fertil. Suppl., 48: 61–73.

Smorąg Z., Gajda B., Jura J., Skrzyszowska M., Pasieka J. (1999). Factors affecting the production of zygotes in superovulated pigs: A seven-year retrospective study. Ann. Anim. Sci., 26 (4): 155–161.

Williams B.L., Skarks A.E.T., Canseco R.S., Knight J.W., Johnson J.L., Velander W.H., Page R.L., Drohan W.N., Kornegaj E.T., Pearson R.E., Wilkins T.D., Gwazdauskas F.C. (1992) Evaluation of systems for collection of porcine zygotes for DNA microinjection and transfer. Theriogenology, 38: 501–511.

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Porównanie metod transfekcji zygot świńskich

STRESZCZENIE

Celem pracy było porównanie efektywności standardowej metody mikroiniekcji DNA w odniesieniu do nowo zaproponowanej metody transfekcji zygot świńskich – lipomikroiniekcji. Porównując efektywność transfekcji (transgenezy) należy stwierdzić, że wypadła ona dużo lepiej przy zastosowaniu metody standardowej mikroiniekcji DNA. Odsetek „transgenicznych" blastocyst uzyskanych z zygot transfekowanych metodą standardowej mikroiniekcji DNA był prawie dwukrotnie większy w porównaniu z odsetkiem „transgenicznych" blastocyst uzyskanych po zastosowaniu lipomikroiniekcji. Ponieważ badania prowadzone były w warunkach *in vitro*, nie można jednoznacznie stwierdzić, która z metod będzie skutkowała mniejszym odsetkiem uzyskanych osobników mozaikowych. Metoda lipomikroiniekcji DNA jest jednak dużo prostsza w wykonaniu i powoduje niższe straty w transfekowanym materiale.