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Effectiveness of liposomes to transfect livestock fibroblasts

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ABSTRACT. The development of an efficient transfection system in livestock cells is an important step towards investigating gene transfer and the functioning and production of transgenic animals. Important factors involved in cationic liposome mediated gene transfer were evaluated through *in vitro* transfection of bovine, caprine and ovine fibroblast cells. Transfection of plasmid DNA complexes of different commercially available liposomes (Lipofectamine, Lipofectin, Cellfectin and DMRIE-C; Gibco-BRL, USA) was evaluated utilizing the following parameters: DNA/liposome ratio, cell density, DNA conformation, and the effect of transfection time on the efficiency of bovine fibroblasts to express a reporter gene. The effects and concentrations of liposomes were also evaluated in caprine and ovine fibroblasts. Lipofectamine alone and Lipofectamine with Plus reagent induced high-frequency expression of β-*galactosidase* and *neo* genes in all cells evaluated (47 and 88.3%, respectively). Regarding phenotype, chromosomal stability was similar in transfected and non-transfected cells. The parameters set in this study will establish a foundation for utilizing transfected fibroblast cells to gen-

erate transgenic animals through nuclear transfer technology and gene function studies.

Key words: Transfection, Liposomes, Fibroblasts, Livestock cells

INTRODUCTION

The transfer of genetic material into mammalian cells is an indispensable technique for investigating function and gene therapy (Sikes et al., 1994; Colosimo et al., 1998, 2000). Diverse industries, including pharmaceutical, food and chemical ones, rely on the production of recombinant proteins (Jebanathirajah et al., 2002). Key advantages of mammalian cells over other expression systems are their ability to carry out proper protein folding, complex *N*-linked glycosylation and authentic *O*-linked glycosylation, as well as a broad spectrum of post-translational modifications (Makrides, 1999). Recently, *in vitro* transfection of cultured cells combined with nuclear transfer was demonstrated to be the most effective procedure to produce transgenic livestock (Schnieke et al., 1997; Polejaeva and Campbell, 2000; Bordignon, et al., 2003).

A variety of methods have been reported for mammalian cell transfection including microinjection (Sikes et al, 1994), particle bombardment (Williams et al., 1991), calcium phosphate (Chen and Okayama, 1988), virus mediation (Kovesdi et al., 1997), and liposomes (Gao and Huang, 1995). However, each technique has its limitations. Some methods require complicated procedures, expensive equipment or present low transfection efficiency with suspension cells. Liposomes along with cationic polymers and $CaPO_4$ precipitation are among the most efficient chemical vehicles for DNA delivery into mammalian cells (Chen and Okayama, 1988; Baker et al., 1997).

Cationic liposome-mediated gene transfer is a useful process for *in vivo* (Seol et al., 2000; Wiseman et al., 2003; Smyth, 2003) and *in vitro* (Nguyen et al., 2003; Yan et al., 2003; Sugiyama et al., 2004) cell transfection. This system allows specific genes to be introduced into mammalian cells, transcribed and subsequently to produce proteins that reflect native molecular structures and biological functions (Colosimo et al., 2000). Easy manipulation and uniform transfection efficiency make liposomes the most utilized transfection process at present. Plasmid DNA mixed with small cationic vehicles such as liposomes, form a DNA/lipid complex, which based on electrostatic interaction is able to interact with target cells and transfer DNA to the cytoplasm. In general, optimization of a DNA-liposome condition depends on its mixing ratio and cellular development stages (Sakurai et al., 2000). The overall ratio between the negative DNA charge and the positive cationic lipid charge may determine the success of gene transfer. It is generally accepted that a direct correlation may exist between the lower gene transfer efficiency at higher charge ratios and cytotoxin induced by the cationic lipids (Jenkins et al., 2003; Trubetskoy et al., 2003).

Transfection time, DNA quantity, and liposome type are some critical factors that influence transfection efficiency (Colosimo et al., 2000). Despite information regarding liposome transfection, there are no reports about the best parameters for livestock primary cell transfection. In this study we evaluated the following parameters: DNA/liposome ratio, cell density,

DNA conformation, and the effect of transfection time of liposome-mediated gene transfer on high-frequency transfection of bovine fibroblasts. The effects of liposomes and concentrations were also evaluated in caprine and ovine fibroblasts. Results will establish a foundation to integrate transfected fibroblasts into gene expression studies and therapy, as well as to generate a nucleus donor to produce transgenic animals.

MATERIAL AND METHODS

Fibroblast cell cultures

Primary fibroblast cell cultures were established from bovine, caprine and ovine ear skin biopsies. Fur and cartilage were removed from 2-mm^2 skin fragments. Samples were subsequently rinsed three times with 1 ml ice-cold PBS supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml) (Sigma, St. Louis, MO, USA). Then, skin fragments were rinsed four times with 1 ml Trypsin solution (Trypsin 0.05%, w/v, and EDTA 0.02%, w/v; Gibco BRL, Carlsbad, CA, USA) and transferred to 25-cm^2 cell culture bottles containing 4 ml D-MEM (Dulbecco's modified Eagle's medium, Gibco BRL), 10% fetal calf serum (FCS) (Gibco BRL), penicillin (100 IU/ml) and streptomycin (100 µg/ml; Sigma). Cells were cultured at 37°C and 5% CO_2 until reaching 70% confluence. Growing cells were trypsinized and transferred to new cell culture bottles without the original skin fragments.

Plasmid DNA vectors

The plasmid vector pCMVβ (7.2 kb), utilized to evaluate transient transfection, was purchased from Clontech (Palo Alto, CA, USA). This vector carries the β-*galactosidase* gene under control of the cytomegalovirus (CMV) promoter sequence and a poly-A sequence. The plasmid vector pCI-*neo* (5,472 pb), utilized for stable transfection evaluations, was purchased from Promega (Madison, WI, USA). This vector carries the neomycin phosphotransferase gene under control of the CMV promoter sequence and a poly-A sequence. Plasmids were amplified in DH5α strain of *Escherichia coli* and purified using a Qiagen Plasmid Giga Kit (Qiagen, Valencia, CA, USA). DNA plasmid concentrations were measured by UV absorption at 260 nm. Plasmid DNA purity was assessed using agarose gel electrophoresis and A_{260}/A_{280} ratio measurements.

Parameters affecting transient transfections of bovine, ovine and caprine fibroblast cells

Diverse parameters affecting transient transfection efficiencies in livestock fibroblast cells were evaluated. 1) Efficiency of DNA/liposome cell transfection was assessed using the pCMVβ vector to transfect established cell cultures of bovine, caprine and ovine fibroblasts with four liposome reagents: Lipofectin (1 mg/ml), Lipofectamine (2 mg/ml), DMRIE-C (2 mg/ml), and Cellfectin (1 mg/ml) (Gibco BRL). Solutions consisted of 150 µl D-MEM with no FCS added to the plasmid vector in a concentration of either 5.3, 2.65, 1.33, 0.66, 0.33 or 0.16 µg/ml (Gibco BRL), mixed with 150 µl D-MEM containing the appropriate liposome reagent for a final concentration of 8.3 µl/ml. After 15 min at 22°C, the DNA/liposome complexes in a final volume

of 300 µl were added to the cell culture wells (3.5 cm in diameter) containing 7 x 10^4 fibroblasts, between the 6th and 8th passages, and cultured at 39°C and 5% CO_2. After 5 h, the DNA/liposome complexes were removed, and a fresh medium with 10% FCS was added to stop transfection. 2) The effect of cell density on transfection rate was evaluated utilizing bovine fibroblast cultures with densities of 3 x 10^4 (30% confluence), 6 x 10^4 (60% confluence), and 9 x 10^4 (sub-confluence ~90%) cells/ml transfected with 2.65 µg/ml of plasmid vector and 8.3 µl/ml Lipofectamine. 3) The effect of plasmid vector form on transfection efficiency in bovine fibroblast cells was evaluated using Lipofectamine liposome and pCMVβ plasmid vector in: a) circular form; b) linear form attained by digesting pCMVβ with *Hind*III and c) fragment form carrying the β-gal gene, CMV promoter, and SV40 poly-A signal, obtained by digesting pCMVβ with *Eco*RI and *Hind*III (4,529 bp). The linear and the fragment forms were gel purified after digest. Fibroblasts were transfected with 37.5 nM of each vector and 8.3 µl/ml Lipofectamine. 4) The influence of time and peptide reagent on transfection rates was evaluated using bovine fibroblast cells transfected with or without the polypeptide cocktail Plus reagent (Gibco BRL). A concentration of 2.65 µg/ml of the plasmid vector was mixed with 13.3 µl/ml Plus reagent in 150 µl D-MEM for 15 min at room temperature, prior to incubation with Lipofectamine (8.33 µl/ml). Fibroblast cells were exposed to DNA-peptides-liposome complexes for 3 or 6 h. Transfection was stopped by replacing the transfection media with fresh D-MEM plus 10% FCS.

Biochemical β-galactosidase assay

β-galactosidase expression was detected 24 h after transfection (Sanes et al., 1986). The percentage of transfected cells was calculated under an optic microscope (100X) and determined by the ratio between the number of β-gal expressing fibroblast cells (blue cells), and the number of total fibroblast cells in the observed field. Five distinct fields were recorded for each sample. All experiments were performed in triplicate, plotted, and transfection percentage values were transformed into Arcsin, and means were compared by the Tukey test ($P < 0.01$).

Stable fibroblast transfection

Bovine fibroblast cells were plated at 2 x 10^5 into 24-well culture dishes and transfected with 0.5 µg pCI-*neo* and Lipofectamine (8.3 µl/ml) with Plus reagent 13.3 (µl/ml). Forty-eight hours after transfection, cells were diluted to 1:10, and G418 was added for a final concentration of 0.5 mg/ml. Transfected cells achieved subconfluence after 7 days of selection. At the third passage, one portion of the cells was diluted 1:10, cultured under antibiotic selective pressure for seven days and cryopreserved as an uncloned population. The other portion was diluted 1:1000 and cultured under antibiotic selective pressure for 7 days. Individual colonies were isolated and expanded for cryopreservation, chromosome counting and PCR analysis.

Chromosome counting

Transfected bovine fibroblast lines, isolated for two weeks after selection with G418, were used for chromosome counting. A cell sample was induced into metaphase by colchicine treatment (0.04 mg/ml), fixed, stained with Giemsa, and the chromosome numbers were counted with optic-microscopy to detect aneuploidies. Cell arrest, fixation, and chromosome staining

with Giemsa were performed to detect aneuploidies as previously described (Freshney, 1992). Chromosome counts were performed in triplicate and represent one sample.

Polymerase chain reaction analysis

Genomic DNA of two lines of transfected and one of non-transfected bovine fibroblasts were extracted and purified as described (Miller et al., 1988). Genomic DNA was used as a template in PCR reactions containing specific *neo* gene primers (410 bp): NPT151 (5'-ATGATTGAAGAAGATGGATTG-3') and NPT941 (5'-GAAGAACTCGTCAAGAAGGCC-3'). The PCR reaction was carried out in a final volume of 25 µl containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 160 µM of each dNTP, 0.4 µM of each primer, and 2 U Taq polymerase (Invitrogen, Carlsbad, CA, USA). The PCR reaction consisted of 35 cycles (94°C/1 min, 60°C/1 min, 72°C/1 min) in a PT-100 thermal cycler (MJ Research, Waltham, MA, USA). Amplified PCR products were analyzed in 1.5% agarose gel stained with ethidium bromide (50 ng/ml), and images were digitalized using eagle eye (Stratagene, La Jolla, CA, USA).

RESULTS

Transfection efficiency in livestock cells was determined by monitoring the blue cell color (Figure 1). Overall, Lipofectamine was the most effective reagent in mediating transfection in all cells analyzed (Figure 2). Bovine fibroblasts have shown the highest transfection efficiency comparing to ovine and caprine fibroblasts that presented similar results (Figure 2A, B and C). Administration of Cellfectin, Lipofectin and DMRIE-C caused a decrease in transfection efficiency (Figure 2). On the other hand, Lipofectamine obtained greater transfection efficiency with plasmid concentration 2.65 µg/ml reaching 30% of all cells; however, all DNA/liposome complexes presented low transfection efficiency (less than 10%) when plasmid concentration was below 1.33 µg/ml (Figure 2).

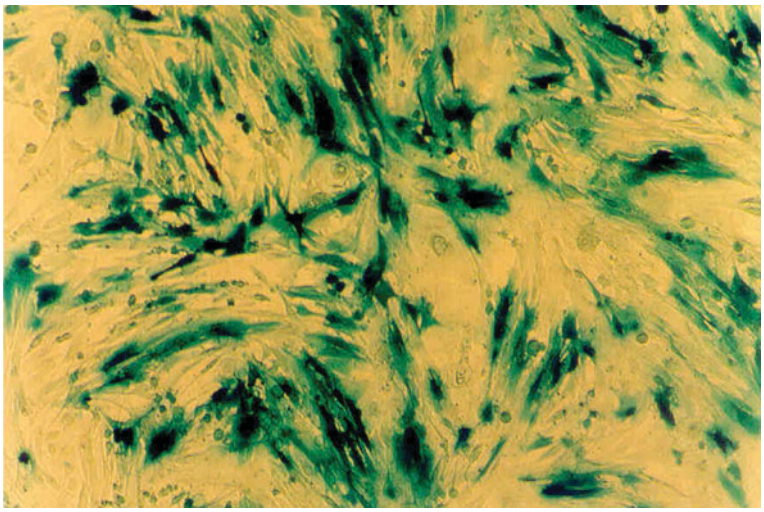

Figure 1. Bovine fibroblasts transfected with pCMVβ, utilizing Lipofectamine (200X).

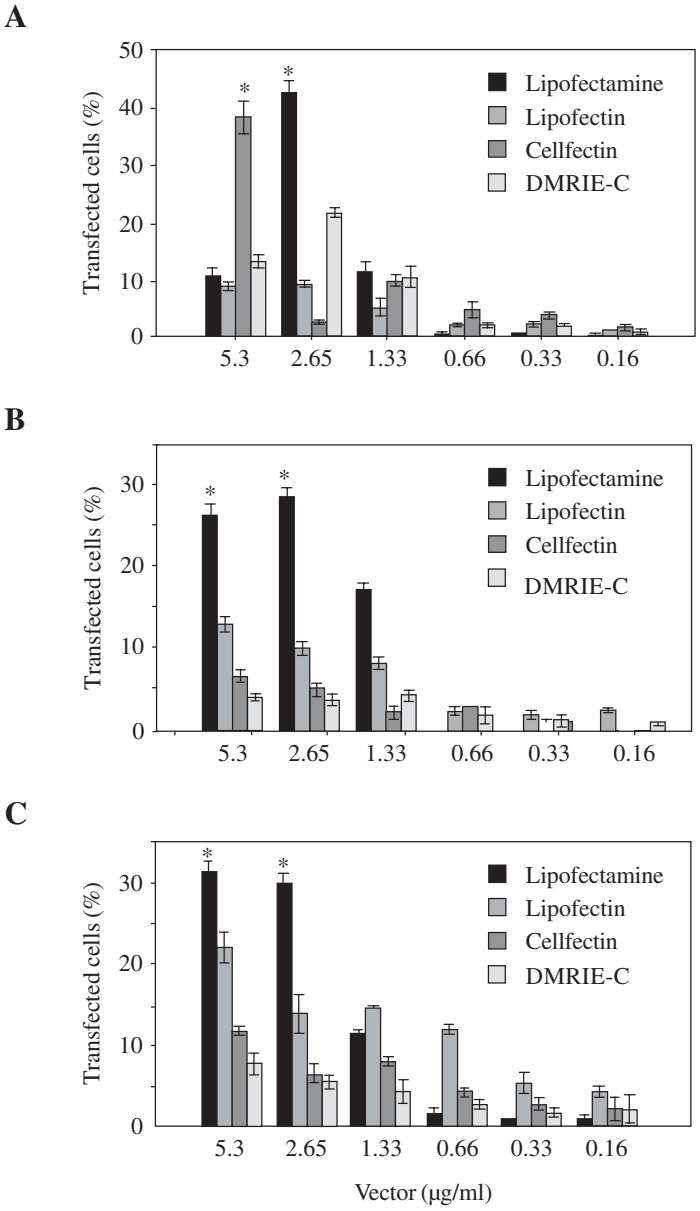

Figure 2. Efficiency of liposome vectors in transfecting bovine (A), caprine (B), and ovine (C) fibroblast cells ($P < 0.01$).

The effect of cell density on transfection efficiency in bovine fibroblasts was evaluated. Lower cell culture densities presented significantly higher transfection rates (45%). Fibroblasts cultured at 6 x 10^4 and 9 x 10^4 cells/ml obtained transfection rates of 27.6 and 6.5%, respectively (Figure 3).

The effect of plasmid vector shape was also evaluated. Transfection frequency was 34% for circular forms and 33% for linear forms. The fragment vector had transfection frequencies of up to 25% (Figure 4).

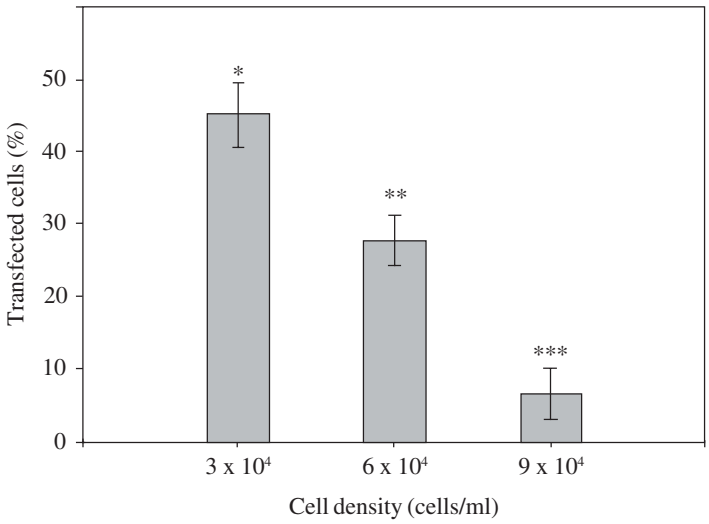

Figure 3. Effect of bovine fibroblast density on transfection rate ($P < 0.01$). *, **, *** - There are significant differences between the values.

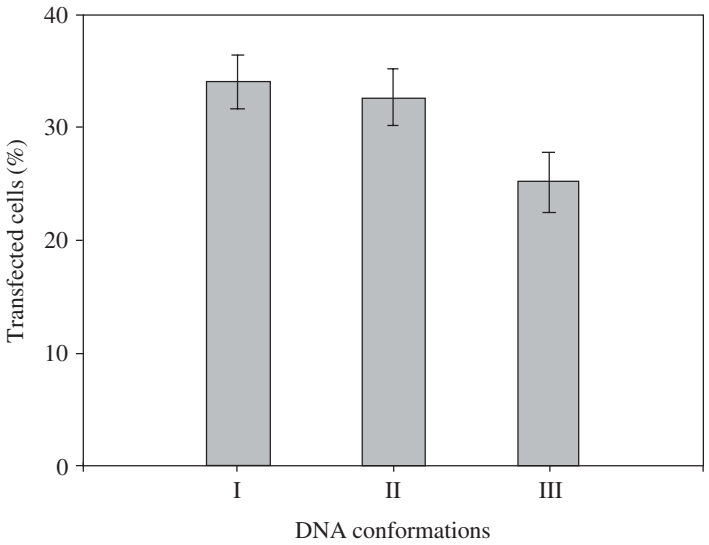

Figure 4. Effect of vector form (3.75 nM circular - I, linear - II, or fragment - III vector) on transfection efficiency in bovine fibroblast cells (7×10^4) ($P < 0.01$).

Utilization of Plus reagent before liposome addition resulted in transfection rates of 88 and 80% for cells incubated for 3 and 6 h, respectively (Figure 5). There was no significant difference in transfection efficiency after either exposure times. Lipofectamine with Plus reagent proved to be more efficient with vector concentration 5.3 µg/ml (data not shown).

Cultured bovine fibroblast cells were utilized to generate stable transfected lines, and their genomic DNA served as a template in PCR reactions to assure the presence of the *neo* gene in their genome (Figure 6). Chromosome number was determined at passage 5, after an

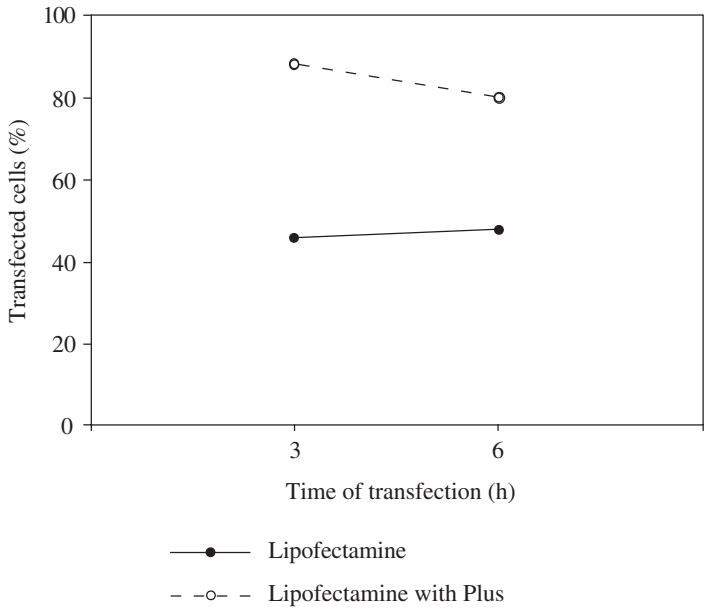

Figure 5. Influence of time and peptide reagent on transfection rates in bovine fibroblasts ($P < 0.01$).

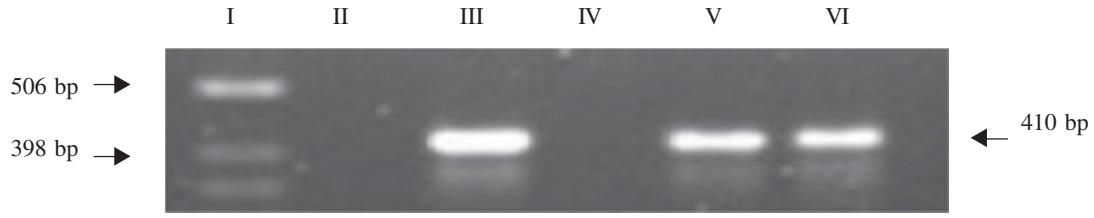

Figure 6. PCR detection of *neo* gene in bovine fibroblast genomic DNA from two transfected cell colonies. I, Molecular weight marker; II, PCR without template, to assure no contamination of the PCR reaction; III, 10 pg of purified pCI-*neo* vector; IV, 200 ng of non-transfected DNA fibroblasts; V, 200 ng of transfected bovine fibroblast DNA, and VI, 200 ng of a second line of transfected DNA cells.

average of 30 days in culture under antibiotic selective pressure. Each individualized transfected line presented a modal chromosome number of 60, indicating an absence of gross chromosomal instability during culture and antibiotic selection. The control of non-transfected bovine fibroblasts cultured at passage 5 had a modal chromosome number of 60, which is the euploid bovine chromosomal complement (Table 1).

DISCUSSION

There are considerable data on physicochemical factors affecting liposome performance in mediating animal cell transfection (Kamiya et al., 2002; Nakanishi, 2003; Trubetskoy et al., 2003). For example, it is known that utilization of liposomes to mediate transfection depends on different factors, such as establishment of physicochemical properties like liposome

Table 1. Chromosome counts of transfected and non-transfected bovine fibroblast lines.

Non-transgenic bovine fibroblast Cytogenetic analysis (N= 600 metaphases)		Transgenic bovine fibroblast Cytogenetic analysis (N= 600 metaphases)	
Aneuploidy	Polyploidy	Aneuploidy	Polyploidy
18.89[a]	5.11[b]	17.55[a]	6.67[b]

[a,b]Lower case letters indicate no significant difference between non-transgenic bovine fibroblasts and transgenic bovine fibroblasts ($P < 0.05$).

type, liposome/DNA mixing ratio, complex concentration, and DNA form among others. The objective of the present study was to establish major factors affecting transient transfection of bovine, ovine and caprine fibroblast cells and to obtain stable transfected fibroblast lines. Experiments utilized commercially available liposomes that are usually adopted in standard, efficient protocols to mediate transfection. Optimal isolation and fibroblast culture conditions were determined *in vitro* prior to initiation of transfection experiments.

Cationic lipids are the most important gene carriers *in vitro* and *in vivo* (Serikawa et al., 2000; Simberg et al., 2001). The above authors observed that rhodamine-labeled DNAs transfected by liposomes in NIH3T3 cell reached the nucleus with a much higher frequency than that expected from cell division alone, suggesting that DNA can enter the nucleus through the nuclear membrane (Kamiya et al., 2002). This same group observed lipid-free DNA in the nucleus suggesting that the cationic lipid and exogenous DNA complex fuse with the nuclear membrane after which naked DNA is released into the nucleus. However, it is not fully understood how the liposome delivers DNA sequences into the cells followed by expression of these genes (Sakurai et al., 2000).

As the association between DNA and lipids occurs primarily through electrostatic interactions, the DNA/liposome ratio did indeed influence the efficiency of transfection (Serikawa et al., 2000; Lampela et al., 2004). Bovine fibroblasts transfected with increasing concentrations of pCMVβ presented reduced percentages of transfected fibroblasts. These data suggest that a certain quantity of DNA can saturate the liposome, and the vector/liposome ratio must be optimized to improve transfection efficiency of livestock fibroblast cells. One possible hypothesis for this result is that the vector solution in 2.65 µg/ml is more efficient in forming a complex with the Lipofectamine lipids due to the equilibrium of positive (lipids) and negative (vector) charges, thus neutralizing the net charge of the DNA/lipids complex. Cells are able to internalize this neutral complex more efficiently. Transfection frequencies obtained with plasmid vectors in circular, linear and fragment conformations (34, 33 and 25%, respectively) showed that total DNA mass and not the number of reporter molecules is the most relevant parameter to be adjusted in transfection assays.

Comparing to ovine and caprine cells, liposomes were more efficient to transfect bovine fibroblasts. It is known that DNA/liposome complexes bind to the cell surface due to an electrostatic interaction between the positive charges of the cationic liposomes and the negative charges on the cell surface (Sakurai et al.., 2000). It was also demonstrated that the number of surface charges differs among cells (Damme et al., 1994). These data support the difference found in the transfection efficiency among bovine, caprine and ovine fibroblasts.

Another important factor in obtaining an efficient transfection system is the exposure

time of cells to the DNA/liposome complex (da Cruz et al., 2001). A short time can result in a reduced interaction between DNA/lipid complex and plasma membrane, reducing the transfection efficiency. However, extended contact between this complex and cells can result in higher cellular mortality during transfection, probably due to a toxic effect of the DNA/liposome complex on cells. No considerable difference in transfection efficiency was observed in the present study when cells were transfected for 3 or 6 h.

Cell density influenced transfection efficiency, with the best result obtained with a cell density of 3 x 10^4 cells/ml. As cell density increased, the number of transfected cells decreased. However, transfection may be improved in cultures with cell densities greater than 3 x 10^4 cells/ml by increasing concentrations of the DNA/lipid complex. Transfection with established cell culture lines such as HeLa, CHO, 293, or BHK cells is commonly accomplished with subconfluent cultures (MacDonald et al., 1996). In contrast, results of the present study have shown that unlike established cell cultures, bovine fibroblasts present high transfection rates (45%) at lower cell culture densities.

Commercially available peptide cocktails improve transfection efficiency by strengthening DNA/liposome formation through interactions that promote tighter DNA-liposome linking (patent No. 5,736,392). Utilization of Lipofectamine with Plus reagent significantly improved transfection efficiency when incubated with pCMVβ vector prior to DNA/liposome mixing. The Plus reagent contains peptide solutions, which include fusogenic lipids to promote fusion between plasma and liposome membranes, and nuclear localization peptides (patent No. 5,736,392). These peptides are covalently linked to a DNA ligant group, such as polyaminic ones. In this case, the nucleic acid/liposome complex has proven to be more efficient in translocating from the cytoplasm to the nucleus, thereby increasing transfection rates. Similar results were found by Byrnes et al., in 2002, utilizing fibroblasts (3T3) in a transfection assay with a liposome. These authors showed that a peptide containing specific amino acid sequences, known as nuclear localization signals, improved transfection efficiency by about twenty times. Ou et al. (2003) reported that an antennapedia peptide (AP) linked to L-4F, a water-soluble, amphipathic α helical peptide that avidly binds lipids (AP - 4F), increases liposome-mediated transfection.

Regarding phenotype, chromosomal stability was similar in transfected and non-transfected bovine fibroblasts with the DNA/liposome complex. One of the most important uses of somatic cell transfections is the production of transgenic livestock by means of nuclear transference technique (Stice et al., 1998). Therefore, in order to produce a healthy organism, transgene integration should not disrupt the normal cell karyotype.

The present study investigated the effect of diverse factors involving liposomes on transfection of fibroblast cells cultured *in vitro*. It was demonstrated that different liposomes can be utilized to mediate high-frequency transient transfection and the liposome Lipofectamine can generate stable transfected lines under selective pressure. The present findings will establish a foundation to utilize transfected fibroblasts in gene expression studies as well as to generate transgenic bovine through nuclear transfer.

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