



Effects of sodium lactate Ringer's injection on transfection of human protein kinase C- α antisense oligonucleotide in A549 lung cancer cells

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ABSTRACT. In the present study, we evaluated the effects of four solutions [Dulbecco's modified Eagle's medium (DMEM), sodium lactate Ringer's injection (SLRI), phosphate-buffered saline (PBS), and NaCl] on the transfection of the human protein kinase C- α antisense oligonucleotide (PKC- α ASO) aprinocarsen in human lung carcinoma A549 cells. Specifically, SLRI, DMEM, PBS, or NaCl were used as the growth solutions for A549 cells, and OPTI-MEM was used as

the PKC- α ASO diluent for transfection. Additionally, SLRI, DMEM, PBS, or NaCl were used as both the growth solutions and diluents for transfection. The cell viability and transfection efficiency were determined. The results demonstrated that when SLRI was used as either the growth solution or both the growth solution and diluent for aprinocarsen transfection in A549 cells, the effects were close to the best effects observed with DMEM as the growth solution and OPTI-MEM as the diluent, which supported the transfection of aprinocarsen into the cells. Moreover, SLRI resulted in higher transfection efficiency than those of PBS and NaCl. In *in vitro* experiments, aprinocarsen effectively induced apoptosis in A549 cells. In conclusion, SLRI may replace PBS or NaCl in clinical trials as a transfection solution readily accepted by the human body. To our knowledge, this is the first report demonstrating the use of SLRI as a transfection solution in lung-cancer cell lines.

Key words: Sodium lactate Ringer's injection; Antisense oligonucleotide; Protein kinase C- α ; Transfection; Lung carcinoma

INTRODUCTION

A large number of gene therapy clinical trials have been aimed at the treatment of cancer (Ginn et al., 2013). Additionally, studies have shown that protein kinase C (PKC) activation is closely related to tumor growth, migration, and invasion in cancer progression (Bosco et al., 2011). PKC is an important enzyme in the intracellular signal transduction pathways of animals. In total, PKC has 12 isoforms (α , β I, β II, γ , δ , ϵ , ζ , η , θ , ι , λ , and μ), and each isoform has different tissue distribution and function (Liu, 1996). Once activated, PKC triggers many intracellular signaling pathways (Xu et al., 1999). Moreover, it was found that over-expression of the PKC- α isoform can cause proliferation of tumor cells (Way et al., 2000).

Inhibitors of PKC activity have played an important role in anti-tumor therapy (Sobhia et al., 2013). Among these inhibitors, antisense oligonucleotide (ASO) sequences are highly effective in inhibiting the effects of PKC (Sanchez-Bautista and Nicolas, 2013). ASOs can specifically inhibit the expression of mRNA sequences of particular PKC isoforms, thus providing precise direction for tumor therapy (Cohen, 1999). For example, Rao et al. (2004) observed anti-tumor activities after 87 cycles of PKC- α ASO intravenous injection were performed in patients with non-Hodgkin's lymphoma. A 21-day continuous intravenous injection of PKC- α ASO combined with 5-fluorouracil and leucovorin also showed partial anti-tumor effects in patients with advanced cancer (Mani et al., 2002; Villalona-Calero et al., 2004). However, in a separate study, it was reported that a single agent such as PKC- α ASO has no significant clinical activity in treatment against advanced ovarian carcinoma (Advani et al., 2004). Some evidence also suggests that PKC- α ASO has no clinical activity in the treatment of refractory metastatic colorectal cancer (Marshall et al., 2004). These conflicting reports indicate that PKC- α ASO exhibits uncertain effects on different tumors types, and that the observed anti-tumor activities of PKC- α ASO differ when used as a single reagent or in combination with other chemotherapy treatments. However, the reasons for these contradictions are still unknown.

The use of PKC- α ASO in the treatment of lung cancer cases has been reported. Furthermore, it has been demonstrated that in the A549 human non-small cell lung cancer cell line, PKC- α ASO reduced PKC- α mRNA expression in a dose-dependent manner (Lee et al., 1997). Dean et al. (1996) found that when PKC- α ASO is dissolved in saline and is administered to nude mice via tail vein injection for 21 consecutive days, it successfully inhibited the growth of human A549 lung, T-24 bladder, and Colo205 colon carcinoma xenografts. Additionally, when patients with advanced non-small cell lung cancer were treated with intravenous injection of PKC- α ASO for 21 consecutive days in combination with cisplatin and gemcitabine, they had relatively good outcomes (Grossman et al., 2005).

Gene therapy protocols in clinical or preclinical trials have shown great promise (Ginn et al., 2013). However, the transfection efficiency is affected by many factors such as liquid ion species, ion component ratio, and ion concentration, among others. Previous studies have often utilized phosphate-buffered saline (PBS) or NaCl as the diluent for ASO. However, it remains unknown whether the variability in the therapeutic effects of ASO is related to the diluent used. Moreover, local *in vivo* clinical transfection trials have reported that sodium lactate Ringer's solution (sodium lactate Ringer's injection; SLRI) can be used as a diluent for ASO in the treatment of chronic myocardial ischemia (Hedman et al., 2003). However, the effect of SLRI as a diluent for PKC- α ASO transfection of lung cancer cell lines has not been reported. Therefore, the aim of the current study was to investigate the effects of SLRI on human PKC- α ASO transfection efficiency into A549 cells. The overall goal was to identify optimal extracellular fluids and reagent concentrations for efficient human PKC- α ASO transfection into human lung cancer cells.

MATERIAL AND METHODS

Cells and reagents

Human lung carcinoma A549 cells of human alveolar epithelial cell origin were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 g/L glucose (Gibco, New York, NY, USA). All culture medium was additionally supplemented with 10% fetal bovine serum (FBS) and antibiotics (0.1 mg/mL penicillin and 0.1 mg/mL streptomycin; Gibco). Cells were treated with PKC- α ASO in the presence of Lipofectamine 2000 (Lipo2000) cationic lipid reagent (Invitrogen, Carlsbad, CA, USA). Aprinocarsen [d(P-Thio)GTTCTCGCTGGTGAGTTTCA deoxyribonucleic acid] is a thio-phosphorylated PKC- α ASO with 20 bases, and was synthesized by TaKaRa Biotechnology (OtsuShiga Company, Tokyo, Japan). A random sequence (scrambled) was used as a control ASO (cont. ASO), which was also from TaKaRa. SLRI was purchased from the Baxter Healthcare Corp. (Shanghai, China). OPTI-MEM I Reduced Serum Medium (OPTI-MEM) was purchased from Gibco.

Cell transfection

One day before transfection, 2×10^5 cells were seeded on 12-well plates in 400 μ L growth medium (DMEM) without antibiotics so that the cells would be 70% confluent at the time of transfection. The ASO aprinocarsen (250 nmol) was diluted in 50 μ L OPTI-MEM

(final concentration of ASO was 500 nM). Then, 1 μ L Lipo2000 was diluted in 50 μ L OPTI-MEM. The mix was incubated for 5 min at room temperature. After the 5-min incubation, the diluted ASO was combined with the diluted Lipo2000 and the final mix was incubated for 20 min at room temperature. Finally, the ASO-Lipo2000 complexes were added to each well containing cells and medium. The plates were rocked gently back and forth. The cells were then incubated at 37°C in a CO₂ incubator for 4-6 h. Next, the cells were incubated in fresh medium for another 48 h until cell imaging.

Cell transfection in different growth solution

When the cells reached 50-75% confluency in DMEM with 10% FBS on 48-well culture plates, the cells were divided into growth solution groups (DMEM, SLRI, PBS, or NaCl) and rinsed three times with the corresponding serum-free DMEM, SLRI, PBS, or NaCl, respectively. For example, cells in the SLRI group were rinsed with SLRI. Then, 150 μ L corresponding growth solution was added to the cells. Another 50 μ L OPTI-MEM was then used to dilute Lipo2000 and 50 μ L OPTI-MEM was used to dilute for the ASO aprinocarsen. The two dilutions were incubated together for 15-20 min and then were added into the corresponding wells. The final concentration of the ASO aprinocarsen was 500 nM. Next, the cells were incubated at 37°C for 4-6 h. After treatment, the growth solutions were removed, then 10% FBS-containing DMEM was added to the cells from each group, and the cells were incubated for another 48 h. Subsequently, the effects of cell transfection on the four growth solution groups were evaluated. The timeline of transfection is shown in Figure 1.

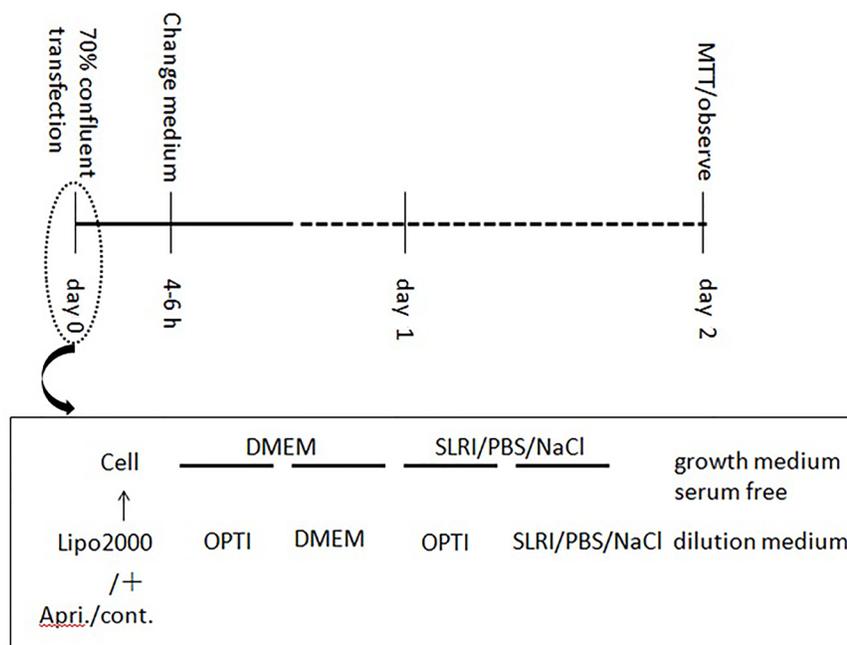


Figure 1. Timeline of study procedures. For abbreviations, see legend to Figure 2.

Cell transfection in consistent growth solution and diluents

The aforementioned four growth solutions were kept constant, while the OPTI-MEM was replaced by serum-free DMEM, SLRI, PBS, or NaCl so that the final growth solution and ASO diluent in each group for cell transfection were the same. Then, the aforementioned described cell transfection procedures were repeated. The cells were then incubated in FBS-containing DMEM for another 48 h after the 4-6 h transfection. The effects of cell transfection on the four extracellular solution groups were then evaluated. During the 4-6 h transfection process, the normal group (N) was only treated with growth solution and diluent.

Determination of cell viability

Cell viability was measured with the MTT (3-(4,5-dimethylthiazol-2-yl)-5-(3,4-diphenyltetrazolium bromide) assay. Briefly, the cells were seeded on 96-well plates in 200 μ L DMEM with 10% FBS at an initial density of 0.6×10^4 cells/well. After 24 h, cell transfection experiments were performed with a final concentration of ASO of 500 nM. The cells were then incubated for 48 h in DMEM with 10% FBS. After 48 h, another 180 μ L fresh DMEM with 10% FBS was added to each well. MTT (20 μ L; 5 mg/mL formulated with PBS, pH = 7.4) was subsequently added to each well and mixed gently for an additional 4-h incubation. The supernatants were carefully aspirated, 150 μ L DMSO was added to each well, and the plates were shaken for 10 min to fully dissolve crystals. Absorbance was measured at 490 nm in each well with an enzyme-linked immunosorbent monitor (Kehua KHB -ST-360, Shanghai, China).

Cell image acquisition

Cell images were obtained with an IX53 microscope (Olympus, Tokyo, Japan) equipped with QImaging camera (QImaging, Surrey, BC, Canada). All images were captured at room temperature. Two to three researchers observed cells at least three times in each case while taking representative photographs.

Statistical analysis

All data are reported as means \pm SD. Significant differences between groups were assessed by one-way analysis of variance (ANOVA) with a *post hoc* Dunnett test. Differences were considered to be statistically significant when $P < 0.05$. Statistical comparisons between groups were performed using the Student *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Aprinocarsen treatment resulted in A549 cell apoptosis

First, the MTT assay was used to assess the effect of the PKC- α ASO aprinocarsen on the apoptosis of A549 cells with DMEM as the growth medium and OPTI-MEM as the ASO diluent. A549 cells were divided into 4 groups and each group was treated according

to the cell transfection protocol described in Material and Methods. The N group represented normal cultured cells; the Lipo. group represented Lipo2000-treated cells; the Lipo.+Apri. group represented cells transfected with Lipo2000 and the PKC- α ASO aprinocarsen; and the Lipo.+Cont. group represented cells transfected with Lipo2000 and control ASO (Figure 2A and B). The results showed that there was a large amount of A549 apoptosis in the Lipo.+Apri. group. The MTT value of this group was 0.107 ± 0.014 , which was significantly lower than that of the Lipo. group (0.192 ± 0.004 , $P < 0.05$) and that of the Lipo.+Cont. group (0.186 ± 0.010 , $P < 0.05$).

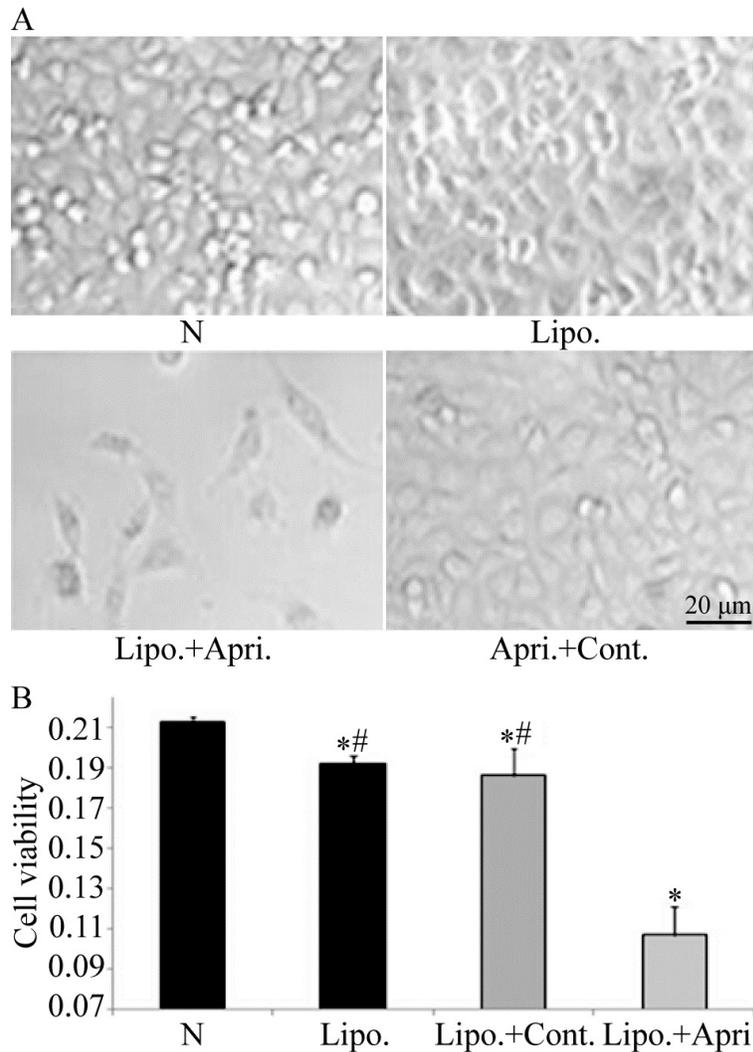


Figure 2. A. Images of A549 cells transfected with aprinocarsen after 48 h. **B.** Effects of aprinocarsen transfection on A549 apoptosis after 48 h. N: normal cultured cells; Lipo.: Lipo2000-treated cells; Lipo.+Apri.: cells transfected with aprinocarsen via Lipo2000; Lipo.+Cont.: cells transfected with control ASO via Lipo2000. * $P < 0.05$ vs N group; # $P < 0.05$ vs Lipo.+Apri. group.

SLRI can be used as a cell growth solution

Next, we performed transfections according to the aforementioned cell transfection procedure with OPTI-MEM as the ASO diluent, but with different growth solutions including DMEM, SLRI, PBS, and NaCl (Figure 3). We found that in the same sized field of view, the number of live cells with normal morphology (cell viability) were different among the groups. DMEM-OPTI, SLRI-OPTI, PBS-OPTI, and NaCl-OPTI were used to denote the groups with different growth solutions with the same OPTI-MEM ASO transfection diluent. When the cells were transfected with Lipo2000 only, the cells in the SLRI-OPTI group (35 ± 2.65) appeared to have a stronger tolerance to Lipo2000 toxicity compared to those of the PBS-OPTI group (17 ± 3.06 , $P < 0.05$) and the NaCl-OPTI group (15 ± 1.15 , $P < 0.05$). The number of live cells in the SLRI-OPTI group was closest to, but lower than that in the DMEM-OPTI group (44 ± 2.00 , $P < 0.05$). When the cells were transfected with Lipo2000 and control ASO (Lipo.+Cont.), a trend similar to Lipo2000 only was observed in the four groups. The number of live cells in the SLRI-OPTI group was 30 ± 2.00 , which was higher than those in the PBS-OPTI group (16 ± 3.06 , $P < 0.05$) and in the NaCl-OPTI group (14 ± 2.00 , $P < 0.05$), but lower than that in the DMEM-OPTI group (42 ± 1.53 , $P < 0.05$). However, when the cells were transfected with Lipo2000 and aprinocarsen, almost no live cells could be found in any of the four groups.

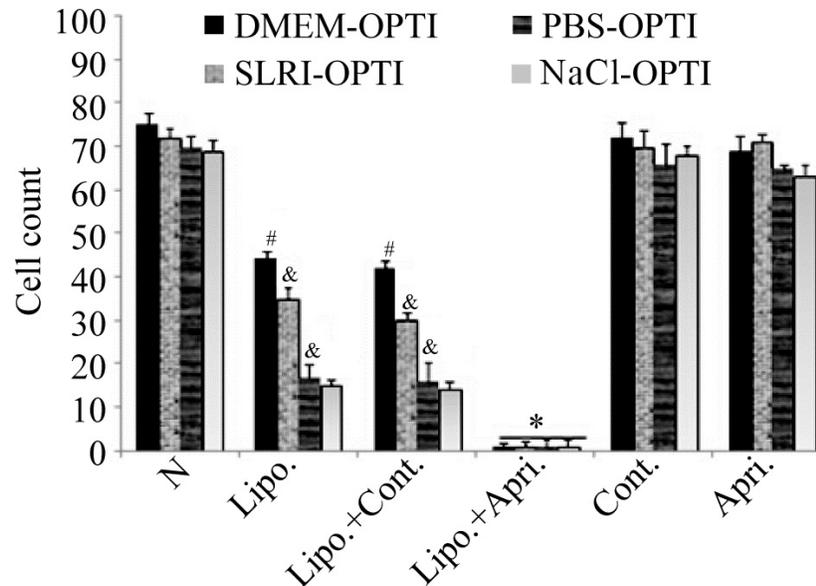


Figure 3. Effects of different growth solutions on aprinocarsen-transfected A549 cells. N: normal cultured cells; Lipo.: Lipo2000-treated cells; Lipo. + Cont.: cells transfected with control ASO via Lipo2000; Lipo. + Apri.: cells transfected with aprinocarsen via Lipo2000; Cont.: control ASO-treated cells; Apri.: aprinocarsen-treated cells. DMEM-OPTI, SLRI-OPTI, PBS-OPTI, and NaCl-OPTI denote that the transfection diluents were OPTI-MEM and the growth solutions were DMEM, SLRI, PBS, or NaCl, respectively, in the different groups. * $P < 0.05$ vs N, Lipo., Lipo. + Cont., Cont., and Apri. groups. # $P < 0.05$ vs N, Lipo. + Apri., Cont., and Apri. groups; & $P < 0.05$ vs N, Lipo. + Apri., Cont., and Apri. group.

SLRI can be used as an ASO diluent for transfection

Lastly, we evaluated the effects of transfection with the same solution for cell growth and dilution on the four groups. Among the groups, DMEM, SLRI, PBS, or NaCl was used as the growth solution, but the OPTI-MEM diluent was replaced by the corresponding growth solution. The DMEM-DMEM group represented cells where both the growth solution and ASO transfection diluents were DMEM, whereas SLRI-SLRI, PBS-PBS, and NaCl-NaCl were used to denote groups where SLRI, PBS, or NaCl was used as both the growth solutions and transfection diluents, respectively (Table 1). We found that when cells were not treated with Lipo2000 or Lipo2000-ASO complexes, the corresponding cell counts were 64 ± 3.61 in the DMEM-DMEM group and 63 ± 3.00 in the SLRI-SLRI group, which were both significantly higher than those in the PBS-PBS group (44 ± 1.73 , $P < 0.05$) and in the NaCl-NaCl group (30 ± 2.00 , $P < 0.05$). When cells were treated with Lipo2000 or Lipo2000 and control ASO, the number of live cells in each group was decreased compared to that of the corresponding untreated group. These results demonstrate the toxicity of Lipo2000 on cells. However, the different groups showed different levels of tolerance to Lipo2000. Specifically, the tolerance to Lipo2000 of cells in the SLRI-SLRI group (the live cell count was 33 ± 2.00) was higher than that of the PBS-PBS group (7 ± 2.65 , $P < 0.05$) and the NaCl-NaCl group (1 ± 1.15 , $P < 0.05$), but was lower than that of the DMEM-DMEM group (42 ± 2.65 , $P < 0.05$). The change in live cell counts resulting from aprinocarsen transfection in the SLRI-SLRI group was closest to that in the DMEM-DMEM group compared to those in the PBS-PBS and NaCl-NaCl groups, although almost no live cells could be found in any group. These results suggest that the tolerance of A549 cells to Lipo2000 toxicity was higher in the SLRI group than that in the PBS and NaCl groups, and that aprinocarsen transfection could have a greater effect with SLRI than with PBS or NaCl.

Table 1. Effects of aprinocarsen transfection on A549 cells when the ASO diluent was the same as the growth solution.

Group	Cell count/field of view			
	DMEM-DMEM	SLRI-SLRI	PBS-PBS	NaCl-NaCl
N	64 ± 3.61	63 ± 3.00	44 ± 1.73^{ab}	30 ± 2.00^{abc}
Lipo.	42 ± 2.65	33 ± 2.00^a	7 ± 2.65^{ab}	1 ± 1.15^{abc}
Lipo.+Cont.	40 ± 4.00	28 ± 4.00^a	7 ± 1.73^{ab}	1 ± 1.00^{abc}
Lipo.+Apri.	1 ± 1.53	1 ± 0.58	1 ± 1.53	1 ± 1.15
Cont.	65 ± 3.61	61 ± 3.61	46 ± 2.52^{ab}	29 ± 3.79^{abc}
Apri.	63 ± 2.00	62 ± 5.29	48 ± 3.61^{ab}	32 ± 2.08^{abc}

N: normal cultured cells; Lipo.: Lipo2000-treated cells; Lipo. + Cont.: cells transfected with control ASO via Lipo2000; Lipo. + Apri.: cells transfected with aprinocarsen via Lipo2000; Cont.: control ASO-treated cells; Apri.: aprinocarsen-treated cells. DMEM-DMEM, SLRI-SLRI, PBS-PBS, and NaCl-NaCl denote both the transfection diluents and growth solutions, which were DMEM, SLRI, PBS, and NaCl, respectively. ^a $P < 0.05$ vs DMEM-DMEM; ^b $P < 0.05$ vs SLRI-SLRI; ^c $P < 0.05$ vs PBS-PBS.

DISCUSSION

Over the last few decades, gene therapies for lung cancer have made many advances in regards to gene regulation and gene drug carriers (Canonico et al., 1994; Verma and Somia, 1997; Bragonzi et al., 2000; Gautam et al., 2000; Lentz et al., 2006). However, problems that limit the clinical applications of gene therapies persist. Clinically speaking, vector-gene drugs

have traditionally been diluted in PBS or NaCl for intravenous infusion, although the clinical effects have not been satisfactory. There are many reasons that may explain these results, such as the impact of blood components on gene-carrier complexes, low doses of the drugs reaching target lesions via blood circulation, or side effects associated with intravenous administration that decrease patient survival rates. The selection of alternative diluents for gene-vector complexes has thus become a particularly important decision. Particularly, studies have shown that changes in certain properties of transgenic solutions such as changes in pH could affect the efficiency of gene transfection (You and Auguste, 2009). Low osmotic pressure was also shown to increase gene transfection efficiency (Rudolph et al., 2005). Further research showed that low osmotic pressure could enhance the uptake of genetic material into cells by increasing the intracellular calcium ion concentration (Sun et al., 2010). Therefore, diluents used for transfection may affect the transfection efficiency of cationic liposome-gene complexes.

It has been reported that Ringer's solution (i.e., SLRI) has been used as a gene drug diluent for clinical transfection (Hedman et al., 2003). Therefore, in order to determine which diluents increase gene transfection efficiency and which may be more similar to the human microenvironment for the treatment of lung cancer, we compared the effects of SLRI, PBS, NaCl, and DMEM on transfection of A549 cells.

The results of the current study showed that when the transfection diluent was OPTI-MEM, SLRI could replace DMEM as the growth medium in the transfection process, and that the transfection efficiency with SLRI was superior to that of PBS and NaCl. Although the SLRI-OPTI group treated with Lipo2000 alone exhibited only a slightly lower tolerance to Lipo2000 toxicity, the cell count of SLRI-OPTI was much closer to that of the DMEM-OPTI group compared to those of the PBS-OPTI or NaCl-OPTI groups. Meanwhile, the NaCl-OPTI group showed the lowest tolerance to Lipo2000 toxicity among the four groups. DMEM is a culture medium that contains a variety of amino acids as well as glucose. It is known that high-sugar medium is conducive for better cell growth of adherent cells, thus DMEM was favorable for the maintenance of cell growth. However, to date, this knowledge is limited to *in vitro* laboratory studies. SLRI, PBS, and NaCl are commonly used in clinical injections because their osmotic pressures are similar to those of plasma and tissue fluids, and hence do not change the normal concentration of ions in the human body. As we know, SLRI contains lactate and calcium ions that have roles in maintaining cellular adhesion and increasing cytoskeletal movement to promote cellular endocytosis. Since PBS and NaCl lack Ca^{2+} , the cellular adhesion ability of cells in these solutions is lower than that in SLRI. Under these conditions, deficient stretch eventually leads to reduced endocytic ability, and even a complete loss of endocytosis.

When PBS and NaCl were used as the diluent to replace OPTI-MEM, the cells lost the OPTI-MEM-provided nutritional support. Additionally, when cells were in a pure NaCl or PBS environment, Lipo2000 toxicity was more prominent, and thus the cell count of the PBS-PBS and NaCl-NaCl groups was lower than that of SLRI-SLRI group. Since some cells had died before the PKC- α ASO aprinocarsen was administered, we could not determine the contribution of aprinocarsen treatment to the final cell death count. Other than Ca^{2+} , SLRI also contained lactate, which could also increase the buffering capacity of cells and provide necessary energy. Therefore, SLRI further maintained the stable state of cells to endure Lipo2000 toxicity, and promoted the transfection process.

In conclusion, SLRI can replace DMEM as the growth solution or OPTI-MEM as the

diluent for ASO transfection, and the effects are superior to those of PBS and NaCl. As a new gene transfection solution, the data herein support the use of SLRI in clinical transfection trials for lung cancer gene therapies. As such, the present study provides insight into a new selection for gene transfection solutions that may be matched to conditions in the human body.

Conflicts of interest

The authors declare no conflict of interest.

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