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Chapter 4

EFFICIENT DNA TRANSFECTION IN PROTISTS MEDIATED BY CELL-PENETRATING PEPTIDE

Betty Revon Liu[1,*], Yue-Wern Huang[2] and Han-Jung Lee[3]
[1]Department of Laboratory Medicine and Biotechnology,
Tzu Chi University, Hualien, Taiwan
[2]Department of Biological Sciences,
Missouri University of Science and Technology, Rolla, MO, US
[3]Department of Natural Resources and Environmental Studies,
National Dong Hwa University, Hualien, Taiwan

ABSTRACT

Developing a useful and efficient DNA transfection method is always a concerning issue and necessary for study of specific molecules and their functions in individuals. However, most transfection methods utilized today are applied in mammalian cells such as embryonic cells

* Corresponding Author's Email: brliu7447@gms.tcu.edu.tw.

and cell lines. Rare transfection studies are found in individual microscopic organisms such as paramecium and rotifer which belong to single-celled and multi-celled individuals, respectively. Here, we introduced cell-penetrating peptides (CPPs) as an efficient tool for DNA transfection. HR9, a designed CPP, containing nona-arginine flanked by cysteine and penta-histidine displayed a high penetrating ability in mammalian cells. Moreover, HR9 was able to internalize paramecia and rotifers which contain the pellicles and cuticles, respectively. DNAs were also delivered into these cells and organisms by HR9 and still contained the bioactivities. High viabilities of organisms and low cytotoxicities after HR9/DNA treatments illustrated that this CPP was harmless and could be a potent tool for DNA transfection.

ABBREVIATIONS

BFP	blue fluorescent protein
CPP	cell-penetrating peptide
CNPT	covalent and noncovalent protein transductions
DMSO	dimethyl sulfoxide
EGFP	enhanced green fluorescent protein
EtOH	alcohol
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
HIV-1	human immunodeficiency virus type *1*
HR9	nona-arginine flanked by cysteine and penta-histidine cell-penetrating peptide
MTT	1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan
PBS	phosphate buffered saline
RFP	red fluorescent protein
SDs	standard deviations
Tat	transactivator of transcription

INTRODUCTION

A practical and efficient DNA transfection method is very important and required to study gene functions and regulations [1]. Multiple techniques of exogenous nucleic acid delivery have been developed, including viral and nonviral vector-mediated methods [2]. Retroviruses, lentiviruses, and adeno-associated viruses are three types of vectors which are commonly used in transient or stable transgene expression in mammalian cells [2]. Nonviral DNA delivery which is the prominent route can be further divided into two groups: chemical-based methods and physical techniques [2]. Calcium phosphate, cationic lipids, polymers, peptides, polysaccharides, and inorganic nanomaterials are utilized as the chemical-based methods, while physical techniques contain ultrasonic nebulization, microinjection, electroporation, and particle bombardment [2, 3]. Pros and cons of these gene delivery methods are being stated and the choice of a proper tool for transfected gene expression depends on the goal of a study and other factors [4]. Most DNA delivery systems were developed and applied in mammalian cells, including totipotent embryonic and pluripotent stem cells [4-6]. Few studies reported that these present transgenic tools were able to be applied in individual microscopic organisms. Therefore, development of safer, more efficient, easy-handled, and widely used methods for gene delivery are next steps for transgenic protists.

Cell-penetrating peptides (CPPs) are defined as short peptides which contained the ability to deliver cargoes to transport across plasma membrane [7]. The first reported CPP was Tat, which came from the transcription activator of the human immunodeficiency virus type *1* (HIV-*1*) [8]. The study of primary structure of the domain responsible for internalization in Tat revealed that the cationic residues, such as lysine and arginine, were the keys for the plasma membrane penetration [9]. Various CPPs were flourishingly developed later and able to be divided into three categories: cationic, amphipathic, and hydrophobic [9, 10]. CPPs were good shutters to take other biomolecules as cargoes into cells in covalent, noncovalent, or covalent and noncovalent protein transductions (CNPT)

manners [11, 12]. The types of cargoes included proteins, DNAs, RNAs, small molecular drugs, and some inorganic particles [7, 9, 10, 12-16]. The sizes of cargoes were able to reach up to 200 nm in diameter [17]. Besides, the dosage of CPPs for bio-application was up to 100 μM and it still would not cause injury to cells [18]. Therefore, CPPs could be the good tools for therapeutic delivery into cells because of their high transduction efficiency, fast transduction rate, and low cytotoxicity.

Various DNA delivery methods were modified and applied to different organisms. Simple heat-shock gene transformations were commonly used in prokaryotes which had to be prepared as competent cells first [19]. Electroporation had to be modified to increase transfection efficiency in *Parabodo causatus* and *Euglena gracilis* [20, 21]. The viral RNA-based transfection method was reported in *Trichomonas vaginalis* [22]. However, no transfection methods could be used in all organisms. Besides, complicated procedures of competent cell preparation and gene-delivery system setting, low efficiency of successful transfection, and high cells/organisms injury become the drawbacks of these gene delivery systems. Our previous studies reported that CPPs possessed the ability to enter different organisms including prokaryotes, yeasts, insect cells, mammalian cells, plant tissues, paramecia, and rotifers [11, 15, 16, 23-31]. Here, we illustrated that one-CPP-one-protocol could be used for exogenous gene deliveries in mammalian cells, paramecia, and rotifers.

Materials and Methods

Culture of Various Cells and Organisms

Human bronchoalveolar carcinoma A549 cells (American Type Culture Collection, Manassas, VA, USA; CCL-185) were grown in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) bovine serum (Gibco). Cells were cultured in a humidified incubator with 5% CO_2 at 37°C as previously described [15].

Paramecia (*P. caudatum*) were grown in the culture medium of 1.25% (w/v) fresh lettuce juice [32] diluted with the Dryl's solution [33]. Young cells with 4–5 divisions were maintained at 23–25°C and seeded at a density of 50–100 paramecia per 100 μl in a well of 24-well plates for one day at room temperature before experiment.

Rotifers (*Brachionus calyciflorus*) (Bioprojects International Co.; Kaohsiung, Taiwan) were cultured in freshwater supplemented with the Fresh Chlorella V-12 (Bioprojects). The culture system was air-pumped at a rate of 0.1~0.3 L/min according to the manufacturer's instructions. Rotifers were seeded at a density of 1×10^5 in each well of 24-well plates and incubated in a shaker incubator at 25–28°C [16].

Preparation of Plasmids and Peptides

The pEGFP-N1 plasmid (Clontech, Mountain View, CA, USA) containing the enhanced green fluorescent protein (*EGFP*) and the cytomegalovirus (CMV) promoter was used for CPP-mediated gene delivery and gene expression in transfected A549 cells. The pGFP-actin1-1 plasmid (kindly provided by Dr. Ilya N. Skovorodkin, University of Oulu, Finland) containing green fluorescent protein (GFP)-actin fusion gene is under the control of the *P. caudatum* α-tubulin promoter. The pCS2+ DsRed plasmid contains the coding regions of red fluorescent protein (*RFP*) (*DsRed1* with GenBank accession number JF330266) under the control of the simian cytomegalovirus immediate-early enhancer/promoter sequence (GenBank accession number U38308) [34, 35].

HR9 (CHHHHHRRRRRRRRRHHHHHC) was synthesized as previously described [14]. The molecular mass of HR9 peptide is 3001.7 Daltons. HR9-FITC peptide containing the fluorescein isothiocyanate (FITC) at the N-terminus was chemically synthesized using solid-phase peptide synthesis and cross-linked with FITC by the FluoroTag FITC conjugation kit (Genomics, Taipei, Taiwan) as previously described [15]. HR9-FITC peptide contains a molecular mass of 3504 Daltons.

Protein Transduction in Organisms

To monitor cellular uptake of HR9-FITC in mammalian cells, human A549 cells were treated with 10 μM of HR9-FITC for 1 h at 37°C followed by the treatment of Hoechst 33342 (Invitrogen) as previously described [14]. The cells were then washed with phosphate buffered saline (PBS) to remove superfluous peptides and dyes. The cells were monitored using a Nikon A1+ confocal fluorescent microscope with a magnification of 600× (Nikon Instruments Inc., Melville, NY, USA).

To observe cellular uptake of HR9-FITC in the protozoa, young paramecia were washed in water before treatment. Cells were treated with 7.15 μM of HR9-FITC for 1 hour at room temperature [25]. The cells were subsequently monitored right after incubation with 1% formaldehyde fixation using the BD Pathway 435 system (BD Biosciences, Franklin Lakes, NJ, USA). Another protozoa, rotifers, were treated with 6 μM of HR9-FITC for 1 h at 28°C as previously described [15, 25]. Live rotifers were washed and monitored without fixation. Fluorescent and bright-field images were recorded using a BD Pathway 435 System (BD Biosciences).

CPP-Mediated Gene Delivery into Various Organisms

To observe gene delivery mediated by HR9 and the functional reporter gene assay, A549 cells were treated with either 3 μg of the pEGFP-N1 plasmid DNA only or the pEGFP-N1 plasmid DNA mixed with HR9 at the N/P ratio of 3. Cells treated with only medium served as a negative control. Cells were incubated for 10 min at 37°C. After that, solution was removed, and cells were washed with PBS thrice. A549 cells were then supplemented with 100 μl of 10% serum-containing medium and incubated at 37°C for 48 h. After 2 days, cells were stained with Hoechst 33342 and observed using a BD Pathway 435 system (BD Biosciences).

Three microgram of the circular pGFP-actin1-1 plasmid were mixed without or with HR9 at the N/P ratio of 3 in water at a final volume of 60 μl and incubated for 1 h at room temperature. The mock, pGFP-actin1-1 plasmid only, or HR9/pGFP-actin1-1 complexes were then dropped into cells and incubated for 30 min at room temperature. After the incubation, the cells were added with 1 ml of culture medium and incubated for additional 3 days at room temperature. Cells were monitored without fixation using the BD Pathway 435 system (BD Biosciences).

Rotifers were treated with water or 3 μg of the pCS2+ DsRed plasmid DNA alone as controls, while rotifers were treated with HR9/pCS2+ DsRed plasmid DNA complexes at the N/P ratio of 3 as an experimental group. After incubation for 10 min at 28°C, solution was removed, and rotifers were washed with freshwater thrice, followed by incubation at 28°C for 24 h. Rotifers were then observed using the Olympus BX51 inverted fluorescent microscope (Olympus, Center Valley, PA, USA).

The transfection efficiency of the HR9-mediated gene delivery and the gene expression intensity were determined as previously described [15, 16, 24, 25]. The relative intensities of fluorescent images from the functional gene assay were converted and quantified using the UN-SCAN-IT software (Silk Scientific, Orem, UT, USA).

Confocal and Fluorescent Microscopy

Bright-field, GFP, and blue fluorescent protein (BFP) images were recorded using a BD Pathway 435 System (BD Biosciences) as previously described [14-16, 24, 25]. Excitation filters were set at 377/50, and 482/35 nm for blue and green, respectively. Emission filters were set at 435 LP (long-pass) and 536/40 nm for BFP and GFP channels, respectively. Transmitted light without the excitation filter, but with 536/40 nm emission filter, was used to observe cell morphology as bright-field images. The Olympus BX51 inverted fluorescent microscope (Olympus) was set with excitation at 531–554 nm and emission at 580-615 nm for RFP channel [16]. In mammalian cells observation, BFP, GFP, and bright-field images were detected using a Nikon A1+ confocal fluorescent microscope (Nikon Instruments Inc.) with excitation at 405 nm and

emission at 450/50 nm for BFP and excitation at 488 nm and emission at 520–568 nm for GFP.

Toxicity Measurement

The 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay was conducted to determine viability of A549 cells for 48 h, paramecia for 3 days, and rotifers for 24 h as previously described [15, 16, 25]. A549 and rotifers without any treatments served as the negative controls, while A549 cells and rotifers treated with 100% dimethyl sulfoxide (DMSO) served as the positive controls. In viability assay of paramecia, cells treated with culture medium alone served as the negative control, while paramecia treated with 70% alcohol (EtOH) served as a positive control. A549 cells, paramecia, and rotifers were treated with DNA only, HR9 only, and HR9/DNA complexes, respectively as the experimental groups

Statistical Analysis

Data are presented as mean ± standard deviations (SDs). Statistical comparisons between the control and experimental groups were performed using the Student's *t*-test. Mean values and SDs were calculated for each groups examined in at least triplicate independent experiments. The level of statistical significance was set at $P < 0.05$ (*, †, α) or 0.01 (**, ††, αα).

RESULTS

To assess whether HR9-FITC can enter mammalian cells, human A549 cells were treated with HR9-FITC peptide. No green signal was observed in the cells treated with PBS as a negative control (Figure 1A). However, strong green fluorescent signal was visualized in the cells treated with HR9-FITC at the GFP channel (Figure 1A). These results showed that this

CPP, HR9-FITC, possesses the cellular internalization activity in mammalian cells.

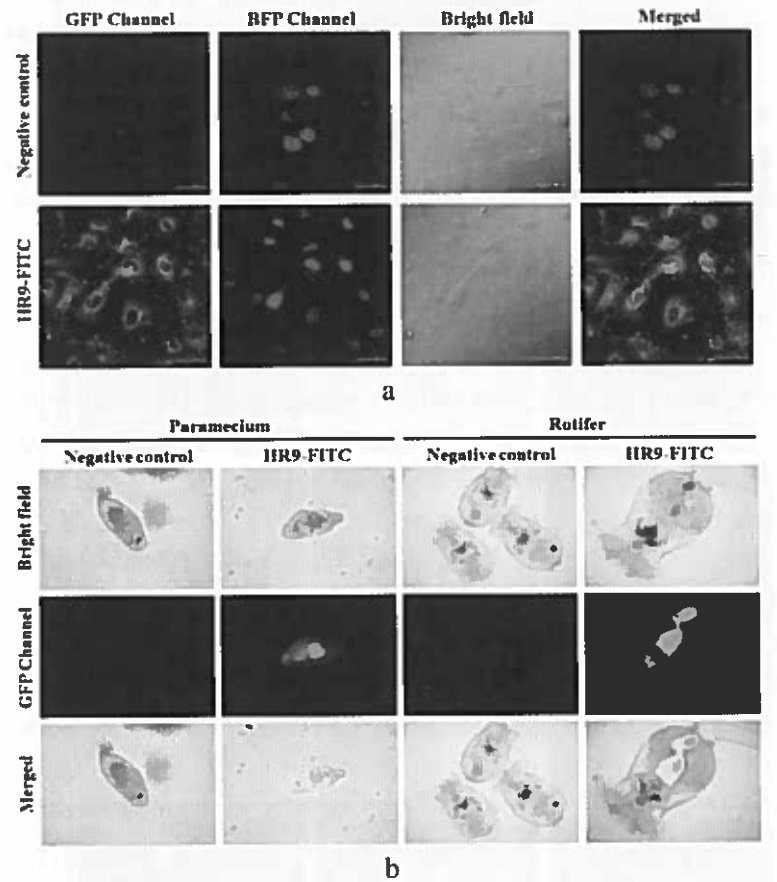

Figure 1. Protein transduction in various species. (A) Protein tranduction in human bronchoalveolar carcinoma A549 cells. Cells were treated with 10 μM HR9-FITC for 1 h. Cells without any treatments were served as the negative control. GFP, BFP channels and bright fields revealed the distributions of FITC-labeled HR9, nuclei, and cell morphologies. All images are obtained using a Nikon A1+ confocal fluorescent microscope with a magnification of 600×. (B) Protein transduction in the protozoa. Paramecia and rotifers were either treated with HR9-FITC or double-deionized water as the experimental groups and the negative control, respectively. GFP channels and bright fields illustrated the distributions of HR9-FITC and cell morphologies, and merged images were overlapped with GFP channels and bright fields. All images are obtained using a BD Pathway 435 confocal microscopic system with a magnification of 200×.

To demonstrate that CPPs can enter live organisms, HR9-FITC was used to incubate with paramecia and rotifers. We found that no signal was observed in the paramecia without any treatments as a control. On the other hand, green fluorescence was visualized in the paramecia treated with HR9-FITC at the GFP channel (Figure 1B). Similar results were shown in rotifers. No signal was detected in the rotifers treated with water as a control. On the contrary, green fluorescence was visualized in the rotifers treated with HR9-FITC (Figure 1B). These results demonstrated that HR9 can be internalized into both paramecia and rotifers (Table 1).

To determine plasmid DNA could be delivered by HR9 into organisms and the cargo DNA can be actively expressed after delivery, pEGFP-N1, pGFP-actin1-1, and pCS2+ DsRed were used for the functional gene assay in A549 cells, paramecia, and rotifers, respectively. Three microgram of plasmid DNA were mixed with HR9 at the N/P ratio of 3 and HR9/DNA complexes were added into these different organisms (Figure 2). After 48 h of incubation in A549 cells, no green fluorescent signal could be detected in the cells treated with PBS as a negative control and DNA only (Figure 2A). In contrast, green fluorescence was displayed in A549 cells treated with CPP/DNA complexes (Figure 2A), indicating that DNA transfection mediated by HR9 is efficient and gene expressed well in A549 cells (Figure 2B and 2C). In paramecia, no signals were observed while cells treated with either mock as a negative control or DNA only after 3 days recovery (Figure 2A). Bright fluorescence was easily observed in cells treated with HR9/DNA complexes (Figure 2A), revealing DNA was efficiently transported into paramecia and still contained its function (Figure 2B and 2C). In rotifers, extremely weak signals were detected in both the control group and the group treated with DNA only (Figure 2A). However, red fluorescence was visualized in the rotifers treated with HR9/DNA complexes (Figure 2A). These results demonstrate that HR9 is an effective transgenic carrier in Rotifera and transfected gene is activate (Figure 2B and 2C, Table 1).

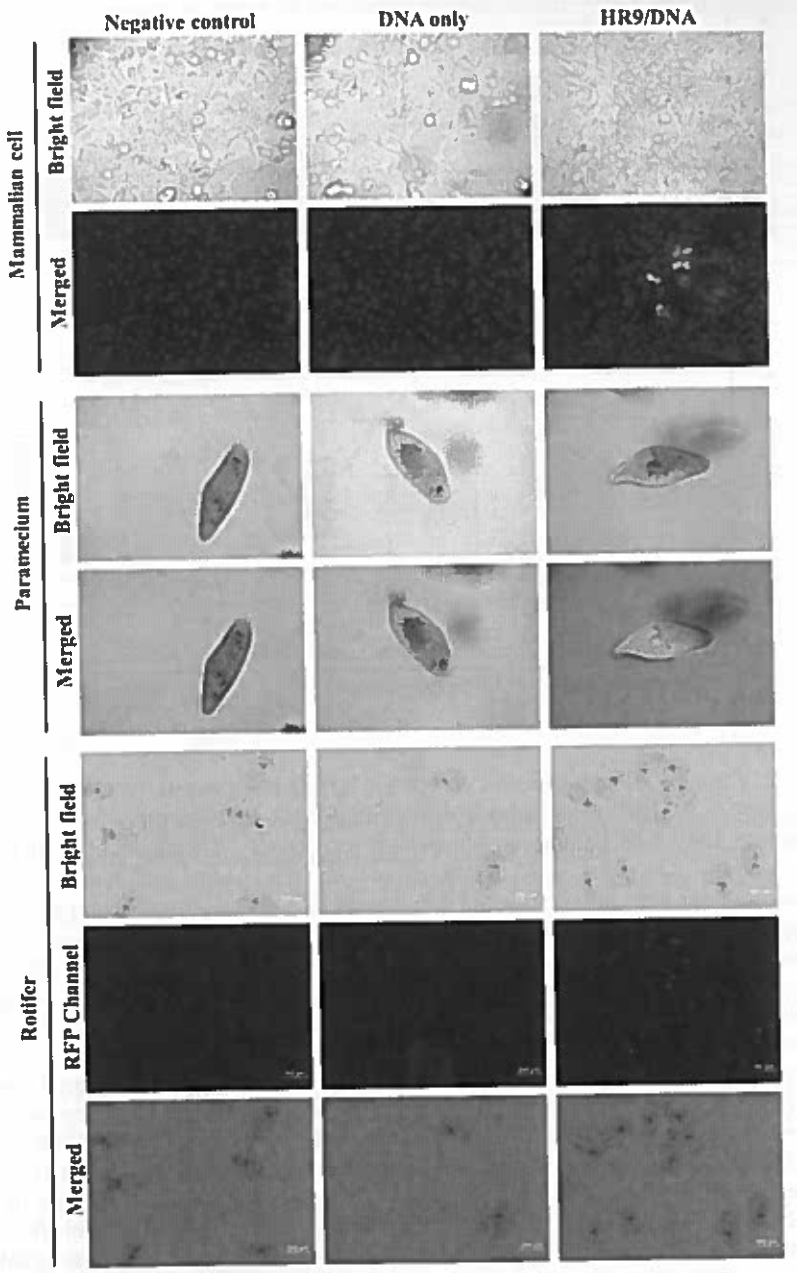

Figure 2. (Continued).

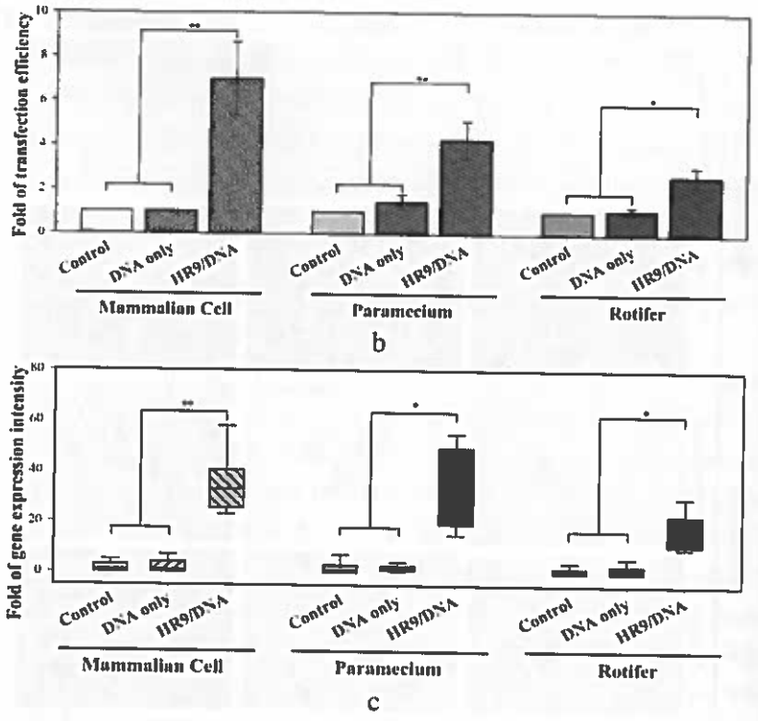

Figure 2. Plasmid DNA transfection via HR9 in different organisms. (A) Fluorescent microscopy of the HR9-mediated delivery of pEGFP-N1, pGFP-actin1-1, and pCS2+ DsRed into A549 cells, paramecia, and rotifers, respectively. The nuclei of A549 cells were stained with Hoechst 33342 and observed with BFP channel; cell-transfected gene expressions were observed with GFP channel in mammalian cells and paramecia. Merged images were obtained by the overlapping of GFP and BFP channels in the groups of mammalian cells as well as overlapping of GFP channel and bright field in the groups of paramecia, respectively. Transfected gene expression of pCS2+ DsRed was observed with RFP channel and merged images were obtained by RFP channel and bright field. Bright fields revealed the cell morphologies, shapes of paramecia, and appearances of rotifers. Images in mammalian cells and paramecia are procured using a BD Pathway 435 system at a magnification of 200×; while images in rotifers are shown using the Olympus BX51 inverted fluorescent microscope at a magnification of 200×. (B) Quantification of HR9-mediated gene transfection efficiency. DNA transfection efficiency was calculated from the fluorescent intensities at the GFP or RFP channels of figure 2A. (C) Quantification of gene expression intensity in HR9-mediated gene delivery. Gene expression intensities were converted from the digital image data of functional gene assay and analyzed by the UN-SCAN-IT software. Each experimental group was compared with the control group. Significant differences were determined at $P < 0.05$ (*) and $P < 0.01$ (**) between the control and various treatments in each organisms.

Table 1. The comparison of protein transduction, DNA transfection, and structures between different organisms

	Mammalian cells	Paramecium	Rotifer
Protein transduction	+	+	+
DNA transfection	+	+	+
Organism type	Single-celled	Single-celled	Multi-celled
Outer structure of cell membrane	–	+ (pellicles)	+ (cuticle)

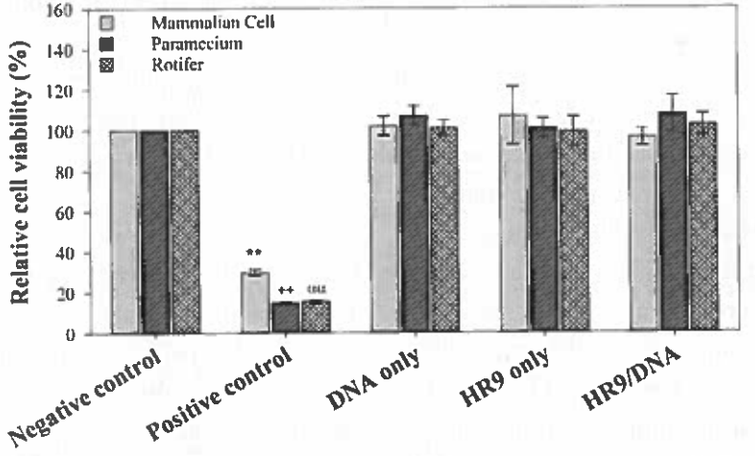

Figure 3. Cell viability of the HR9/DNA treatment. A549 cells, paramecia, and rotifers were treated with DNA only, HR9 only, and HR9/DNA complexes. Three types of specimens without any treatments served as a negative control. A549 cells and rotifers were treated with DMSO as a positive control, while paramecia were treated with 70% alcohol (EtOH) as a positive control. The MTT assay was used to evaluate cytotoxicity after these treatments. Each group was compared with the negative control. Significant differences were determined at $P < 0.01$ (**, ††, and αα) between the negative control and various treatments in each organisms.

To evaluate any cytotoxicity caused by HR9 or HR9/DNA complexes, A549 cells, paramecia, and rotifers were treated with the plasmid DNA only, HR9 only, and HR9/DNA complexes and subjected to the MTT assay (Figure 3). Organisms treated with 100% DMSO in A549 and rotifers or 70% alcohol in paramecia as the positive control illustrated distinct reduction of viabilities compared to the negative control (Figure 3). However, viability in treatment groups was not different from those in

negative controls (Figure 3). These results suggested that neither HR9 nor HR9/DNA complexes cause cytotoxicity in these organisms.

DISCUSSION

In this study, we demonstrated that HR9 could not only enter mammalian cells, paramecia, and rotifers by itself, but also deliver plasmid DNAs as cargoes into these different organisms. HR9-delivered DNAs still contained their bioactivities after cellular internalization. Potent transfection efficiencies, significant gene expression intensities, and harmless transfection processes revealed that CPPs (HR9) would be a good tool for transgenic protists studies.

Macromolecules, such as DNAs, RNAs, and proteins, were prevented to get into cells by plasma membranes because of the hydrophobic bilayer structures forming the perfect barrier. Paramecium, a ciliated unicellular organism, contains the extracellular structure called pellicles surrounding the plasma membrane (Table 1) [36-38]. This cortical ultrastructure made by elastic proteins forms three 2-dimentionally arrayed system and provides extra protections to stabilize cellular form and integrity [37, 38]. Similar to paramecium, rotifers possess outer structures of plasma membrane named cuticle (Table 1) [39-42]. Cuticles which are composed of sclerotized proteins to form multiple plates combine with a syncytial hypodermis to form the major integuments of rotifers. Maintaining shapes and osmosis, excluding external molecules and danger, providing basal laminate to spines, ridges, or other ornamentation, and offering a morphological diagnosis and identification among species are the roles and functions that cuticles play [41, 42]. Theoretically, it should be harder for exogenous molecules to penetrate plasma membrane in paramecia and rotifers. However, we indicated that CPPs displayed a complete departure from these limits and delivered macromolecules into cells/organisms (Figure 1 and 2; Table 1) [12, 15, 16, 23-28]. Pellicles, cuticle, and even multi-celled structures are not the keys for blocking CPPs' penetration.

Transfected genes in cells are easily degraded and lose their bioactivities via endocytosis-mediated internalization. Exogenous nucleic acids will be degraded by *lysosomal acidification* mechanisms unless they are able to escape from lysosomes or bypass endocytosis pathway while entering [43]. Likewise, paramecia and rotifers, single-celled and multi-celled *aquatic protozoa*, respectively, utilize similar intake routes and digest exogenous molecules by their vacuoles and stomachs [44, 45]. However, HR9 entered cells by the direct translocation mechanism and the cargoes that HR9 took and HR9 itself would not be trapped in lysosomes [14, 15]. Besides, HR9/cargo complexes would enter and stay in nuclei at the end of intracellular trafficking [46]. This is the good news for DNA transfection because exogenous genes could be transported into the inherited center directly and decrease risks of functional loss. CPP/DNA complexes internalized by cells via endocytosis showed lower transfection efficiencies, which supports this hypothesis [14-16, 24, 25, 46]. Here, we exhibited that cationic CPPs (HR9)-mediated gene delivery was able to increase transfection efficiencies, upregulate the gene expression, and decrease the injuries and immune responses [47] of transgenic targets with only one and simple transfection protocol in all tested organisms (mammalian cells, paramecia, and rotifers). Therefore, CPPs were potent tools for transfection and could be widely applied in the future.

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