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EVALUATION OF THE INFLUENCE OF THREE DIFFERENT METHODS OF TRANSFECTION ON SURVIVAL OF BOAR SPERMATOZOA

Joanna Jurkiewicz[1], Daniel Lipiński[2,3],
Barbara Szczęśniak-Fabiańczyk[1], Ryszard Słomski[2,3]

[1]Department of Animal Reproduction Biotechnology, National Research Institute of Animal Production, 32-083 Balice n. Kraków, Poland
[2]Department of Biochemistry and Biotechnology, University of Life Sciences, Wołyńska 35, 60-637 Poznań, Poland
[3]Institute of Human Genetics, Polish Academy of Sciences, Straszyńska 32, 60-479 Poznań, Poland

Abstract

The use of spermatozoa as vectors for introduction of exogenous DNA into the oocyte during fertilization is an alternative method for generation of transgenic animals. This procedure is based on spontaneous ability of spermatozoa to bind exogenous DNA. For spermatozoa to serve as appropriate vectors for exogenous DNA, they should exhibit good motility and viability, because only such cells are capable of binding exogenous DNA and fertilization. Boar semen was subjected to transfection involving direct incubation of spermatozoa with plasmid DNA, incubation with plasmid DNA in the presence of dimethyl sulfoxide (DMSO), and lipofectant-aided lipofection. Sperm cell membrane motility and damage was evaluated before and after centrifugation and following transfection. Spermatozoa motility varied according to the transfection method used. A statistically significant difference was observed between transfection methods using DMSO and lipofection (P<0.05). The motility and percentage of live spermatozoa of the boar were found to decrease after centrifugation. The decrease in boar spermatozoa motility was the lowest after the direct incubation with plasmid DNA and the highest after lipofection. There was no correlation between the decrease in boar spermatozoa motility and percentage of spermatozoa with damaged cell membrane. No significant effect of decreased spermatozoa motility after lipofection was found.

Key words: transfection, spermatozoa, transgenic animals

The possibility of binding exogenous DNA by mammalian spermatozoa was first reported 29 years ago (Brackett et al., 1971) when spermatozoa were shown to bind foreign DNA and transfer it to the oocytes during fertilization, causing them to trans-

form. Rabbit spermatozoa were incubated with DNA isolated from simian virus 40 (SV40), radioactively labelled with [^{3}H]-thymidine. However, this information was disregarded. It was reported again in 1989 when two research teams described the binding of exogenous DNA by sea urchin (Arezzo, 1989) and mouse sperm (Lavitrano et al., 1989). These findings aroused great interest but were also the subject of much controversy, mainly because of difficulties in obtaining repeatable results (Brinster et al., 1989). However, in the next years many authors published optimistic results suggesting that exogenous DNA can be bound by insect (Atkinson et al., 1991), amphibian (Habrova et al., 1996), fish (Khoo et al., 1992) and mammalian spermatozoa (Camaioni et al., 1992). Despite the initial controversy, this method is now considered promising and providing a real possibility to produce transgenic farm animals. Literature suggests that binding of exogenous DNA molecules to sperm cells and further internalization in nuclei does not occur haphazardly, but is a strictly controlled process. DNA binding site is always localized in the post-acrosomal segment of the sperm head. The binding event is driven by the negative charge of the molecule. The interaction is ionic, reversible, sequence independent; it is not limited to DNA but may involve other, negatively charged macromolecules. In addition, the ability to bind exogenous DNA is only shown by plasma-free spermatozoa (Lavitrano et al., 1992; Zani et al., 1995). The most popular method for integration of DNA with spermatozoa involves direct incubation of plasma-free sperm with exogenous DNA. The incubation is performed with appropriately adjusted temperature and duration parameters. This results in spermatozoa carrying foreign genetic information that are further used for *in vitro* fertilization, and standard or laparoscopic insemination (Lavitrano et al., 2002). However, preliminary studies showed that this method has low efficiency. To make the uptake and internalization of exogenous DNA by sperm more efficient, different methods of sperm transfection were proposed using electroporation, lipofection, restriction enzyme, monoclonal antibody (mAb C), dimethyl sulfoxide (DMSO) or magnetic nanoparticles (MNPs). A recent method proposes microinjecting sperm incubated with DNA into oocyte cytoplasm (ICSI). Another alternative method using spermatozoa as DNA vectors is microinjection of exogenous DNA directly into seminiferous tubules or the rete testis. In addition, this method has to be optimized to make it more efficient. One of the most important aspects is selection of semen donors. Only highly motile sperm (at least 80%) guarantees the binding of exogenous DNA and fertilization. It is very important to select boars as sperm donors which are characterized by perfect seminal parameters because of individual differences in semen quality. The ability to bind exogenous DNA is only shown by spermatozoa with no plasma, which is why it has to be removed. It is essential that centrifugation parameters ensure complete removal of plasma and centrifugation does not reduce semen motility. When optimizing procedure conditions, special consideration must be given to the moment when the interaction between exogenous DNA and sperm cells begins. It is also important to determine the appropriate amount of exogenous DNA used in the reaction and the parameters of spermatozoa incubation with exogenous DNA. Literature reports indicate that about 90% of exogenous DNA is bound to sperm cells, but only 20% is internalized in the sperm nucleus. It is unfavourable to use excessive amounts of

exogenous DNA because this may damage sperm cells, thus making them incapable of binding exogenous DNA and fertilization (Sciamanna et al., 2000).

The aim of this study was to evaluate the influence of three different methods of transfection on survival of boar spermatozoa subjected to co-transfection with two plasmids containing albumin and GFP. Additionally the plasmids were labelled with rhodamine.

Material and methods

Preparation of semen

Semen collected from sexually mature boars was used as experimental material. After preliminary macroscopic and microscopic examination (motility, concentration, pH), fresh semen was diluted 1:10 in Biosolwens Plus extender (Biochefa, Poland). Semen was centrifuged twice (800 × g for 10 min., 25ºC; 800 × g for 10 min., 17ºC) to remove plasma. After removal of supernatant, the sperm precipitate was diluted with extender to required volume and semen concentration was calculated using a Bürker chamber after 100-fold dilution with 3% NaCl.

Genetic construct

We used two plasmids in equal concentrations: 1. *pWAPAlb* gene construct containing human albumin cDNA in the pBluescript SK+ vector at *Cla*I and *Sph*I restriction sites and WAP regulatory sequence introduced into *Sph*I and *Eco*RI restriction sites, and 2. pTracer-EF/Bsd A containing GFP (Invitrogen). Both plasmids were labelled with rhodamine (Mirrus Bio).

Transfection of sperm

The following boar sperm transfection methods were used:

1. Direct incubation of spermatozoa with plasmid DNA

Boar spermatozoa at a concentration of 1×10^6 sperm in 200 µl of Biosolwens Plus with 0.4 µg (concentration 1µg/1µl) of plasmid DNA were incubated. The mixture was incubated at 17°C for 2 h.

2. Incubation in the presence of dimethyl sulfoxide (DMSO), followed by thermal shock

Samples containing boar spermatozoa at a concentration of 1×10^6 sperm in 500 µl of Biosolwens Plus were supplemented with 1% DMSO. Next, 0.4 µg (concentration 1µg/1µl) of plasmid DNA was added to the mixture, gently stirred and incubated for 10 min at room temperature. After incubation, the mixture was again supplemented with 3% DMSO. The mixture was incubated at 4°C for 20 min., at 42°C for 2 min. and at 17°C for 2 h.

3. Incubation in the presence of lipofection mixture

0.4 µg of plasmid DNA (1µg/1µl) was dissolved in 50 µl of Biosolwens Plus and 0.8 µl of lipofectant was also dissolved in 50 µl of the same extender. Both solutions were incubated for 30 min. at room temperature. Following incubation, the solutions were combined, gently stirred and incubated for 10 min. at room temperature. After

incubation, the mixture was added to the samples of boar spermatozoa at a concentration of 1 × 10^6 sperm in 500 µl of extender and incubated at 37°C for 4–5 h.

Evaluation of sperm motility

Sperm motility was evaluated on a microscope slide heated to 38°C. Boar sperm samples were evaluated before centrifugation, after centrifugation and after transfection. The evaluation was performed daily until sperm motility reached 30%.

Cytometric evaluation of sperm cell membrane damage

Damage to cell membranes of boar spermatozoa was evaluated by flow cytometry using double staining with SYBR-14/propidium iodide. These stains distinguish live from dead sperm. The method is based on the ability of low-molecular-weight SYBR-14 to enter the cell through the intact plasmalemma and to bind to genetic material contained in the cell nucleus, and the ability of high-molecular-weight propidium iodide to enter the cell and bind to the DNA of dead cells or to the strongly disturbed structure of plasmalemma. The staining procedure was as follows: to 1 ml of diluted sperm was added 5 µl of SYBR-14 (1 mM in DMSO), incubating sperm for 10 min. at 35°C, and then 5 µl of propidium iodide was added (24 mM in H_2O), incubating the mixture for 10 min. at room temperature, which was followed by cytometric analysis. Boar sperm samples were evaluated before and after centrifugation, and following transfection.

Statistical analysis

Statistical significance of the differences was analysed using Duncan's test.

Results

The study was performed with 4 ejaculates from 4 boars in 3 repeats. Sperm motility and cell membrane damage were evaluated before centrifugation, after centrifugation and after transfection using 3 transfection methods: direct incubation in the presence of plasmid DNA, incubation with plasmid DNA in the presence of DMSO, and lipofection.

The motility of boar sperm samples averaged 67.5% before centrifugation and decreased to an average of 55% after centrifugation. Transfection caused a further reduction in motility. Sperm motility averaged 40% after direct incubation with foreign DNA, 27.5% after incubation in the presence of DMSO, and 22.5% following lipofection.

In the analysed samples containing sperm, intact cell membrane was found in 85.3% of sperm before centrifugation and in 72.1% of sperm after centrifugation. Intact cell membrane was observed in 71.6% of sperm after direct incubation with foreign DNA and in 74.9% of sperm after incubation in the presence of DMSO. The lower proportion of sperm with intact cell membrane (49.2%) was found after lipofection (Figure 1).

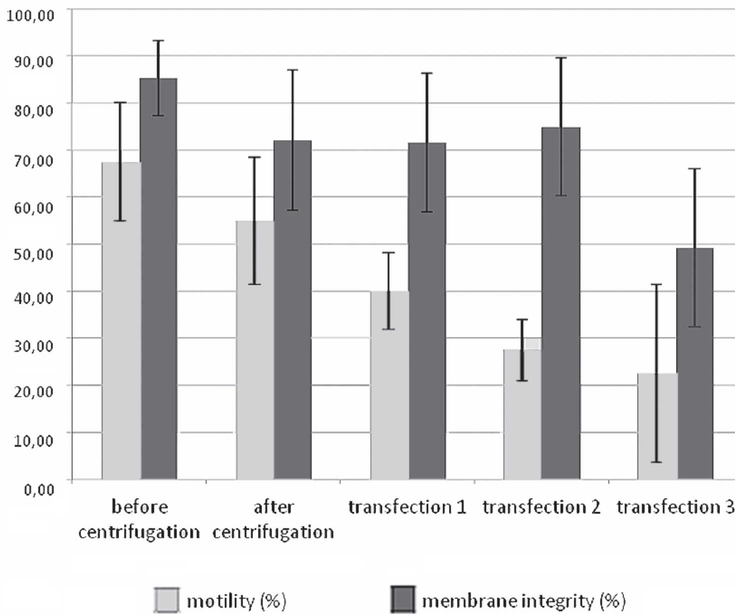

Figure 1. Effect of the centrifugation procedure and incubation of exogenous DNA on motility and membrane integrity of boar spermatozoa
*Transfection 1 – incubation of exogenous DNA with spermatozoa.
Transfection 2 – incubation of exogenous DNA with spermatozoa and DMSO.
Transfection 3 – incubation of exogenous DNA with spermatozoa and liposomes.

Discussion

The main barrier to the use of spermatozoa as vectors of exogenous DNA in animal transgenesis is the lower survival of transfected sperm, which limits their application in insemination or *in vitro* fertilization. In view of the considerable interest in this method in recent years and the possibility of using it in transgenesis of farm animals, we assessed survival of boar semen subjected to transfection. The results obtained confirm the reports that the critical moment of sperm transfection is sperm centrifugation to remove plasma, and incubation with exogenous DNA. These stages of the method reduced sperm motility in the boar (Kang et al., 2008; Lavitrano et al., 2002; Wu et al., 2008), cattle (Anzar and Buhr, 2006) and other species of animals (Castro et al., 1990). Centrifugation was found to reduce sperm motility and proportion of live cells. Thus, removal of plasma, which is a necessary stage of the method, lowers sperm motility and viability (Kang et al., 2008). As a result of transfection, sperm survival varied according to transfection method. The three transfection methods had differing effects on boar sperm motility and cell membrane damage. Most reports on transfection of boar sperm concern the direct incubation with plasmid DNA. This method resulted in the binding and transmission of the transgene dur-

ing fertilization to produce genetically modified embryos and transgenic animals with modified genetic characteristics (Lavitrano et al., 2002; Webster et al., 2005; De Cecco et al., 2010). No such reports on transfection of boar spermatozoa using DMSO or liposomes are available. In other species of animals, sperm transfection using DMSO and liposomes made the binding and internalization of exogenous DNA much more efficient (Bachiller et al., 1991; Kuznetsov et al., 2000). In the presence of liposomes, over 80% of mouse spermatozoa were found to bind exogenous DNA, with 60% fertilization success when appropriate DNA and liposome concentrations were applied (Bachiller et al., 1991). Meanwhile, incubation of rabbit spermatozoa with exogenous DNA in the presence of DMSO in combination with thermal shock increased the number of embryos with confirmed transgene presence from 19 to 62% (Kuznetsov et al., 2000). Both DMSO and liposomes make it easier for exogenous DNA to penetrate sperm cell membrane without showing any toxic effect. In our study, a statistically significant ($P<0.005$) difference was found between the transfection methods using DMSO and lipofection in the boar. In the samples of sperm transfected with DMSO a higher proportion of live sperm cells than after centrifugation was found. These results were not statistically significant. From among the three transfection methods proposed, lipofection reduced boar sperm motility and proportion of live cells to the highest extent, one possible reason being that liposomes need to be incubated for several hours at a minimum of 37°C and incubation of boar spermatozoa with liposomes at this temperature weakens the sperm of this species. Direct incubation with plasmid DNA proved to be the most beneficial for boar sperm, which is consistent with reports from the world literature (Lavitrano et al., 2002; Lavitrano et al., 2003). As a result of direct incubation of boar spermatozoa with plasmid DNA, the decline in boar sperm motility and proportion of live sperm cells was the lowest. What is more, no correlation was found between the decline in boar sperm motility and the proportion of sperm with damaged cell membrane. It can therefore be speculated that spermatozoa with poor motility still have an intact cell membrane and can be used for transfection, whereas generally weaker motility and viability of sperm after transfection can be attributed to activation of endonucleases that cause cell apoptosis (Maione et al., 1997).

The present study established the survival of boar sperm subjected to different transfection methods, and pinpointed those transfection factors and stages that determine survival of boar sperm. Further attempts at boar sperm transfection should concentrate on selection of ejaculates characterized by high motility, the use of appropriate centrifugation parameters and optimum amounts of plasmid DNA introduced, as well as incubation time and temperature. A thorough understanding of these aspects will result in better survival of boar semen, which is strictly related to the capacity for binding exogenous DNA and fertilization.

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JOANNA JURKIEWICZ, DANIEL LIPIŃSKI, BARBARA SZCZĘŚNIAK-FABIAŃCZYK, RYSZARD SŁOMSKI

Ocena wpływu trzech różnych metod transfekcji na przeżywalność plemników knura

STRESZCZENIE

Wykorzystanie plemników jako wektorów do przekazywania egzogennego DNA do oocytu podczas procesu zapłodnienia jest alternatywną metodą uzyskiwania zwierząt transgenicznych. Metoda oparta jest na spontanicznej zdolności wiązania egzogennego DNA przez plemniki. Aby plemniki mogły spełniać rolę odpowiednich wektorów dla egzogennego DNA, muszą charakteryzować się dobrą ruchliwością i żywotnością, gdyż tylko takie komórki są zdolne do wiązania egzogennego DNA i zapłodnienia. Nasienie knura poddano transfekcji polegającej na bezpośredniej inkubacji plemników z plazmidowym DNA, inkubacji z plazmidowym DNA w obecności DMSO (dimetylosulfotlenek) oraz lipofekcji wspomaganej użyciem lipofektanta. Oceniano ruchliwość i uszkodzenie błony komórkowej plemników przed i po wirowaniu oraz po transfekcji. Zaobserwowano różnice w przeżywalności plemników w zależności od stosowanej metody transfekcji. Statystycznie istotną różnicę zaobserwowano między metodami transfekcji z użyciem DMSO i lipofekcji ($P<0,05$) Stwierdzono spadek ruchliwości i odsetka żywych plemników knura po wirowaniu. Najniższy spadek ruchliwości nasienia knura zaobserwowano po bezpośredniej inkubacji z plazmidowym DNA, a najwyższy po lipofekcji. Nie stwierdzono korelacji pomiędzy spadkiem ruchliwości plemników knura a odsetkiem plemników z uszkodzoną błoną komórkową. Nie zaobserwowano statystycznie istotnego obniżenia ruchliwości plemników po lipofekcji.