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Combined delivery of the adiponectin gene and rosiglitazone using cationic lipid emulsions

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ABSTRACT

For the combined delivery of an insulin-sensitizing adipokine; *i.e.*, the *ADN* gene, and the potent PPARγ agonist rosiglitazone, cationic lipid emulsions were formulated using the cationic lipid DOTAP, helper lipid DOPE, castor oil, Tween 20 and Tween 80. The effect of drug loading on the physicochemical characteristics of the cationic emulsion/DNA complexes was investigated. Complex formation between the cationic emulsion and negatively charged plasmid DNA was confirmed and protection from DNase was observed. The *in vitro* transfection efficiency and cytotoxicity were evaluated in HepG2 cells. The particle sizes of the cationic emulsion/DNA complex were in the range 230–540 nm and those of the rosiglitazone-loaded cationic emulsion/DNA complex were in the range 220–340 nm. Gel retardation of the complexes was observed when the complexation weight ratios of the cationic lipid to plasmid DNA exceeded 4:1 for both the drug-free and rosiglitazone-loaded complexes. Both complexes stabilized plasmid DNA against DNase. The ADN expression level increased dose-dependently when cells were transfected with the cationic emulsion/DNA complexes. The rosiglitazone-loaded cationic emulsion/DNA complexes showed higher cellular uptake in HepG2 cells depending on the rosiglitazone loading, but not depending on the type of plasmid DNA type such as pVAX/ADN, pCAG/ADN, or pVAX. The drug-loaded cationic emulsion/plasmid DNA complexes were less cytotoxic than free rosiglitazone. Therefore, a cationic emulsion could potentially serve as a co-delivery system for rosiglitazone and the adiponectin gene.

1. Introduction

Adiponectin (ADN) is an adipocyte-produced protein hormone that circulates in the blood. ADN acts via the activation of AMP-activated protein kinase (AMPK) and decreased circulating ADN concentrations are associated with insulin resistance, obesity, and type 2 diabetes (Liu et al., 2008). Plasma ADN is reduced markedly in obese human and those with type 2 diabetes, suggesting that circulating ADN is related to the development of insulin resistance. ADN plays an important role in insulin sensitization in mammals (inhibiting gluconeogenesis and stimulating fatty acid oxidation) by activating AMP-activated protein kinase and peroxisome proliferator-activated receptor (PPAR) proteins in skeletal muscle, liver, and adipocytes (Astapova and Leff, 2012; Lustig et al., 2012).

Rosiglitazone is a member of the thiazolidinedione (TZD) class of agents used to treat type 2 diabetes. This class of agents improves glycemic control by improving insulin sensitivity. Rosiglitazone is a selective PPARγ agonist. The activation of PPARγ receptors regulates the transcription of insulin-responsive genes in the tissues in which they are found which include adipose tissue, skeletal muscle, and liver. PPARγ-responsive genes are involved in the control of glucose production, transport, and utilization, as well as in the regulation of fatty acid metabolism (Yamauchi et al., 2001). Additionally, TZDs have been shown to rapidly stimulate AMPK (Fryer et al., 2002; LeBrasseur et al., 2006) and may indirectly activate AMPK through the effects of PPARγ to stimulate ADN secretion (Suzuki and Eguchi, 2006; Kubota et al., 2006).

Therefore, both TZDs and ADN have been known to activate AMPK (Coughlan et al., 2014). Moreover, TZDs increase the production and plasma concentration of ADN. TZDs have weaker

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antidiabetic effects in ob/ob mice lacking the *ADN* gene than in ob/ob mice with it, and the activation of AMPK by TZDs is also attenuated in these mice, suggesting that ADN is required for the activation of AMPK by TZDs (Nawrocki et al., 2006). If rosiglitazone were available in combination with ADN, then the ADN might alleviate the side effect of rosiglitazone via dose control (Wiradharma et al., 2009). The combined delivery of a drug and gene is feasible because we previously combined drug-loaded lipid carriers and nucleic acids (Jeong et al., 2009, 2014; Davaa et al., 2010).

Cationic lipid emulsions have been prepared to overcome problems related to the solubility, encapsulation efficiency, and release of rosiglitazone (Davaa and Park, 2012). The encapsulation efficiency of a drug in a vehicle is of pharmaceutical importance, especially for optimizing efficacy and cost effectiveness. The cellular uptake of a drug is another factor to consider when maximizing its therapeutic efficacy. If a positive charge is introduced to the nanoparticles, the interaction between the positively charged vehicle and negatively charged cell surface would increase. Furthermore, cationic lipid emulsions have been used to transfer target genes into cells (Choi et al., 2004; Kang et al., 2009; Kim et al., 2014). Rosiglitazone would be loaded in the inner section of the emulsion, while plasmid DNA could interact electrically with the surface of a cationic emulsion. Therefore, the combination of rosiglitazone and the ADN gene is feasible for achieving synergistic or additive effects.

Here, we prepared a cationic emulsion that could efficiently co-deliver rosiglitazone and the ADN gene to a target cell with good physicochemical properties and high drug loading efficiency. The complexation ratios of the plasmid DNA and drug-loaded cationic emulsion were examined to optimize the co-delivery system. Then, the expression of ADN mRNA and cellular uptake of rosiglitazone using the optimized co-delivery system were compared.

2. Materials and methods

2.1. Materials

Rosiglitazone maleate was purchased from Masung & Co. (Seoul, Korea). 1,2-Dioleoyl-*sn*-glycero-3-trimethylammonium propane (DOTAP) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Polyoxyethylene sorbitan monooleate (Tween 80) was from Junsei Chemical (Japan). Castor oil, polyoxyethylene sorbitan monolaurate (Tween 20), potassium phosphate monobasic, sodium acetate, hydrochloric acid, chloroform, dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and solubilization solution were purchased from Sigma–Aldrich (St. Louis, MO). Methanol and acetonitrile for high-performance liquid chromatography (HPLC) were obtained from SKYSOLTECH® from SK Chemicals (Seongnam, Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were purchased from Gibco® BRL (Grand Island, NY). Lipofectamine™ 2000 and TRIzol® were purchased from Invitrogen (Carlsbad, CA). Anti-adiponectin was from Cell Signaling Technology, Inc. (Beverly, MA, USA) and anti-β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-mouse and goat anti-rabbit antibodies were from Abfrontier (Seoul, Korea). All other chemicals were reagent grade and were used without further purification. The distilled and deionized water was used after sterilization.

2.2. Cell culture

Human hepatocellular carcinoma cells (HepG2) were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 unit/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator supplied with 5% CO_2.

2.3. Purification of plasmid DNA

The plasmid DNA encoding adiponectin (pVAX/ADN and pCAG/ADN) (Nan et al., 2010; Davaa et al., 2013) was amplified in *Escherichia coli* DH5α and purified with a Plasmid Mega kit (QIAGEN, CA), according to the manufacturer's instructions. The integrity of the DNA preparation was confirmed on a 1% agarose gel.

2.4. Preparation of cationic lipid emulsions

The cationic lipid emulsion was prepared using the sonication method described by Choi et al. (2004). DOTAP and DOPE were used as the cationic and helper lipid, respectively. Rosiglitazone was dispersed in a mixture of castor oil and surfactants with constant stirring until the pre-emulsion concentrate became clear. An aliquot of rosiglitazone pre-emulsion concentrate was added to DOTAP and DOPE at a defined ratio. In brief, DOTAP, DOPE, castor oil, Tween 20, and Tween 80 were mixed at a weight ratio of 3:3:3:0.75:0.75 dissolved in chloroform (Davaa and Park, 2012). The organic solvent was removed on a rotary evaporator (RE-47 Yamato Scientific, Japan). The dried lipid film was flushed with nitrogen gas to remove traces of organic solvents and hydrated with 10 mL of phosphate-buffered saline (PBS, pH 7.4). Then, the hydrated lipid solution was vortexed and sonicated in a bath-type sonicator (Bransonic, Branson Ultrasonic, Danbury, CT) at 37 °C for 2 h. Finally, the prepared cationic emulsion was stored at 4 °C. To remove the unloaded rosiglitazone, lipids, and surfactants from the emulsion, the formulation was ultrafiltered through a 0.45-μm PVDF syringe filter (Leur Lock type; NSW Norm-Ject®, Whatman Inc.) (Davaa et al., 2010; Davaa and Park, 2012). The loading concentration of rosiglitazone in cationic emulsion was 662.3 ± 47.9 μg/mL.

2.5. Preparation of cationic lipid emulsion/DNA complexes

Drug-loaded or unloaded cationic lipid emulsions/DNA complexes were prepared by mixing plasmid DNA (pVAX/ADN or pCAG/ADN) and the cationic lipid formulation in various ratios in serum-free medium. The mixtures were incubated for 15 min at 37 °C to facilitate complex formation.

2.6. Measurement of droplet size and zeta potential

The droplet size distribution of the cationic emulsions and complexes was measured using a dynamic light-scattering spectrophotometer (ELS-8000, Otsuka Electronics, Japan) at a fixed angle of 90° at room temperature. The samples were diluted with deionized water, and then transferred into a quartz cuvette in the ELS-8000. The system was used in auto-measuring mode at 80 mV.

The zeta potential of the cationic emulsions and complexes was measured using an electrophoretic light-scattering spectrophotometer (ELS-8000, Otsuka Electronics) at an angle of ∼20° at 40 mV to assess the surface charge of vesicles after dilution with deionized water at room temperature. The data were analyzed using the ELS-8000 software supplied by the manufacturer.

2.7. Agarose gel retardation

Complex formation between the plasmid DNA and cationic emulsion was assessed using agarose gel electrophoresis. Various ratios (0.5–32, w/w) of lipid/DNA mixtures with a fixed amount of

DNA (1 μg of pVAX/ADN or pCAG/ADN) were incubated for 15 min to facilitate complex formation and loaded onto 1% agarose gels. As a control, Lipofectamine®/DNA complex at a Lipofectamine® to DNA ratio of 6 (w/w) was also loaded on the gel. Gel electrophoresis was carried out in TBE buffer (45 mM Tris, boric acid, 1 mM EDTA) at 50 mV. Ethidium bromide stained DNA was visualized using an ultraviolet (UV) illuminator.

2.8. Stability of drug-loaded or unloaded emulsion/DNA complexes

The storage stability of rosiglitazone-loaded or unloaded cationic emulsions and complexes was evaluated in terms of the change in droplet size. The samples were stored at 4 °C or room temperature. At predetermined times, the mean particle size was measured with an electrophoretic light scattering spectrophotometer. At completion of the study, the samples were transferred into a clear test tube for visual observation.

The cationic emulsions were mixed with 0.2 μg of DNA (pVAX/ADN or pCAG/ADN) at a DOTAP to DNA ratio of 32 (w/w) and allowed to form complexes for 15 min at room temperature or 37 °C. For DNase I digestion, the cationic emulsions were prepared in water instead of PBS to avoid the interfering effect of high salt on the DNase I activity. Naked DNA (0.2 μg) or cationic emulsion/DNA complex containing the same amount of DNA was exposed to DNase I for 30 min at 37 °C to investigate the stability of the complex against nuclease digestion. DNA was retrieved by phenol/chloroform extraction followed by ethanol precipitation and visualized on an agarose gel containing ethidium bromide.

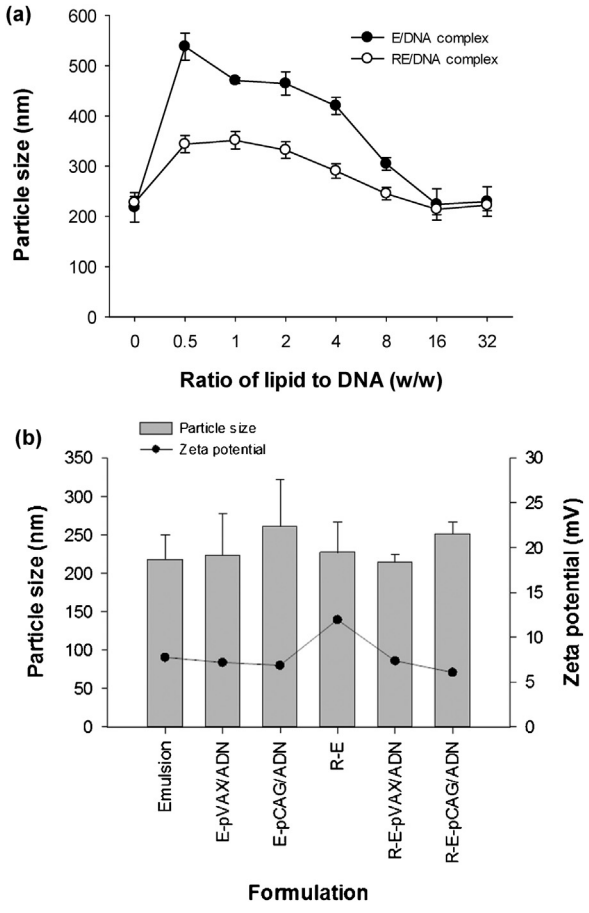

Fig. 1. (a) Particle size of rosiglitazone-loaded and unloaded cationic lipid emulsion/DNA complexes at various ratio ($n = 3$). E/DNA complex means the complex of drug-unloaded cationic emulsion and pVAX/ADN. RE/DNA complex represents the complex of rosiglitazone-loaded cationic emulsion and pVAX/ADN. (b) Particle size and zeta potential of various lipid formulation with or without rosiglitazone and DNA lipoplexes ($n = 3$). The complexes were prepared at weight ratio of plasmid DNA: lipid = 1:16. E and RE mean drug unloaded cationic emulsion and rosiglitazone-loaded cationic emulsion, respectively.

2.9. In vitro *transfection*

Cells were seeded at a density of 5×10^5/well in six-well plates (Nunc, Denmark) and grown to 70–80% confluence. The cells were washed with PBS and the transfection complex was added to each well. After transfection for 3 h, the transfection complex was removed and the cells were fed with 2 mL of fresh DMEM containing 10% FBS and incubated for 24 h at 37 °C. Lipofectamine® 2000 was used as a control. All transfection experiments were performed in triplicate. ADN mRNA expression in cells was measured by RT-PCR at 24 h post-transfection.

2.10. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The transfected cells were lysed with TRIzol® and protein was extracted with chloroform. Total RNA was precipitated with isopropanol and washed twice with 70% ethanol diluted with diethylpyrocarbonate-treated water. The RNA purity and concentration were analyzed using a spectrophotometer at 260 and 280 nm. cDNA was synthesized from the total RNA in a reaction mixture containing 5× reaction buffer (250 mM Tris–HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT), 100 μg/mL oligo-dT, 10 mM dNTP, and 100 U M-MLV reverse transcriptase (Promega, Madison, WI). The RT reaction product was amplified by PCR with specific primers (forward: 5′-GGA ATT CAT GCT ACT GTT GCA AGC TCT-3′, reverse: 5′-GCT CTA GAT CAG TTG GTA TCA TGG TAG AGA AG-3′) for ADN (Davaa et al., 2013) and (forward: 5′-CTG TCT GGC GGC ACC ACC AT-3′, reverse: 5′-GCA ACT AAG TCA TAG TCC GC-3′) for human β-actin as a housekeeping gene. The human ADN was amplified for 30 cycles of 95 °C for 30 s, 59 °C for 40 s, 72 °C for 1 min. The PCR products were separated on 1% agarose gels and visualized under UV. The amount of cDNA was measured using the ChemiDoc™ XRS System (Quantity One®, Bio-Rad) to quantify ADN or β-actin mRNA in separate reactions.

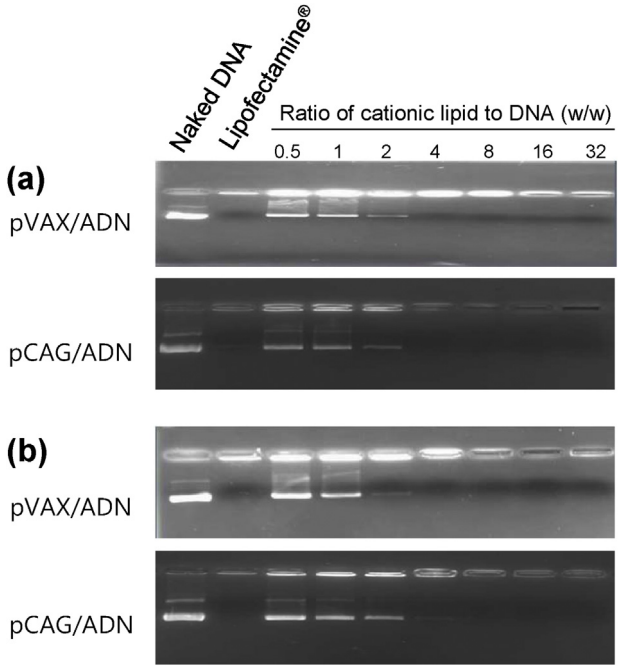

Fig. 2. Agarose gel retardation of (a) drug free complexes and (b) rosiglitazone-loaded complexes with plasmid DNA.

2.11. Western blot analysis

After transfection, HepG2 cells were washed twice in ice-cold PBS and lysed with RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1.0% NP-40, 2 mM EDTA, 5 mM NaF, 1 mM PMSF, 1 mM sodium orthovanadate, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate). Lysates were incubated at 4 °C for 30 min and then centrifuged at 8000 × g for 10 min at 4 °C. The extracted protein concentrations were determined by BCA protein assay kit (Pierce, Rockford, IL, USA). Cell lysates (15 μg of protein) were resolved on 13.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene fluoride (PVDF) membranes (ATTO Corp., Tokyo, Japan) at 200 mA for 1.5 h. The blots were blocked with 5% BSA in TBS-T (20 mM Tris-Base, 137 mM NaCl at pH 7.4 and 0.05% Tween-20) at 4 °C for 1.5 h and incubated with adiponectin or β-actin primary antibodies at 4 °C overnight and subsequently with an appropriate secondary antibody. Protein bands were detected using the ECL kit (ATTO Corp., Tokyo, Japan), and the intensities of bands were quantified using the Quantity One software (Bio-Rad Hercules, CA, USA).

2.12. Cellular uptake of rosiglitazone

To evaluate the cellular uptake of rosiglitazone, cells were seeded at 1×10^5/well and cultured in 12-well plates for 24 h. All of the wells were treated with 100 μM of rosiglitazone-loaded emulsion for each formulation and the cells were harvested at designated times. Then, the cells were incubated with rosiglitazone-loaded emulsion or lipoplexes (drug concentration 100 μM) in serum-free DMEM for designated times. After the cells were washed with PBS, 100 μL of trypsin were added. Following further incubation for 5 min, the cells were harvested by adding 900 μL of the mobile phase. The cells were lysed using an ultrasonic Vibra-Cell™ (VCX130, SONICS®, Newtown, CT) and centrifuged at 10,000 rpm for 10 min. The rosiglitazone concentration was determined by HPLC, as reported previously (Davaa and Park, 2012). In brief, the separation was performed on a Capcell PAK C_{18} column (150 × 4.6 mm I.D., 5 μm particle size, Shiseido, Tokyo, Japan) maintained at 30 °C. The mobile phase was an acetonitrile: methanol:acetate buffer at pH 4.0 (20:10:70 v/v/v) and was delivered at a flow rate of 1 mL/min. The injection volume of the sample was 20 μL. The eluate was analyzed at a wavelength of 214 nm.

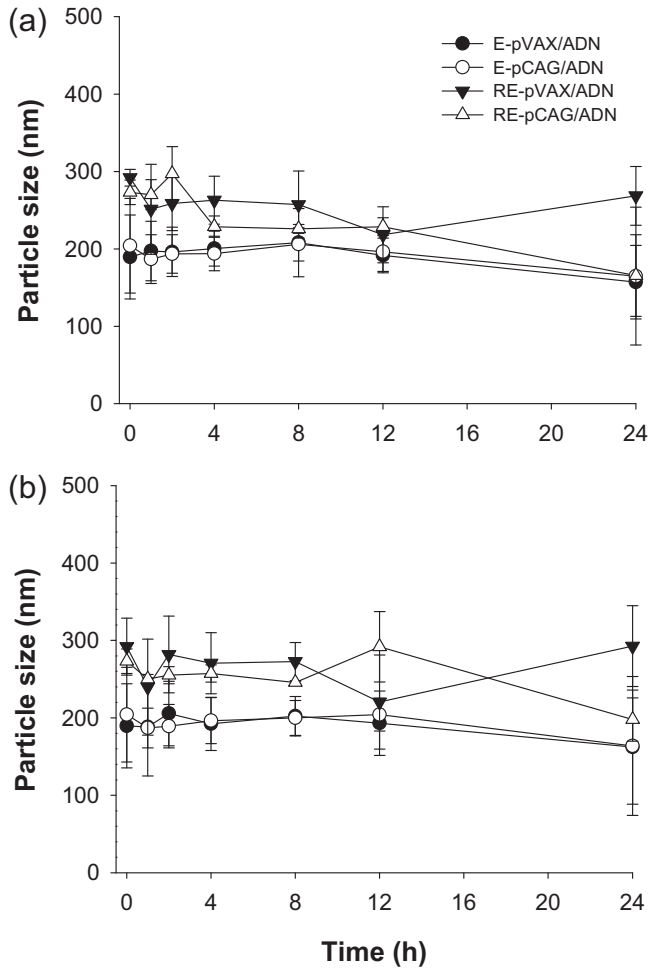

Fig. 3. Physical stability of lipoplexes at (a) room temperature and (b) 4 °C ($n = 3$). The complexes were prepared at weight ratio of 1:16.

2.13. Cytotoxicity

The cytotoxicity of rosiglitazone alone, drug-loaded and unloaded emulsions, and emulsion/DNA complexes was determined to demonstrate the viability and proliferation of cells against transfection complexes. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a yellow salt that is reduced in the mitochondria of viable cells to a blue formazan product with maximum absorbance at 570 nm. HepG2 cells were seeded at 1×10^5/mL in 96-well plates. After overnight incubation, the cells were exposed to rosiglitazone or rosiglitazone-loaded emulsion/DNA complexes containing medium for 24 h. Then, the medium was removed and 100 μL of MTT-containing medium (5 mg/mL) were added to the wells. Following a 4-h incubation at 37 °C, the MTT-containing medium was aspirated carefully to avoid disturbing any formazan crystals formed and 100 μL of MTT solubilization solution were added to each well. The plates were incubated at room temperature for 30 min and the optical densities were recorded at 570 nm using a Microplate reader (Sunrise, Tecan Trading, Switzerland). Cell viability was expressed as a percentage of the untreated control cells.

2.14. Statistical analysis

Statistical analysis of the data was performed using Student's *t*-test or one-way analysis of variance (ANOVA). A *p*-value of <0.05 was considered to indicate significance. Data were expressed as the means ± the standard error of the mean (SEM) of three independent experiments.

3. Results and discussion

3.1. Particle size and zeta potential of complexes

The particle sizes of the cationic emulsion/DNA complexes were compared between drug-loaded and unloaded emulsions (Fig. 1a). The size of the complex decreased as the ratio of cationic lipid emulsion to DNA increased. The particle size was 240 nm for the cationic emulsion and 245 nm for the rosiglitazone-loaded cationic emulsion, in the absence of plasmid DNA. For the cationic emulsion, castor oil as a core can form small nanoparticles despite the high viscosity (Chung et al., 2001). Based on the increased amount of plasmid DNA, the particle size of the complex decreased as the weight ratio of plasmid DNA to lipid increased. The mean particle sizes of the emulsion/DNA complexes and rosiglitazone-loaded emulsion/DNA complexes decreased from 540 to 230 nm and from 340 to 220 nm, respectively. The surfactants Tween 20 and Tween 80 prevent the large formation of cationic emulsion/

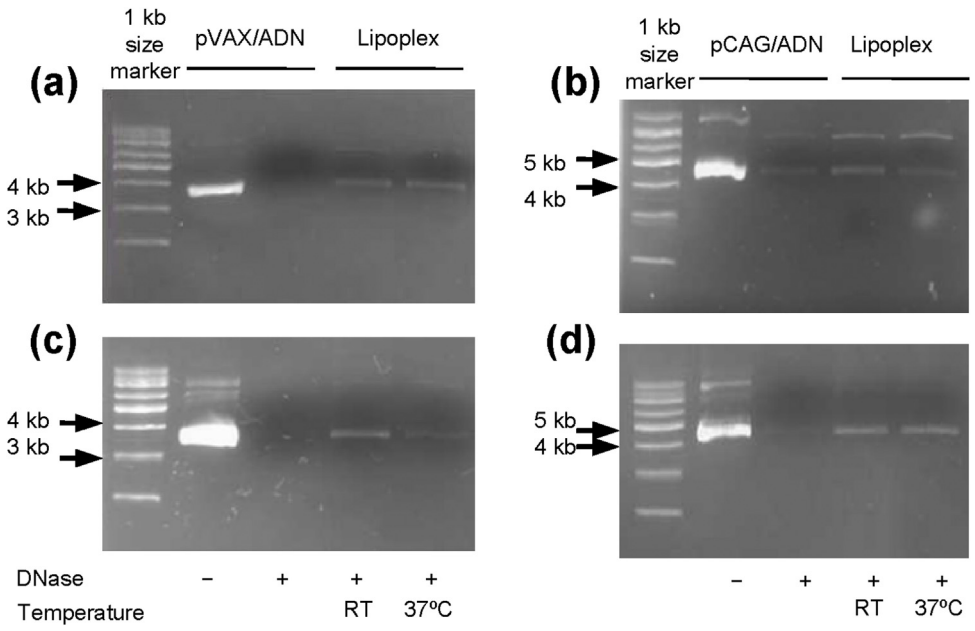

Fig. 4. Stability against DNase I of plasmid DNA by (a, b) drug free complexes and (c, d) drug-loaded complexes.

DNA complexes (Choi et al., 2004). Based on the results of complexation, we chose a complexation ratio of DNA to lipid of 1:16 (w/w). The combination complexes of drug-loaded or unloaded cationic emulsion and plasmid DNA were prepared using this ratio.

To investigate the effect of drug loading, we determined the particle size and zeta potential of the cationic emulsion and rosiglitazone-loaded cationic emulsions, and plasmid DNA complexes, at a lipid to DNA complexation ratio of 16:1. Fig. 1b shows that the particle size increased slightly depending on drug loading and complexation with the plasmid DNA. The zeta potential of the drug-loaded and unloaded cationic emulsions did not differ among the formulations, and all of the lipid formulations showed positive charges. The particle sizes and zeta potential had values suitable for intravenous injection.

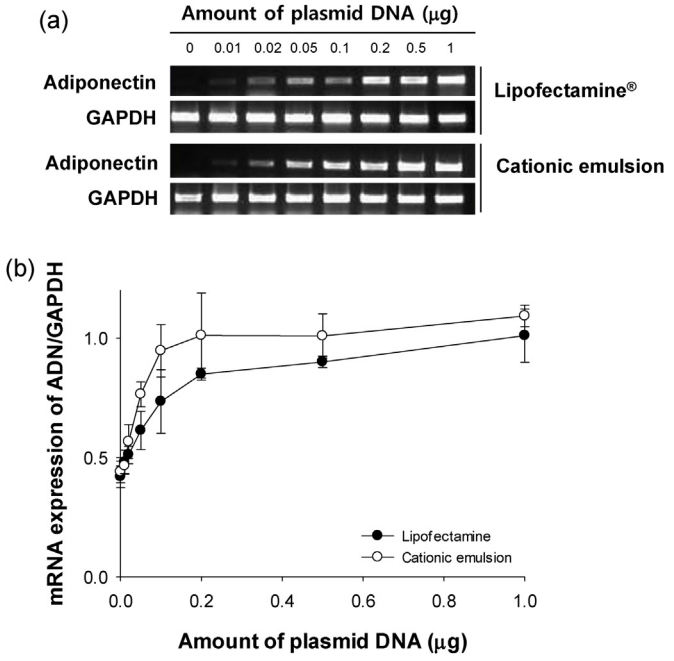

Fig. 5. (a) Representative image and (b) quantitative comparison of ADN mRNA expression in HepG 2 cells after transfection of cationic emulsion/DNA complexes at a ratio of plasmid DNA: lipid = 1:16. The amounts of plasmid DNA were increased 0.01–1.0 μg. Bars represent mean ± s.d. ($n = 3$).

3.2. Gel retardation assay

The DNA binding ability of rosiglitazone-loaded or unloaded cationic emulsions was studied using a gel retardation assay (Jeong et al., 2009; Davaa et al., 2013). To verify the drug-loaded or unloaded cationic emulsions/DNA complex formation, agarose gel electrophoresis was performed after the complexes were formed with 0.5 μg of pVAX/ADN or pCAG/ADN (Fig. 2). Free DNA that had not formed a complex with the cationic emulsion was clearly visible at complex ratios of 0.5–32 (μg:μg). However, no such free DNA was evident at a DOTAP to DNA ratio >4 (μg:μg). Lipofectamine® formed complexes with DNA without a trace of free DNA at a 6:1 ratio (Lipofectamine®:DNA, w/w), according to the manufacturer's instructions. The movement of plasmid DNA on 1% agarose gels was retarded as the amount of emulsion increased. At ratios over 8, the cationic emulsions bound DNA efficiently, and the complete retardation of DNA mobility was achieved for both pVAX/ADN and pCAG/ADN. However, the complexes that formed at under ratios less than 4 showed incomplete complexation for both pVAX/ADN and pCAG/ADN. Therefore, the complexes at a ratio of 16 showed good complexation with small particle size and a suitable zeta potential.

3.3. Stability of drug-loaded or unloaded cationic emulsion/DNA complexes

Previously, we reported that cationic lipid and rosiglitazone-loaded emulsions had good physicochemical properties and stability (Davaa and Park, 2012). The rosiglitazone-loaded cationic emulsion showed a high efficiency (>90%) of rosiglitazone encapsulation. In this study, we prepared combination complexes of rosiglitazone-loaded or unloaded cationic emulsion and plasmid DNA, based on the previous results. The particle size of the rosiglitazone-loaded or unloaded cationic emulsion/plasmid DNA (pVAX/ADN or pCAG/ADN) complexes at a ratio of 16 (lipid/DNA,

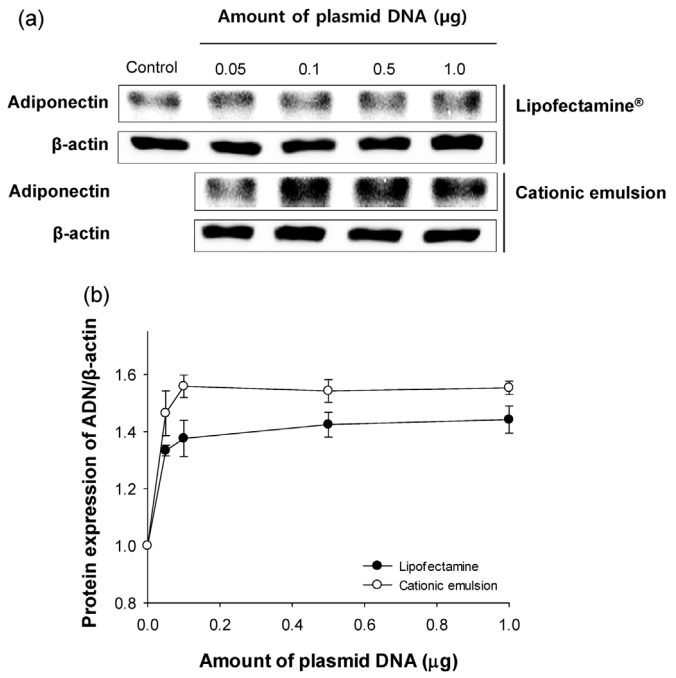

Fig. 6. (a) Representative immunoblotting image and (b) quantitative comparison of ADN protein expression in HepG2 cells after transfection of cationic emulsion/DNA complexes at a ratio of plasmid DNA: lipid = 1:16. The amounts of plasmid DNA were increased 0.05–1.0 μg. Bars represent mean ± s.d. ($n = 3$).

w/w) with a fixed amount (1 μg) of pVAX/ADN or pCAG/ADN did not change considerably at room temperature, even after 24 h after complexation (Fig. 3a). In addition, the physical stability of both lipoplexes was maintained for 24 h at 4 °C after complexation (Fig. 3b). No aggregation was observed under either storage condition.

We also examined whether DNA in the rosiglitazone-loaded and unloaded complex would be protected from DNase I digestion (Jeong et al., 2009), since complexes cannot function *in vivo* owing to the interrupting effect of nuclease. Naked DNA (pVAX/ADN and pCAG/ADN) was digested completely by 0.25 units of DNase I (Fig. 4), while the DNA complexed with the cationic emulsion was protected from DNase I at room temperature and 37 °C. Drug loading did not affect the stability against DNase I of the complexes DNA. Therefore, the cationic drug-loaded and unloaded emulsions formed complexes with DNA. We confirmed that cationic lipid emulsions could facilitate co-delivery of plasmid DNA (pVAX/ADN or pCAG/ADN) by protecting the complexes from nuclease.

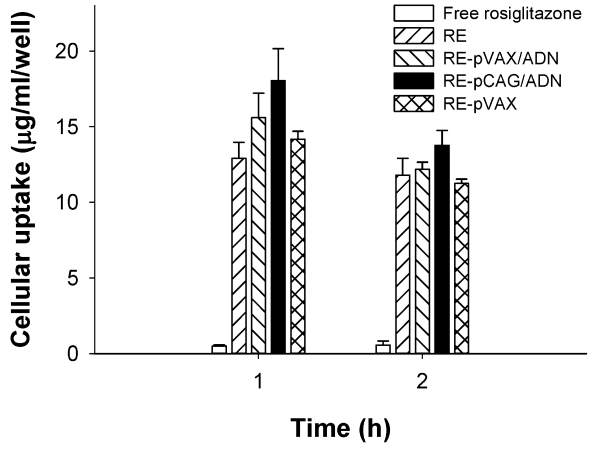

Fig. 7. Cellular uptake of rosiglitazone by emulsion and plasmid DNA complexed emulsion in HepG2 cells ($n = 3$).

3.4. In vitro *transfection of cationic emulsion/DNA complexes*

To quantify ADN mRNA expression, HepG2 cells were transfected with combination complexes using a drug-unloaded cationic emulsion. The cationic lipid to DNA complex ratio was fixed at 16:1 (w/w) and the amount of DNA (pCAG/ADN) was increased. As shown in Fig. 5, the ADN mRNA expression level increased in a dose-dependent manner in HepG2 cells transfected with plasmid DNA for both the cationic emulsion/DNA complexes and Lipofectamine®/DNA complexes. The ADN expression level after transfection of the cationic emulsion/DNA complexes was higher than that for the Lipofectamine®/DNA complexes. Moreover, to measure the expression of ADN protein, immunoblot for human ADN was performed using total proteins adjusted 15 μg. The results shown in Fig. 6 represents that the ADN protein expression level increased dose-dependently manner in HepG2 cells transfected with plasmid DNA. Similar to the results of mRNA expression, the ADN protein expression level after transfection the cationic emulsion/DNA complexes was higher than that of Lipofectamine®/DNA complexes. Therefore, it is suggested that this cationic emulsion would be more effective in the transfection than commercial Lipofectamine®. Moreover, as dual carrier for rosiglitazone and ADN gene, cationic emulsion has potential to increase the expression of ADN. Although we did not observe direct increase of AMPK activation, it is supposed that the increased ADN could potently activates AMPK activity, reduce body weight, and improve insulin sensitivity (Coughlan et al., 2014).

3.5. Cellular uptake of rosiglitazone

The therapeutic effects of drug-loaded cationic emulsions are dependent on internalization and retention of the cationic emulsions in target cells. *In vitro* investigation can provide some preliminary evidence of the advantages of an emulsion formulation *versus* free drug. Fig. 7 shows that the cellular uptake of the rosiglitazone-loaded cationic emulsions had the highest value. Then, the difference between two ADN-encoding plasmid DNAs was compared. Although not significant, the complex of the rosiglitazone-loaded cationic emulsion and pCAG/ADN had the highest value, of 18%, at 1 h after treatment with 100 μM rosiglitazone. These results showed that the cationic emulsion complexes can co-deliver rosiglitazone to targeted HepG2 cells. The cellular uptake of rosiglitazone was enhanced by loading in the cationic emulsion. Moreover, plasmid DNA did not significantly affect the cellular uptake of rosiglitazone loaded in the cationic emulsion.

Table 1
Cytotoxicity of formulations with or without rosiglitazone and plasmid DNA ($n = 3$). The experiments were performed on HepG2 cells and complexes were prepared at weight ratio of lipid to DNA, 16:1.

Formulation	Cell viability (%)
Free rosiglitazone	80.6 ± 14.9
Blank emulsion	85.5 ± 21.3
Rosiglitazone emulsion	94.5 ± 17.4
Lipofectamine-pVAX/ADN	67.6 ± 18.4
Lipofectamine-pCAG/ADN	69.7 ± 16.4
Emulsion-pVAX/ADN	81.4 ± 19.8
Emulsion-pCAG/ADN	86.4 ± 14.9
Rosiglitazone emulsion-pVAX/ADN	89.9 ± 16.0
Rosiglitazone emulsion-pCAG/ADN	98.5 ± 13.9

3.6. Cytotoxicity

The cytotoxicity of various formulations was examined using the MTT assay. The viability of cells exposed to rosiglitazone, emulsion, rosiglitazone-loaded emulsion, and plasmid DNA complexes exceeded 80% (Table 1). The viability of cells exposed to the rosiglitazone-loaded cationic emulsion was considerably higher than that of those exposed to the free rosiglitazone; moreover, the rosiglitazone-loaded cationic emulsion was less toxic to HepG2 cells than were the blank cationic emulsions. Moreover, the rosiglitazone-loaded cationic emulsion/DNA complexes showed higher cell viability than the cationic emulsion/DNA complexes or Lipofectamine®/DNA complexes. All of the rosiglitazone-loaded, unloaded cationic emulsions and the various DNA-complexed formulations showed low cytotoxicity. Therefore, the combination of a rosiglitazone-loaded emulsion and plasmid DNA complexes has low toxicity.

4. Conclusion

Cationic emulsions have the ability to co-deliver the adiponectin gene and rosiglitazone to target cells. In this study, the cationic emulsion formulation was found to result in higher ADN expression and rosiglitazone uptake. In conclusion, cationic lipid emulsions show potential for the combined delivery of newly subcloned plasmid DNA (pVAX/ADN and pCAG/ADN) and rosiglitazone. Further studies are needed to investigate the pharmacokinetic and pharmacological profiles of rosiglitazone and the *adiponectin* gene *in vivo*.

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