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Mycoplasma contamination-mediated attenuation of plasmid DNA transfection efficiency is augmented via L-arginine deprivation in HEK-293 cells*#

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Mycoplasma infection is the most prevalent contamination in cell culture. Analysis of cell culture in laboratories from different countries shows that mycoplasma contamination ranges from 15% to 80% and, in some cases, even reaches 100% (Chernov et al., 2014). Whilst mycoplasma infection is not visible to the naked eye in cell culture, the consequences of mycoplasma contamination have been shown to induce a number of cellular changes, for example, increased resistance to chemotherapeutic drugs. Therefore, any results obtained from tissue culture studies, in the presence of mycoplasma contamination, potentially render the data invalid (Kim et al., 2015; Gedye et al., 2016). As such, mycoplasmas are not harmless bystanders and cannot be ignored in in vitro studies.

Human embryonic kidney 293 (HEK-293) cells are frequently used in the laboratory because of their reliable growth and propensity for transfection. They are utilized as an efficient factory to produce viral vectors such as recombinant adeno-associated virus (rAAV) and bioactive proteins following plasmid DNA transfection (Wang et al., 2014; Zhang et al., 2014; Lin et al., 2017; Strobel et al., 2019). Consequently, they are often cultured in biological research laboratories as well as biotechnology companies. However, the effect of mycoplasma contamination on plasmid DNA transfection efficiency has rarely been reported. Hence, the aim of this study is to identify whether mycoplasma contamination has any effect on plasmid DNA transfection efficiency in HEK-293 cells.

In order to detect mycoplasma contamination in HEK-293 cells, 4',6-diamidino-2-phenylindole (DAPI; 1 μg/mL; Sigma, D-8417, Germany) staining was used. As shown in Fig. 1a, the nucleus was stained blue, and abundant blue fluorescence was clearly observed out of nucleus in the cytoplasmic compartment, indicating mycoplasma contamination. Additionally, the presence of mycoplasma genome was assessed by polymerase chain reaction (PCR) using the Mycoplasma Detection Kit (TaKaRa, Japan) in the presence of appropriate controls to eliminate the possibility of false-negative and false-positive results. As illustrated in Fig. 1b, PCR products were present in the positive control lanes with a size of 810 bp in the first run and 590 bp in the second run. The absence of bands in the negative control (H_2O) indicated the accuracy of this experiment. PCR analysis of mycoplasma-contaminated cells led to the generation of PCR products ranging in size from 400 to 700 bp in the first run and from 200 to 300 bp in the second run.

Several methods have been employed to eliminate mycoplasma including physical (e.g., autoclaving), chemical (e.g., treating with detergent), immunological

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(e.g., in vivo passage of cells in nude mice), and chemotherapeutical (e.g., antibiotic) procedures. In this study, antibiotic treatment was used, which is the simplest and the most effective approach (Uphoff and Drexler, 2014). Plasmocin™ has been frequently utilized by researchers due to its outstanding performance in eliminating mycoplasma (Uphoff et al., 2012; Boslett et al., 2014). Therefore, we applied Plasmocin™ to HEK-293 cells to eliminate the mycoplasma contamination. Following the administration of Plasmocin™ (25 μg/mL; InvivoGen, France) for two weeks, the extra-nuclear punctate pattern of blue fluorescence in HEK-293 could no longer be observed (Fig. 1a). Additionally, mycoplasma-specific PCR products were barely detectable in samples generated from Plasmocin™-treated cells (Fig. 1b), confirming the successful elimination of mycoplasma in these cells.

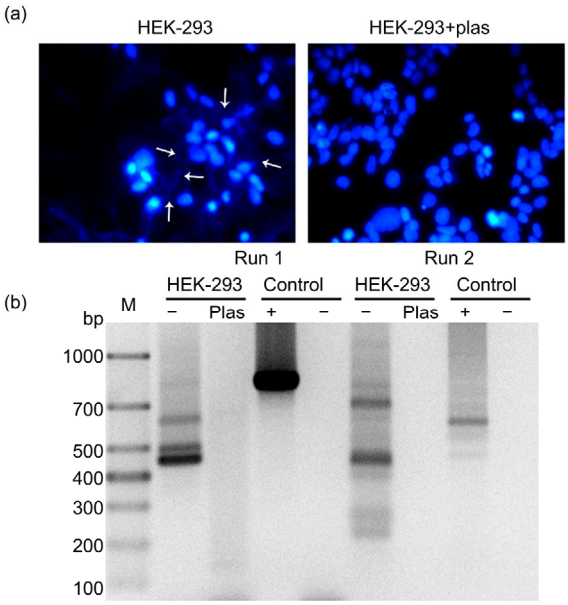

Fig. 1 Detection of mycoplasma contamination and its eradication by Plasmocin™ in HEK-293 cells

The original HEK-293 cells, as well as HEK-293 cells treated with anti-mycoplasma treatment, Plasmocin™, for two weeks, were analyzed for mycoplasma contamination by DAPI staining (a) and PCR (b). Plas, Plasmocin™; M: mark

To determine the effect of mycoplasma contamination on plasmid DNA transfection efficiency, three plasmids (pdsAAV-CBAp-EGFP, pssAAV-CBAp-Fluc-EYFP, and pdsAAV-CBAp-Gluc) that are frequently used in packaging rAAV vectors were tested. These plasmids were added to mycoplasma-contaminated and Plasmocin™-treated (confirmed as mycoplasma-free) HEK-293 cells using the standard protocol for the common transfection reagent, polyethylenimine (PEI). Transfection efficiency was initially assessed by fluorescence microscopy. Fig. 2a showed that the number of visible green dots was lower, and the intensity of green fluorescence in mycoplasma-infected cells was apparently weaker than that in mycoplasma-eradicated cells. Further analysis indicated that the area of green fluorescence in mycoplasma-contaminated cells was approximately 20% of that observed in mycoplasma-free cells (Fig. 2b). Consistent with the findings with the enhanced green fluorescent protein (EGFP)-expressing plasmid, the levels of the two luciferases (firefly luciferase (Fluc) and Gaussia luciferase (Gluc)) were significantly decreased in mycoplasma-infected cells, with only 6% Fluc and 5% Gluc of those observed in mycoplasma-eradicated HEK-293 cells (Figs. 2c and 2d). Therefore, the protein expression post-transfection in mycoplasma-contaminated cells was all dramatically lower than that in mycoplasma-eradicated cells. These data suggest that the attenuated transfection efficiency of plasmid DNA can be attributed to mycoplasma infection in HEK-293 cells.

L-Arginine deprivation is a proven consequence of mycoplasma contamination in tissue culture cells. The microbial enzyme, arginine deiminase (ADI), is abundant in mycoplasma and has a high affinity for L-arginine and catalyzes L-arginine to citrulline (Riess et al., 2018). Because of this particular characteristic of mycoplasma, L-arginine consumption and citrulline accumulation has been confirmed as an indicator of mycoplasma infection in cultured cells in vitro (Capiaumont et al., 1995). Therefore, we collected the cell medium and lysate from mycoplasma-contaminated and mycoplasma-eradicated cells and measured the concentrations of L-arginine and citrulline by high-performance liquid chromatography (HPLC) (Fig. 3a). The results showed that in mycoplasma-infected HEK-293 cells, the L-arginine levels in cell medium and lysate fell significantly by approximately 30% (Fig. 3b). Conversely, the citrulline in cell medium and lysate was remarkably increased by 1- and 2-fold, respectively (Fig. 3c).

Next, we evaluated whether L-arginine supplementation in mycoplasma-contaminated HEK-293 cells

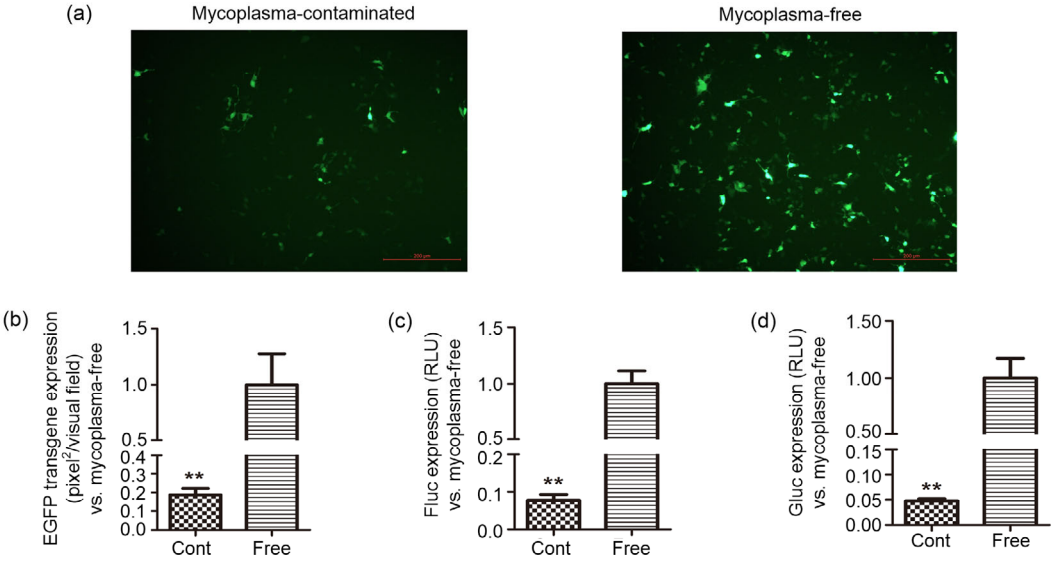

Fig. 2 Effect of mycoplasma contamination on the transfection efficiency of plasmid DNA

Mycoplasma-contaminated and mycoplasma-free HEK-293 cells were seeded, followed by transfection with the plasmids pdsAAV-CBAp-EGFP, pssAAV-CBAp-Fluc-EYFP, and pdsAAV-CBAp-Gluc encoding different reporter genes. Twenty-four hours post-transfection the efficiency of expression of these genes was analyzed. For pdsAAV-CBAp-EGFP, cells were observed under the fluorescence microscope (scale bar=200 μm) (a), and the pixels of green fluorescence were calculated (b). The levels of Fluc (c) and Gluc (d) were measured by a spectral scanning reader after adding their specific substrates. Data are expressed as mean±standard deviation (n=3). $^{**}P$<0.01, vs. mycoplasma-free cells. RLU, relative luciferase unit; Cont, contaminated

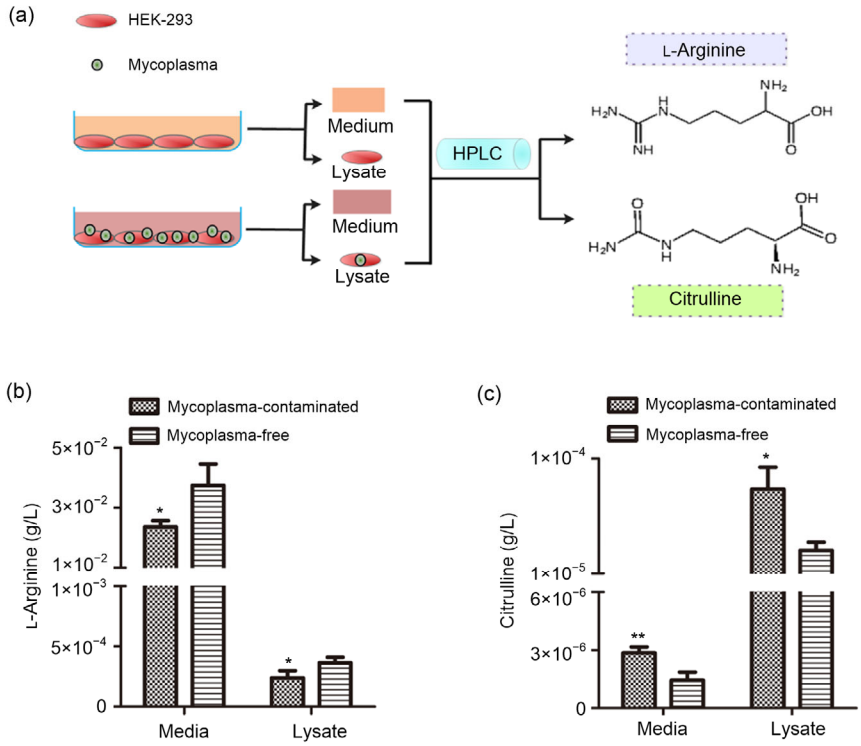

Fig. 3 Identification of L-arginine consumption and citrulline accumulation in mycoplasma-contaminated HEK-293 cells

Schematic representation (a) of measuring the concentrations of L-arginine (b) and citrulline (c) in both cell media and lysates from mycoplasma-contaminated and mycoplasma-free HEK-293 cells. Data are expressed as mean±standard deviation (n=3). $^{*}P$<0.05, $^{**}P$<0.01, vs. mycoplasma-free cells

could improve the transfection efficiency. To this end, we added L-arginine (24 h pre- and post-transfection) at concentrations ranging from 0.1 to 1.0 g/L, which showed no toxicity on cell viability (Fig. S1). As shown in Fig. 4a, when mycoplasma-contaminated cells were transfected with a plasmid driving the expression of EGFP, more green dots were observed in L-arginine-treated cells. Additionally, L-arginine (1.0 g/L) significantly increased the green fluorescence by almost one-fold (Fig. 4b). Consistent with the results obtained with plasmid pdsAAV-CBAp-EGFP, when mycoplasma-positive cells were transfected with plasmids driving the expression of luciferase, the Fluc (Fig. 4c) and Gluc (Fig. 4d) levels in the 1.0 g/L L-arginine-treated cells were slightly augmented by 30% and 60%, respectively. When mycoplasma-contaminated HEK-293 cells were treated with 1.0 g/L L-arginine, which is almost 10-fold higher than that in conventional Dulbecco's modified Eagle medium (DMEM; 84 mg/L), the transgene expression was elevated by less than one-fold. These data, therefore, suggest that L-arginine supplementation was less effective than Plasmocin™ to enhance transfection efficiency in mycoplasma-infected cells.

Collectively, our results highlight that mycoplasma, a notorious cell culture contaminant, can remarkably diminish plasmid DNA transfection efficiency in HEK-293 cells. Mycoplasma contamination, as well as the resulting poor plasmid DNA transfection efficiency, can be effectively reversed by Plasmocin™, a commercially available anti-mycoplasma reagent. This catastrophic effect of poor transfection efficiency is related to L-arginine deprivation in mycoplasma-contaminated cells, but L-arginine supplementation is not a valid strategy to boost transfection efficiency (when measured by transgene expression). Thus, for researchers who utilize HEK-293 cells to produce therapeutic proteins and viruses based on plasmid

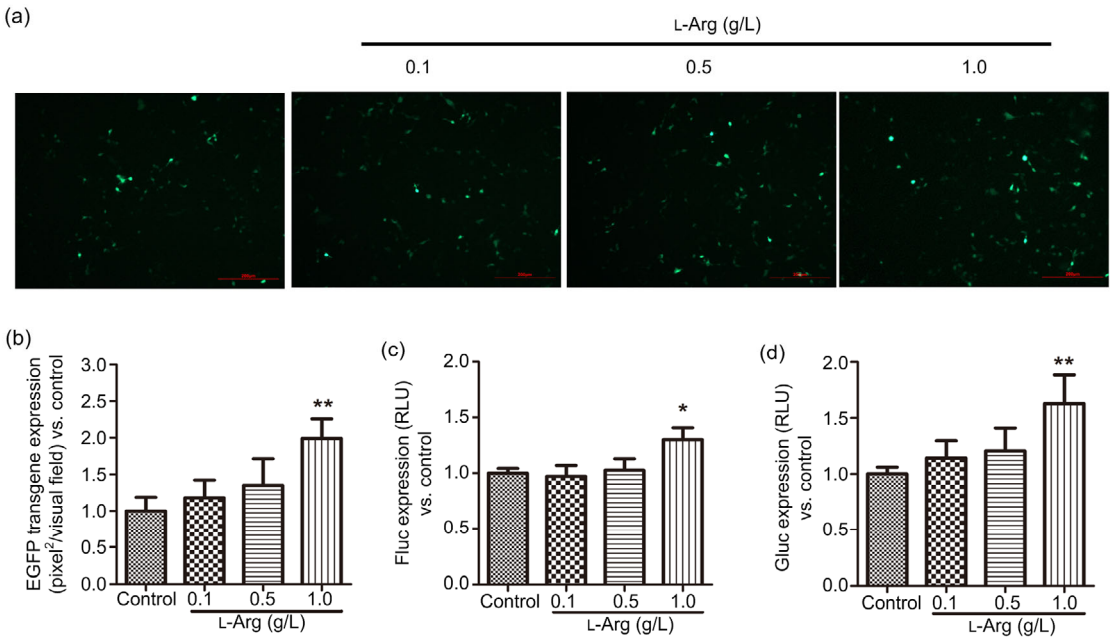

Fig. 4 Effect of L-arginine supplementation on plasmid DNA transfection efficiency in mycoplasma-contaminated HEK-293 cells

Mycoplasma-contaminated HEK-293 cells were transfected with plasmids pdsAAV-CBAp-EGFP, pssAAV-CBAp-Fluc-EYFP, and pdsAAV-CBAp-Gluc. Twenty-four hours pre- and post-transfection, cells were administrated with L-arginine (0.1, 0.5, and 1.0 g/L). The transfection efficiencies of these reporter genes were analyzed 24 h post-transfection by measuring the transgene expression. For pdsAAV-CBAp-EGFP, cells were observed under the fluorescence microscope (scale bar=200 μm) (a) and the pixels of green fluorescence were calculated (b). The levels of Fluc (c) and Gluc (d) were measured using a spectral scanning reader. Data are expressed as mean±standard deviation (n=3). * P<0.05, ** P<0.01, vs. control cells. L-Arg, L-arginine; RLU, relative luciferase unit

DNA transfection, we highly recommend the regular and necessary monitoring for the presence of mycoplasma, as well as the prompt and adequate elimination by anti-mycoplasma treatment if necessary, in order to sustain stable and efficient plasmid DNA transfection efficiency.

Contributors

Zi-fei YIN, Ya-ni ZHANG, Juan DU, and Bin-bin CHENG designed this study. Zi-fei YIN and Ya-ni ZHANG performed the experiments. Shu-fang LIANG and Sha-sha ZHAO contributed to data analysis. Zi-fei YIN wrote the manuscript, while Juan DU and Bin-bin CHENG edited the manuscript. All authors read and approved the final manuscript. Therefore, all authors had full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Zi-fei YIN, Ya-ni ZHANG, Shu-fang LIANG, Sha-sha ZHAO, Juan DU, and Bin-bin CHENG declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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List of electronic supplementary materials

Fig. S1 Cytotoxicity of L-arginine in HEK-293 cells

中文概要

题　目：支原体污染通过耗竭 L-精氨酸降低 HEK-293 细胞中质粒 DNA 转染效率

目　的：明确支原体污染对 HEK-293 细胞质粒 DNA 转染效率的影响，并从支原体对细胞精氨酸代谢的角度探究其机制。

创新点：支原体是细胞培养中的常见污染源。HEK-293 是目前常用的生产蛋白、包装病毒的常用细胞系。然而，目前支原体污染对于质粒 DNA 转染效率的影响未见报道。本研究首次报道支原体污染对 HEK-293 细胞质粒 DNA 转染效率的影响，并揭示其机理。

方　法：采用 4',6-二脒基-2-苯基吲哚（DAPI）和聚合酶

链反应（PCR）方法鉴定 HEK-293 细胞中的支原体污染情况以及支原体抗生素 Plasmocin 对支原体污染的清除效果。通过聚乙烯亚胺（PEI）方法对支原体污染的 HEK-293 细胞及支原体清除后的 HEK-293 细胞转染质粒，比较转染效率差异。通过高效液相色谱法（HPLC）分析支原体污染的和支原体清除后的 HEK-293 细胞的细胞裂解产物和细胞上清中的 L-精氨酸、瓜氨酸含量变化。在支原体污染的 HEK-293 细胞中补充 L-精氨酸，观察质粒转染效率的改变情况。

结　论：支原体污染能大大降低 HEK-293 细胞中质粒 DNA 的转染效率，且其原因与支原体能耗竭细胞中的 L-精氨酸有关。

关键词：支原体；质粒；转染效率；精氨酸；HEK-293 细胞