TRANSIENT TRANSFECTION OF PORCINE GRANULOSA CELLS AFTER 3D CULTURE IN BARIUM ALGINATE CAPSULES

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Three-dimensional culture systems in barium alginate capsules can be employed to maintain primary granulosa cells in an undifferentiated state for almost 6 days. This is due to a self-organization of cells in a pseudofollicular structure. The transfection of primary granulosa cells is a necessary condition when employing these culture systems for several purposes, for example as an in vitro toxicity test or the development of oocytes or zygotes. In this work, the feasibility of two transient transfection techniques (liposome-mediated and electroporation) was assessed in primary porcine granulosa cells after a 6-day culture in an artificial extracellular matrix (barium alginate membrane). Human recombinant green fluorescent protein was chosen as a molecular readout, and protein expression was assessed after 48 hours from transfection. Liposome-mediated transfection gave low transfection levels, with increasing yields from 2 to 12 μgDNA/ml of medium; the maximum percentage (85.7%) was reached at 12 μgDNA/ml of medium. Electroporation-mediated transfection yields were higher: the best results (81.7% of transfected cells) were achieved with two 50V pulses and 12 μg/ml DNA. The application of a single or double pulse (50V) at 4 mgDNA/ml gave negligible results. These results indicate that primary granulosa cell cultured in barium alginate capsules can be transfected by electroporation with high transfection yields.

Granulosa follicular cells (GC) have been extensively studied in the last decade: the challenges are to obtain suitable *in vitro* structures able to promote competent oocyte development and maturation in order to improve the *in vitro* production of human and animal embryos (1-2). In developing follicles, GC are partially endowed with stem-like behaviour, with an intramural replication probably caused by the presence of an extracellular matrix (ECM) (3); when cultured in monolayer or suspension GC undergo morphological and functional evolution and epithelial-mesenchymal transition (luteinisation) with increased synthesis of progesterone and a reduction in aromatase activity (4-6). These features are related to the lack of a three-dimensional (3D) cell arrangement and/or the absence of a suitable ECM, mainly basal lamina; on the other hand, the reduction in oestradiol (E_2) synthesis leads to failure of oocyte maturation, since oocytes need to be primed with E_2 to develop Ca^{++} oscillations during maturation (7).

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Recent results indicate that 3D co-culture of follicular wall cells with immature oocytes is a good methodological approach, since oocyte development depends on interactions between somatic cells and germinal cells (3, 8-11). A 3D system of culturing GC cells encapsulated in barium alginate/protamine membranes has recently been proposed: the capsules allow self-organisation of round-shaped GC into clusters; the luteinisation index (progesterone to oestradiol ratio) is significantly lower in encapsulated GC than in monolayer cultures (12). Transfection of these 3D cultured GC is the first step to enable this culture system to be employed for various new purposes, such as studies of cytotoxicity, oocyte-cumulus interactions and zygote development. The aim of this work was to verify the feasibility and the yields of two techniques of transient transfection (cationic liposomes and electroporation) of primary porcine GC after 6 days of 3D culture in barium alginate gel capsules.

MATERIALS AND METHODS

Cell preparation

Ovaries at different stages of development were taken from sows (aged 6-11 months) in an abattoir; samples were washed at 30°C with NaCl 0.9%. Follicles with a diameter of 2-6 mm were identified on the surface of the ovaries, and follicular fluids containing GC were aspirated using a polypropylene disposable syringe. These cellular suspensions were then centrifuged and washed twice with 10 ml of tissue culture medium 199 (TCM199) + 10% fetal calf serum (FCS) + 1% penicillin/streptomycin. The cell concentration in the resulting GC pellet was determined using a Makler counter. Cell concentrations in the pellets were generally in the range between $1 \cdot 10^5$-$5 \cdot 10^5$/ml.

Cell encapsulation and culture

A method previously described for living cell encapsulation was employed for the 3D immobilisation of the cells (12-13). The centrifuged cell suspension was diluted in a xanthan gum solution (Satiaxane®, SKW Biosystems, France) 0.5% in TCM199, containing Earle salts, L-glutamine and sodium bicarbonate (Sigma-Aldrich, Milan, Italy) to provide a suspension: xanthan gum ratio of 1:3. A saturated solution of $BaCl_2$ was added to the suspension to a concentration of 20 mM. The suspension was then extruded at 25°C through a 25-gauge needle and dropped into a medium viscosity (3500 cP) sodium alginate solution (Sigma-Aldrich, Milan, Italy) 0.5% in culture medium stirred at 30 rpm. The resultant capsules were collected, washed twice with TCM199 containing Earle salts, L-glutamine and sodium bicarbonate and suspended in the same medium. Capsules were treated with a solution of protamine sulphate 1% in TCM199 (containing Earle salts, L-glutamine and sodium bicarbonate) for 30 min at 25°C. All employed solutions were sterilised by filtration. Each single capsule was put into a well for cell culture and suspended in 600 µl of culture medium (TCM199 + 10% FCS + 1% penicillin/streptomycin + 100 mg l 3,17-androstenedione). All culture wells were maintained in an incubator for 6 days at 38.5°C, 5% CO_2 and 90% humidity. The capsules' contents were then aspirated by a pipette with a glass tip, centrifuged and diluted to $2 \cdot 10^6$ cells/ml in TCM199. Cells were evaluated for viability under a microscope (100x) using the trypan blue exclusion method. At least 200 cells were counted in two replicates for each sample. Results are reported as percentage of live cells.

Cell transfection

Construction of plasmid vectors

The gene for the expression of human recombinant green fluorescent protein (hrGFp) was employed. Plasmids encoding for the hrGFp gene were digested with two restriction enzymes: *BamHI*, and *Hind III*. The digestion mix was composed of R^+ Buffer, *Bam HI*, *Hind III*, and distilled water. The mixture was incubated for 60 min at 37°C, followed by 20 min at 65°C to stop the reaction. All reagents and kits were supplied by Stratagene (La Jolla, CA, USA).

The hrGFP gene was isolated by gel electrophoresis (40 ml Tris-acetate-EDTA buffer + 400mg agarose + 20 µl ethidium bromide (Sigma)) for 30 min at 95 mV, followed by gene purification with NucleoSpinExtract Kit (Clontech, Palo Alto, CA, USA). The gene was ligated on pCMVScript expression vector (Stratagene, La Jolla, CA, USA); the presence and size of a DNA insert in the pCMV-Script vector was determined by PCR amplification of DNA (PTC 150 Minicycler). XL10-Gold ultracompetent cells (Stratagene, La Jolla, CA, USA) were employed for the replication of the expression vector, following the manufacturer's instructions. Plasmid DNA was purified using the Spin-column system (DNANucleoSpin Plasmid Kit, Clontech, Palo Alto, CA, USA) and the NucleoSpin Extract kit (Clontech, Palo Alto, CA, USA).

Liposome-mediated gene transfer

A TFX™ Reagents Transfection trio was used as a model cationic lipid packing agent. The reagent is multilamellar vesicles formulated with different molar ratios of a cationic lipid moiety (N,N,N',N'-tetramethyl-N,N'-bis(2-hydroxyethyl)-2,3,-di(oleoyloxy)-1,4-

butanediammonium iodide) and dioleoyl phosphatidylethanolamine (DOPE). Samples were prepared by diluting a solution of purified hrGFP gene containing plasmids into the TFX™ solution at a ratio of 3:1, following the manufacturer's recommendations. DNA-TFX™ complex was added to the cell cultures to achieve final concentrations of 2, 4, 6, 8, 10, 12, and 14 μgDNA/ml, incubated for 90 minutes at room temperature, washed with TCM 199, centrifuged, and then diluted to a final concentration of $1.66x10^5$ cells/ml; 900 μl of cell suspension ($1.5x10^5$ cells) were put into culture wells and then incubated at 38.5°C in 5% CO_2 for 48 hours.

Electroporation-mediated gene transfection

Seventy μl of cell suspension ($1.5x10^5$ cells) were added to 50 μl of DNA solution at 4 μg/ml or 12 μg/ml and transferred to a 2-mm gap cuvette with parallel-plate aluminium electrodes (BTX, Genetronics, San Diego, CA, USA). Electroporation was carried out with a BTX Electro Square Porator ECM 830 (Harvard Apparatus Inc.); one or two 50 V exponential decay pulses were applied at room temperature, with a 20 ms time constant and 20 sec inter-pulse spacing. After recovery at 37°C for 10 min, the cell suspension was processed as reported above for liposome-mediated transfection.

Assessment of transfection efficiency

The number of cells expressing green fluorescent protein in each well was evaluated by fluorescence microscopy (Leica DM LB30 FLUO) imaging with a FISH filter at 450-490 nm and manual counting (at least 500 cells). Cell viability was evaluated by the trypan blue exclusion method. At least 200 cells were counted in two replicates for each sample. Results are reported as percentage of transfected or live cells.

Statistical analysis

Differences between liposome transfected cell percentages depending on DNA concentrations were assessed by one-way ANOVA, followed by the Scheffé test for multiple comparisons. The effects of DNA concentration and pulse number on electroporation-mediated transfection were evaluated by two-way ANOVA, considering DNA and pulses as fixed factors and the percentage of transfected cells as the dependent variable. Because of the nature of variables (mean of percentages), data were submitted to an arcsin transformation prior to the ANOVA procedure.

RESULTS

The efficiency of liposome-mediated transfection as a function of DNA concentration is illustrated in Figure 1: in no case did the transfection yield exceed 7%.

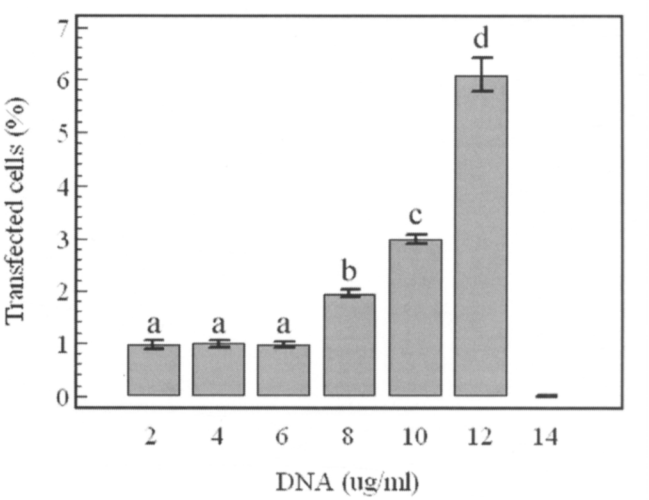

Fig. 1. *Liposome-mediated transfection yields as a function of DNA concentration. Different letters indicate a $p<0.05$ difference between groups.*

There was a significant enhancement of transfection efficiency up to a concentration of 12 μgDNA/ml, but efficiency dropped to zero (i.e. no transfection occurred) when 14 μgDNA/ml were added.

The results of electroporation-mediated transfection are illustrated in Figure 2. The transfection efficiency was much higher, reaching a mean value of 81.7±11%. Two-way ANOVA highlights a significant effect of DNA concentration and pulse number; the peak mean value was obtained at 12 μgDNA/ml with 2 pulses. Figure 3 is a fluorescence image of cells transfected by electroporation under these conditions. A granulosa monolayer with numerous pale grey cytoplasms can be seen, indicating the expression of hrGFP gene.

DISCUSSION

The non-proportional effect or bell-shaped response curve of DNA concentration on transfection efficiency with cationic liposome preparations has been well documented. Felgner *et al.* (14) hypothesised that such a phenomenon is due to a toxic effect of the cationic lipid molecules as they became incorporated into cellular membranes. The cationic lipid content of cellular membranes may be sufficient to significantly alter the net charge of the membranes and adversely affect the activity of ion channels, membrane receptors as well as enzymes. Liposome-mediated transfection of

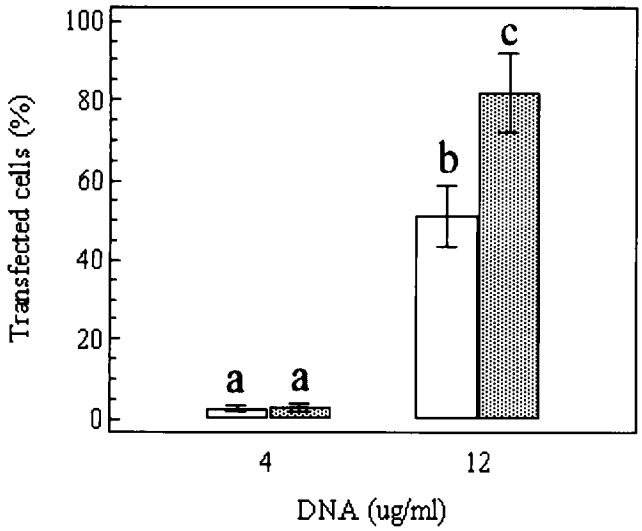

Fig. 2. *Electroporation-mediated transfection yields for a single (white bar) or double pulse (dotted bar) as a function of DNA concentration. Different letters indicate a $p<0.05$ difference.*

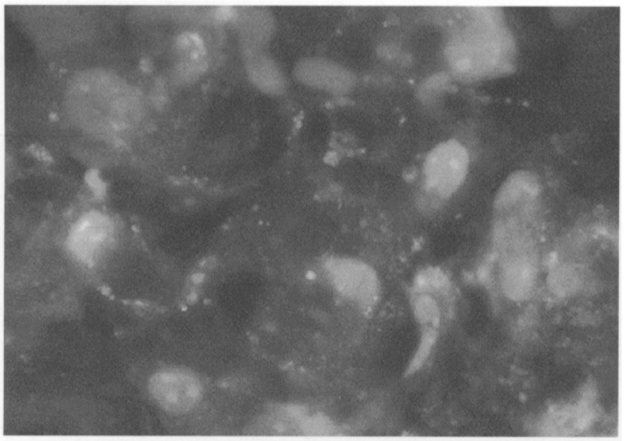

Fig. 3. *Fluorescence microscope photograph of electroporation-mediated transfected GC (12 μgDNA/ml concentration, 2 pulses). Magnification 40x.*

immortalised KK-1 murine GC was described by Paukku *et al.*, (15) who employed DOPE, sphingosine and some of its derivatives, obtaining good results in terms of transfection efficiency. In this work the same kind of cells were transfected, but in primary culture, usually considered refractory to most of the common transfection systems: such transfection yields are compatible with the results reported by Uyttersprot *et al.* for quiescent primary dog and human thyroid follicles transfected with FuGENE 6 transfection reagent, with yields being 8-15% (16). Electroporation is able to deliver molecules into GC by exposing cells to an electric pulse that generates a transmembrane voltage of a few hundred millivolts for microseconds to milliseconds. This is believed to create transmembrane pores with variable lifetimes: exogenous molecules, such as DNA, can enter cells through these pores by diffusion and/or electrically driven transport (17). Orly *et al.* (18) reported that electroporation is the method of choice for transfecting DNA into bovine ovarian cells, but luteinised cells lose 90% of their ability to take up plasmid DNA.

In conclusion, the high GC transfection levels by electroporation after 6 days of 3D culture in alginate membranes can be related to the non-luteinic status of the GC: a follicle-like structure has been obtained by promoting polarisation of the cells and their self-assembly into tissue-like structures that can be transfected with widely used techniques. A GC 3D culture system allowing low luteinisation levels for 6 days seems to be a valid basis for facilitating transfection activity. This is an important feature when primary cell lines, mainly derived from a single subject, are used. By disposing of "slow-luteinising" GC it is easier to produce cell batches with high transfection yields expressing suitable types of proteins, following the different phases of oocyte maturation. The transfected cellular suspension can also be submitted to a further encapsulation process in the same device or a new one, by means of microinjection or other suitable methods. The use of this 3D co-culture system is therefore a promising approach for *in vitro* maturation of competent female gametes, as well as for the culture of stem-like and stem cell lines.

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