Decompression Process of Glycerol Shock Treatment Can Overcome Endo-Lysosomal Barriers for Intracellular Delivery

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ABSTRACT: The glycerol shock treatment has been used to improve the calcium phosphate transfection efficacy for several decades because of its high effectiveness and low toxicity. However, the mechanism of glycerol shock treatment is still obscure. In this study, the endo-lysosomal leakage assay demonstrated that the decompression process of glycerol shock treatment could enhance endo-lysosomal membrane permeabilization, which resulted in facilitating endo-lysosomal escape for effective intracellular delivery. The enhanced decompression treatment derived from glycerol shock treatment could increase the change of osmotic pressure further, which showed higher efficacy for intracellular delivery. Herein, we speculated that the endo-lysosomal swelling originated from the decompression process of glycerol shock treatment could cause endo-lysosomal damage.

INTRODUCTION

In 1973, Graham and van der Eb found that cells could be transfected with herpes simplex virus DNA by the calcium phosphate (CaP) precipitate. This research initiates the widespread application of CaP transfection for introducing foreign DNA into cultured cells. Jordan et al. subsequently have optimized a CaP transfection method, including glycerol shock treatment, which refers to the operation where cells are transfected with the CaP/DNA precipitate for several hours first, following the removal of the precipitate, and then the cells are exposed to about 15% (v/v) glycerol solution for several minutes. This method is modified by widely used Cold Spring Harbor protocols, which state that the uptake of DNA could be increased after glycerol shock treatment. However, the mechanism of how glycerol shock treatment could increase the uptake of DNA after removal of the CaP/DNA precipitate is still not clear. In this study, we attempted to explore the underlying mechanism of glycerol shock treatment.

Glycerol has been widely used in the biomedical field, for instance, as an osmotic agent to treat cerebral edema or a cryoprotective agent. The history of glycerol shock treatment has been shown in Figure S1. As previously reported, Jordan confirmed a glycerol shock did not affect the uptake of DNA into the cells in CaP transfection by labeled DNA. They speculated that the shock treatment appeared to act on cell-endocytosed DNA, which might favor the conjecture that DNA might escape from endo-lysosomes, but no direct evidence was provided. Until now, glycerol shock treatment has been used in CaP transfection for about 4 decades; could more direct evidence explain the mechanism?

In the present study, we have demonstrated that the decompression process of glycerol shock treatment can enhance endo-lysosomal membrane permeabilization to promote endo-lysosomal escape. As shown in Scheme 1, when cells are exposed to a 15% glycerol solution for several minutes, endo-lysosomal membrane permeabilization does not change. Then, cells return to an isotonic solution, the osmotic pressure is from hypertonicity to isotonicity, and the endo-lysosomal membrane permeabilization can be enhanced significantly. Therefore, not the hypertonic glycerol solution but the decompression process from hypertonicity to isotonicity mainly contributed to enhancing endo-lysosomal membrane permeabilization. Glycerol shock treatment also might be applied to other areas related to endo-lysosomal membrane permeabilization, such as autophagy and cancer therapy.

RESULTS AND DISCUSSION

Effect of Glycerol or NaCl Shock Treatment on CaP Transfection. The mechanism of glycerol shock treatment in CaP transfection has long been a mystery. Jordan et al. demonstrated that glycerol shock treatment could not enhance the uptake of CaP/DNA. We also verified that the shock treatment did not improve cellular uptake efficacy of YOYO-1-.
labeled DNA in CaP transfection (Figure S2), so the shock treatment might influence other processes of transfection. As previously reported, either glycerol or dimethyl sulfoxide shock treatment could enhance CaP transfection effectively.11,12 Moreover, it was reported that polyethylene glycol shock also could improve transfection efficiency.13

We speculated that glycerol shock treatment was related to hypertonicity; to test our hypothesis, we performed NaCl shock treatment. We observed that either glycerol or NaCl shock treatment improved the transfection efficacy of CaP transfection, which was verified in two different cell lines, Hela and 5637 cells (Figures 1 and S3). For Hela cells, the percentage of positive enhanced green fluorescent protein (EGFP) cells increased from 20.7 to 53.1% after a 15% glycerol shock and from 20.7 to 48.2% after a 6% NaCl shock. For 5637 cells, the percentage of positive EGFP cells increased from 2.15 to 6.97% after a 15% glycerol shock and from 2.15 to 5.98% after a 6% NaCl shock. The findings suggested that improved transfection efficacy might be highly related to hypertonicity. Next, we investigated the role of hypertonic shock treatment in transfection.

**Effect of Hypertonic Glycerol Shock Treatment on Endo-Lysosomes.** Generally, endo-lysosomal entrapment is a significant barrier to effective intracellular DNA delivery,14−16 which can be observed by the intracellular distribution of labeled DNA (a green fluorescent dye, YOYO-1) and LysoTracker Red-stained endo-lysosomes. Here, we investigated the effect of glycerol shock treatment on the intracellular DNA and endo-

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**Figure 1.** Transfection efficacy of CaP transfection in Hela and 5637 cells. Comparison of glycerol shock and NaCl shock treatment on transfection efficacy in (A,B) Hela cells and (C) 5637 cells after 48 h, respectively. Control is the group treated with 1× PBS. Scale bar, 200 μm.
lyosomes. As shown in Figure 2A, LysoTracker Red-stained endo-lysosomes were more dispersive after the shock treatment, which indicated that hypertonic shock treatment (a 15% glycerol shock or a 6% NaCl shock) appeared to have more impact on endo-lysosomes than YOYO-1-labeled DNA. These results suggested that glycerol shock treatment might destabilize and destroy endo-lysosomes.

Endo-Lysosomal Leakage Assay. To further confirm glycerol shock treatment has an impact on endo-lysosomes, we applied Gal-3 reporter cells to perform the endo-lysosomal leakage assay for more clear evidence. The Gal-3 reporter system has been used to visualize macropinosome leakage and lysosomal membrane permeabilization. In this system, Gal-3 is a soluble protein that generally existed in the cytosol, which can bind β-galactoside sugar-containing carbohydrates. These carbohydrates are usually present on the interior of endocytic vesicles. The permeabilization of vesicles results in cytosolic mAG-Gal-3 being accessible to carbohydrates and accumulating at ruptured vesicles, and fluorescent spots can be observed (Figure 2B).

As shown in Figure 2C,D, glycerol shock treatment made Hela-mAG-Gal3 cells produce bright fluorescent spots, which showed high colocalization with endo-lysosomes. In addition, we performed siramesine (a lysosomal membrane permeabilization inducer) treatment, and either effect of glycerol shock or siramesine treatment was distinct (Figure S4). The result indicated that the endo-lysosomal leakage assay was an excellent tool to visualize endo-lysosomal membrane permeabilization. This result also suggested that glycerol shock treatment could overcome endo-lysosomal barriers by enhancing endo-lysosomal membrane permeabilization.

Decompression Process in Glycerol Shock Treatment. Inspired by the results above, we noticed that a glycerol shock could be divided into two procedures. The first procedure is to expose cells to a hypertonic glycerol solution. The second procedure is to recover isotonicity (as shown in Figure 3A). To determine each procedure’s role in a hypertonic glycerol shock, we used Hela-mAG-Gal3 and S637-mAG-Gal3 cells to perform the endo-lysosomal leakage assay. Contrary to our expectations, endo-lysosomal membrane permeabilization usually occurred in the second procedure, not the first procedure. As shown in Figure 3B,C, when Hela cells were exposed to a 15% glycerol or 6% NaCl solution, the endo-lysosomal membrane permeabilization did not change; few fluorescent spots were observed. However, when cells were exposed to an isotonic solution again, many fluorescent spots appeared immediately. The phenomenon was also observed in S637 cells (Figure S5).

The endo-lysosomal change caused by the decompression process may be similar to the post-hypertonic injury of human spermatozoa. The hypertonic solution makes sperm severely shrink, but the sperm appears to maintain membrane integrity in the hypertonic solution. Severe membrane damage only be detected after the sperm returns to isotonic conditions. The hypertonic glycerol or NaCl solution can cause cells to lose water to shrink. Because of intrinsic membrane permeability, endo-lysosomes are affected by the ambient hypo-osmotic environment, causing dehydration and shrinking. When cells are exposed to an isotonic solution again, endo-lysosomes will swell theoretically. In this study, we found that the endo-lysosome appeared to maintain membrane integrity in the shrunken state. The permeability of the endo-lysosomal membrane was enhanced after the cell was in isotonic conditions again. Maintaining membrane integrity in the shrunken state may be the character of a biological membrane responding to the pressure change. However, irreversible changes may happen on the membrane during the dehydration process. The membrane could be damaged in the reswell process. We speculated that the endo-lysosomal swelling originated from the decompression process of glycerol shock treatment could cause endo-lysosomal damage and rupture (Figure 3D).

Enhanced Decompression Treatment in CaP Transfection. Furthermore, how to improve endo-lysosomal membrane permeabilization for higher transfection? Only
increasing the concentration of a glycerol solution is not a good idea, which may kill cells because of severe dehydration. As shown in Figure 4A, cells and endo-lysosomes lose water to shrink in a hypertonic environment. The exchange from a hypertonic solution to an isotonic solution leads to an increase in the influx of water to maintain osmolarity. The influx of water could make the endo-lysosome swell and eventually cause the endo-lysosomal rupture. The exchange from a hypertonic solution to a hypotonic solution makes more water rush into endo-lysosomes, which could be damaged by more intense swelling. Herein, we proposed that the enhanced decompression treatment, a hypertonic glycerol treatment combined with a hypotonic treatment, might improve endo-lysosomal membrane permeabilization for higher transfection efficacy.

To test our hypothesis that the enhanced decompression treatment could achieve higher transfection efficacy than only hypertonic glycerol shock treatment. The treatment of a 15% glycerol shock treatment combined with a 0.6× DMEM hypotonic shock treatment achieved the highest transfection efficacy. Cells with reduced viability may gain a relatively low level of transfection efficiency. The lower percentage of EGFP positive cells of group 0.4× DMEM than the group 0.6× DMEM may be attributed to the decreased cell viability of group 0.4× DMEM (Figure 4C). These results suggested that enhanced decompression treatment is an efficient and biocompatible method to enhance transfection in CaP transfection.

**Decompression Treatment in Chitosan/DNA Delivery.**

Inspired by the results above, decompression treatment was applied to improve chitosan/DNA transfection efficiency. The group of a 15% glycerol shock combined with a 0.6× DMEM hypotonic shock treatment also achieved excellent gene transfection efficacy. The percentage of EGFP-positive cells of the group above was 8.6-fold higher than the group without shock treatment (Figure 5). The results suggested that endo-lysosomal escape was a limited step in chitosan/DNA delivery, which also could be promoted by glycerol shock treatment.

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**Figure 3.** Endo-lysosomal leakage assay. (A) Schematic representation of a hypertonic shock. A hypertonic shock includes two steps. The first step is from isotonicity to hypertonicity. The second step is to return to isotonicity. (B) Fluorescence images of Hela-mAG-Gal3 cells treated with a 15% glycerol shock or a 6% NaCl shock. The decompression process enhanced endo-lysosomal membrane permeabilization. The arrows indicate ruptured endo-lysosomes. Scale bar: 50 μm. (C) CLSM images of Hela-mAG-Gal3 cells treated with hypertonic shock treatment. Scale bar: 20 μm. (D) Proposed mechanisms of decompression treatment. The dark background represents the hypertonic state; the light background represents the hypotonic state. The hypertonic solution can cause cells to lose water to shrink. Endo-lysosomes are also affected by the cell’s hyperosmotic environment, causing dehydration and shrinking. However, endo-lysosome still appeared to maintain membrane integrity. Once cells return to an isotonic solution, endo-lysosomes will swell because of the influx of water; eventually, endo-lysosomes rupture.
CONCLUSIONS

In this work, we showed the newly observed mechanism of glycerol shock treatment for intracellular delivery. The endo-lysosomal leakage assay provided essential proofs, which demonstrated that the decompression process of glycerol shock treatment could enhance endo-lysosomal membrane permeabilization to promote endo-lysosomal escape for effective delivery. Moreover, we found that glycerol shock treatment, combined with a hypotonic shock, might further overcome endo-lysosomal barriers by more intense swelling. Gene transfection is a complicated biological process, and the efficiency of gene transfection may be affected by many factors.
Future works will be focused on investigating other mechanisms of glycerol shock treatment on gene transfection. In a word, glycerol shock treatment is an efficient and easy-to-operate method to facilitate endo-lysosomal escape quickly, and this method may have applications in many areas related to endo-lysosomal membrane permeabilization.

### EXPERIMENTAL SECTION

#### Materials

CaCl₂, glycerol, HEPES, and Na₂HPO₄·12H₂O were purchased from Sigma (USA). YOYO-1, LysoTracker Red, and Hoechst 33342 were purchased from Invitrogen (USA). Chitosan (80% deacetylated, low-molecular weight) was purchased from TCI (Japan). NaCl, cell counting kit-8 (CCK-8) was obtained from Sangon Biotech (China). The chemicals were used as received without further purification.

Plasmid mAG-Gal3 was amplified by Escherichia coli DH5α (Sangon, China), and plasmid mAG-Gal3 was amplified by E. coli Stbl3 (Sangon, China) according to the manufacturer’s protocols. The amplified plasmids were purified by using the HiPure Plasmid Maxi Kit (Magen, China) according to the product’s protocol. The concentration and integrity of purified plasmids were confirmed by spectrophotometry (NanoDrop, USA) and DNA electrophoresis, respectively.

Cell Culture. Hela cells (a human cervical cancer cell line, ATCC) were cultured in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/mL streptomycin, and 100 U/mL penicillin at 37 °C with 5% CO₂. Hela-mAG-Gal3 and 5637-mAG-Gal3 reporter cell lines were made by infecting cells with CaP/DNA mix containing the plasmid mAG-Gal3. Hela-mAG-Gal3 and 5637-mAG-Gal3 were used as the control. The CaP/DNA precipitate was used to select the cells that are stably expressing mAG-Gal3 proteins. The expression of EGFP was observed by fluorescent microscopy (Zeiss, Germany). The concentration and integrity of purified plasmids were confirmed by spectrophotometry (NanoDrop, USA) and DNA electrophoresis, respectively.

Generation of Gal3 Reporter Cell Lines. The lentiviral particles were produced by a lentiviral transduction system containing the plasmid mAG-Gal3. Hela-mAG-Gal3 and 5637-mAG-Gal3 reporter cell lines were made by infecting cells with the lentiviral particles. After 2 days of lentiviral infection, the cells were transfected in complete medium supplemented with 1 µg/mL of puromycin at 37 °C with 5% CO₂. The addition of puromycin was used to select the cells that are stably expressing mAG-Gal3 proteins. The effect of lentiviral infection could be observed by fluorescent microscopy (Zeiss, Germany).

Calcium Phosphate Transfection. The cells were inoculated at 5 × 10⁶ cells/well in 24-well plates for 24 h before transfection. 100 μL of 2.5 M CaCl₂ and 25 μg of plasmid EGFP were adjusted to 1 mL with water; this 2X CaCl₂/DNA mix was added into an equal volume of 2X HEPES-buffered saline, as previously described. The CaP/DNA solution was incubated for 2 min. The solution was immediately added to the medium. A 50 μL of the solution was used for each 500 μL of the medium in a well. After incubation for 4 h at 37 °C and 5% CO₂, each well was washed with 1× PBS and supplemented with 500 μL complete medium for another 42 h before transfection efficiency analysis.

Shock Treatment. After the CaP/DNA precipitate was incubated with the cells for 4 h at 37 °C and 5% CO₂, the medium was removed and each well was washed with 1× PBS. The shock treatment was performed at this point. For hypotonic treatment, the cells of each well were exposed to 200 μL of glycerol (15 or 5%, v/v) or NaCl (6 or 2%, w/v) in 1× PBS for 2 min. For hypertonic treatment, cells were exposed to 200 μL of DMEM medium diluted with water for 2 min. Each well was washed with 1× PBS and supplemented with 500 μL complete medium for another 42 h before transfection efficacy analysis. In this study, decompression treatment was defined as the process from hypertonicity to isotonicity (or hypotonicity).

Transfection Efficacy Analysis. The expression of EGFP protein was observed by fluorescent microscopy (Zeiss, Germany) with a 10x objective. The percentage of EGFP-positive cells and mean fluorescence intensity was quantitatively measured by flow cytometry assay (BD, USA).

Effect of Glycerol Shock Treatment on Endo-Lysosomes. To observe the effect of shock treatment on the intracellular delivery, Hela cells were seeded in 15 mm glass-bottom cell culture dishes (NEST, China) at a density of 2 x 10⁴ cells/well for 24 h. DNA was labeled with YOYO-1 as described previously, and the CaP/DNA precipitate formed, as mentioned above. After cells were incubated with the CaP/DNA precipitate for 4 h, endo-lysosomes and cell nuclei were stained with LysoTracker Red and Hoechst 33342, respectively. The cells were treated with glycerol or NaCl shock treatment. The dishes were observed by confocal laser scanning microscopy (CLSM, Zeiss, Germany) with a 40x objective for changes of the intracellular distribution before and after the shock treatment.

Endo-Lysosomal Leakage Assay. Hela-mAG-Gal3 and 5637-mAG-Gal3 cells were seeded on cell culture dishes for 24 h, respectively. Then, endo-lysosomes were stained with LysoTracker Red according to the standard protocol. The changes of endo-lysosomes were observed by fluorescent microscopy or CLSM before and after shock treatment.

Cytotoxicity Analysis. The cytotoxicity of shock treatment or decompression treatment was measured by CCK-8 assay. Hela cells were seeded in 24-well plates at a density of 5 x 10⁴ cells/well for 24 h. Hela cells were treated with shock treatment in CaP transfection, as described above. CaP transfection without shock treatment was used as the control. The CCK-8 assay was performed at 48 h post-transfection according to well-established protocols. The data were given as mean ± standard deviation based on triplicate repeats.

Chitosan/DNA Delivery. The cells were seeded in 24-well plates as mentioned above. The chitosan solution (200 μg/mL in 5 mM sodium acetate buffer, pH 5.5) was prepared as previously described. The transfection media for chitosan (DMEM, 5 mM MES, pH 6.5) was prepared for further use. An equal volume of the plasmid EGFP solution (100 μg/mL in water) and the chitosan solution were mixed and vortexed for 30 s. Then, chitosan/DNA polyplexes were incubated for 20 min at 25 °C. The medium in each well was replaced with 500 μL of the transfection medium, and chitosan/DNA polyplexes were added into each well at the dose of 1.5 μg DNA/well. After incubation for 4 h at 37 °C and 5% CO₂, the shock treatment was performed as described above. Then, the cells were washed with 1× PBS and replaced in complete medium for another 42 h before transfection efficacy analysis.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04771.

History of glycerol shock treatment in CaP transfection; cellular uptake of YOYO-1-labeled DNA in Hela cells
with or without glycerol shock treatment; flow cytometry analysis for CaP transfection combined with hypertonic shock treatment in Hela cells; and fluorescence images of Hela-mAG-Gal3 cells treated with a 15% glycerol shock or siramesine treatment (PDF)

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**Notes**

The authors declare no competing financial interest.

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