Journal of Pharmaceutical Research International

32(35): 13-21, 2020; Article no.JPRI.63507
ISSN: 2456-9119
(Past name: British Journal of Pharmaceutical Research, Past ISSN: 2231-2919, NLM ID: 101631759)

An Efficient and Inexpensive PEI-mediated Transfection for Transient Overexpression of CYP2C9 in WRL 68 Normal Liver Cells

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Authors' contributions

This work was carried out in collaboration among all authors. Author NAL designed the study. Authors NAL and HH provided supervision as grant holders. Author AAD conducted part of data collection and wrote the protocol. Author NAM conducted part of data collection, performed data and statistical analysis, managed literature searches and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2020/v32i3530975
Editor(s):
(1) Dr. Sawadogo Wamtinga Richard, Ministry of Higher Education, Scientific Research and Innovation, Burkina Faso.
Reviewers:
(1) E. M. Sutrisna, Universitas Muhammadiyah Surakarta, Indonesia.
(2) Paolina Crocco, University of Calabria, Italy.
Complete Peer review History: http://www.sdiarticle4.com/review-history/63507

Original Research Article

Received 28 September 2020
Accepted 03 December 2020
Published 16 December 2020

ABSTRACT

Aims: This study aims to develop an efficient, and inexpensive transfection procedure using low cost polyethylenimine (PEI) as transfection reagent for overexpression of CYP2C9 in WRL 68 host cells.
Introduction: Overexpression of CYP450 isozymes such as CYP2C9 has been a reliable approach in developing a high specificity *in vitro* tool for inhibition study of this liver enzyme. Transient or stable transfection are used to insert plasmid DNA into a mammalian host cell in order to produce proteins. There are many efficient transfection reagents available in the market for this purpose, however, it can be expensive, and a low-cost alternative is favorable.
Methodology: Cytotoxicity of PEI was screened on four cell lines namely WRL 68, HepG2, MCF-7 and A549 using MTS assay. WRL 68 was transiently transfected with CYP2C9 plasmid using PEI

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and DNA concentration as well as transfection time was optimized for the best efficiency and expression. Expression of CYP2C9 protein was measured using qPCR and further evaluated with western blot analysis.

Results: IC50 values of PEI are 0.327 ± 0.013 mg/mL (WRL-68), 0.395 ± 0.037 mg/mL (HepG2), 1.159 ± 0.032 mg/mL (A549) and 1.281 ± 0.000 mg/mL (MCF-7). Combination of 3ug plasmid DNA with 2.8 µM of PEI and 10.5 mM NaCl resulted in the highest transfection efficiency and expression after 48 hours.

Conclusion: A low-cost and efficient PEI transient transfection procedure was optimized for CYP2C9 overexpression and useful for the purpose of further development of cell-based enzyme inhibition model.

Keywords: Polyethylenimine; transfection; WRL 68; CYP2C9; overexpression.

1. INTRODUCTION

In the past recent years, overexpression of CYP450 isozymes in mammalian cells has been a go-to approach in developing in vitro model for the purpose of studying the enzyme's inhibition or metabolism. CYP2C9 is one of the most important CYP450 isozymes contributing to the biotransformation of approximately 15% of drugs that undergo CYP450-mediated metabolism [1]. Genetic engineering of cell line enhances CYP expression and previous successful generation of a genetically modified HepG2 cells with CYP3A4 overexpression has demonstrated an appreciable and specific isozyme activity [2]. Another recent study has reported that by optimizing transfection and supplementing conditions, a heterologous expression system using 293FT cells was successfully developed for evaluation of three CYP isoforms (CYP1A2, CYP2C9, and CYP3A4) enzymatic activities [3].

Non-viral gene delivery methods such as transfection is the keystone of modern gene delivery. Transfection is a technique that incorporates foreign nucleic acids into host cells to produce either stably or transiently genetically modified cells [4]. Chemical-based transfection methods make use of synthetic or natural carriers to deliver genes into cells such as polymers, liposomes, dendrimers, and cationic lipid systems [5]. Despite of having various options of commercially available transfection reagents, current products are quite expensive and high cost has become an obstacle to conduct experimentation involving a lot of transfection procedure, especially in a large scale [6].

Polyethylenimine (PEI) is an inexpensive, non-viral as well as non-liposomal reagent with a high cationic charge density potential which efficiently condenses DNA to form stable complexes known as polyplexes that promotes gene delivery into cells [7]. The polymer constructed from repeating units of ethylamine in linear or branched forms with molecular weights ranging from 700 Da to 1000 KDa and is highly cationic due to of the presence of a protonable amino nitrogen at every third atom of the polymeric backbone [8]. Electrostatic interactions between the positively charged PEI and the negatively charged phosphate groups of DNA allows PEI to condense the DNA in solution forming PEI-DNA complexes with positive surface charges that interact with the negatively charged cell membrane promoting endocytosis by the cell [9].

Optimization of transfection efficiency is the most crucial step in achieving successful gene delivery because efficiency affected by many factors, including concentrations of reagent and DNA, DNA purity, transfection media, incubation time, cell type, as well as cell toxicity and viability [10,11]. Without proper optimization, non-viral transfection methods usually resulted in less efficient gene transfer, low levels of gene expression or reduced cell viability [12]. Therefore, this study aims to optimize an efficient PEI-mediated transfection procedure in order to achieve an optimal transient overexpression of CYP2C9 enzyme in WRL-68 cell line for future development of an *in vitro* metabolism-based cell model.

2. MATERIALS AND METHODS

2.1 Cell Culture

A549 lung cancer cell line was purchased from ATCC (ATCC® CCL-185™), while WRL 68, MCF7 and HepG2 cell lines were obtained from Pharmacology-Toxicology Laboratory at Faculty of Pharmacy, Universiti Teknologi MARA (Puncak Alam, Selangor, Malaysia). All cell lines were cultured in Dulbecco's modified Eagle

medium (DMEM; Gibco), supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin antibiotic (Invitrogen). The cells were maintained in an incubator at 37°C and 5% CO^2.

2.2 MTS Assay

A serial dilution of PEI solutions was prepared at 50, 10, 2, 0.4, 0.08 and 0.016mg/mL by diluting the stock solution with culture medium. Cells were seeded at 5000 cells/well in a 96-well plate, then incubated for 24 hours. Each cell lines were treated with various concentration of PEI solution for 24 hours, with untreated cells act as negative control. After treatment incubation, 20uL of MTS solution (Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay) were added into each well and incubated for an hour, then the microplates were read at 490nm using spectrophotometer.

2.3 Transfection

$1x10^5$, $2x10^5$ and $3x10^5$ cells seeded in each well of 6 well plate. After 24 hours incubation in humidified atmosphere of 95% air plus 5% CO^2 in a 37°C incubator, media was replaced with DMEM without serum. Mixture of 2.8 µM final concentration of PEI (Polysciences, USA), different concentrations of NaCl (0.00 mM, 5.25 mM and 10.50 mM) and various concentrations (1 µg, 2 µg and 3µg) of cDNA containing CYP2C9 gene, pCMV-CYP2C9-gfpsparks or control vector, pCMV-C-gfpsparks, (Sinobiological, China) was incubated at room temperature for 10 mins before added into well. The plate was incubated for 4 hours before the media replaced with DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. Transfection efficiency was observed at 24, 48 and 72 hours post-transfection. Transfection using commercial jetPRIME® transfection reagent (Polyplus-transfection, France) was done according to manufacturer's protocol. All fluorescence images was analyzed using Image J software to measure the mean fluorescence intensity which is the mean grey value; the average grey value for all pixels within the indicated area [13,14].

2.4 Western Blot Analysis

Western blot analysis was conducted using previously described protocol with modifications [15]. Cell lysates total protein were measured using nanodrop A280. After sample normalization and loading, SDS-PAGE was done for 120 mins at 120 V. The proteins then transferred onto 0.2 µm PVDF membranes (Merck, USA) using wet transfer technique. The blots were blocked using Tris-buffered saline (TBS) containing 0.01% Tween 20 and 5% BSA at room temperature for 1 hour. The blots then incubated with the primary antibodies (1:1000 dilution) of CYP2C9 or β-actin (loading control) for 1 h at room temperature. Then, the blots were washed with TBS containing 0.01% Tween 20 followed by incubation for 1 hour with horse radish–peroxidase-conjugated secondary antibodies (1:10000 dilutions). After further washing with TBS containing 0.01% Tween 20, blots were incubated in chemiluminescence reagents (Azure Biosystems, USA). Bands intensities were detected using Molecular Imaging System (Amershams, USA).

2.5 qRT-PCR

According to manufacturer's protocol, mRNA was extracted from transfected cell using NucleoSpin® RNA Kit (Macherey-Nagel, USA). Template DNA was produced from mRNA using ReverTra Ace® qPCR RT Kit (Toyobo, Japan). qRT-PCR reactions were performed using THUNDERBIRD® SYBR® qPCR Mix (Toyobo, Japan). In a total volume of 20 µL, each reaction contained 5 µL template DNA, 10 µL sterile ddH2O, 0.3 µM final concentration of each forward and reverse primer, 2 µL THUNDERBIRD® SYBR® qPCR Mix and 0.4 µL 50x ROX reference dye. The temperature profile includes the following steps: pre-denaturation (1 min at 95°C), 40 cycles of 15 sec at 95°C and 1 min at 60°C. Two housekeeping control genes (β-actin and GAPDH) were included for each run [16]. Primers sequence were as follows: CYP2C9 fwd: 5'-TCCTATCATTGATTACTTCCCG-3', CYP2C9 rev: 5'-AACTGCAGTGTTTTCCAAGC-3', BACTIN fwd: 5'-CACCATTG GCAATGAGCGGTTC-3', BACTIN rev: 5'-AGGTCTTTGCGGATGTCCACGT-3', GAPDH fwd: 5'-GTCTCCTCTGACTTCAACAGCG-3', GAPDH rev: 5'-ACCACCCTGTTGCTGTAGCCAA-3',

3. RESULTS AND DISCUSSION

Linear PEI with molecular weight of 25000 g/mol was used as transfection reagent and the parameters to achieve optimal transfection efficiency and protein expression were optimized. Linear form of PEI is preferred in this study compared to branched PEI, although the latter

has a substantial chemical reactivity and able to form smaller polyplexes with DNA in salt-containing conditions, linear PEI is generally lower in toxicity and more likely resulted in higher transfection efficiency [17]. Cytotoxicity of PEI was screened on several cell lines namely WRL-68 (normal liver), HepG2 (liver cancer), A549 (lung cancer) as well as MCF-7 (breast cancer). Fig. 1 demonstrates IC50 values of PEI on all mentioned cell lines. A549 and MCF-7 cells are both less susceptible to PEI cytotoxicity in comparison to HepG2 and WRL 68. Previous study reported that variability in PEI toxicity seen in different cell lines is possibly due to the difference in physiology of the cells [18]. WRL 68 and HepG2 are both derived from liver exhibiting morphologic structures similar to hepatocytes [19,20]. Both liver derived cell lines are more affected by PEI toxicity possibly due to their comparable physiology. Nevertheless, working concentration of PEI for the purpose of transfection is lesser than these IC50 values, thus, there will be no significant problem in terms of toxicity to transfect any on these cell lines using PEI. WRL 68 however is chosen as the cell host for further optimization of transfection efficiency and overexpression of CYP2C9 due to its optimal liver marker genes expression in our previous preliminary studies.

Cell density, DNA concentration, and salt condition in transfection process are crucial parameters that have been optimized to achieve superior efficiency. Fig. 2 illustrates the effects of variation in cell density, DNA concentration and salt condition on fluorescence intensity that reflects transfection efficiency on WRL 68 cell line. Inoculation of $3x10^5$ cells has achieved around 70% confluency after 18 hours incubation. A previous study suggested that high cell density used during transfection could spike the probability of cell exposure to PEI-DNA polyplexes [21]. However, over-confluent culture may induce contact inhibition that leads to poor uptake of DNA resulting in reduced expression of the gene, yet, too little cell density in the culture may result in poor growth due to lack of cell-to-cell contact [22]. Therefore, it is important to achieve 50-80% confluency of the culture prior to transfection for optimal efficiency [23]. In addition, it is important to perform transfection on actively dividing cells because nuclear translocation of DNA rapidly occurs at this stage due to breakdown of nuclear membrane during cell division [24].

Culture media used in transfection process was made serum-free. This is to avoid interference by serum proteins that may hamper the transfection efficiency [11]. A recent study has reported that the use of commercially available Opti-MEM, a reduced serum media for transfection process has resulted in destabilized protein expression and stressed cells with reduced viability as well as altered morphology [25]. Therefore, in this study, serum-free DMEM media was used and other parameters were optimized to achieve good transfection efficiency and protein expression. This can simplify the materials needed for transfection process as well as reducing the cost. PEI-DNA complexing time was set constant for 10 minutes. Longer incubation lead to bigger polyplex formation and may affect transfection efficiency. A previous study suggested that as the complexes become larger, there will be an increasing difficulty in their cellular uptake during the transfection process contributing to lower efficiency [26]. The final concentration of PEI in transfection medium was kept constant at 2.8 uM when evaluating other parameters at variable concentrations. Evaluation of transfection efficiency was done at 48 hours post-transfection. From the optimization, 3 ug DNA with the presence of final concentration of 10 mM NaCl has resulted in the highest fluorescence intensity or transfection efficiency. This result suggests that the presence of salt contributes to better formation of DNA-PEI complexes, thus, improve transfection efficiency in comparison to salt-free condition. This is supported by a previous study which reported that sodium and chloride ions promote the aggregation of PEI–DNA by an electrostatic process and salt-free condition causes the complexes to become unstable leading to low transfection efficiency in vitro [27].

CYP2C9 protein expression was evaluated by using qPCR and western blot analysis at 24, 48 and 72 hours post-transfection. As shown in Fig. 3, fluorescence intensity indicating the transfection efficiency is the highest at 48 hours post-transfection. The optimum transfection efficiency has translated into the highest protein expression as illustrated in Fig. 4. The protein expression was low at 24 hours, peak at 48 hours and reduced at 72 hours post-transfection. This finding is consistent with a recent study which reported that 48 hours incubation period post-transfection was necessary to obtain saturated CYP isozymes expression levels [3]. Fig. 4 illustrates the morphology of the cells at 48 hours post-transfection. Transfected cells have

shown no alteration in morphology in comparison to control cell without transfection. This indicates that the cells were not in stressed condition and not adversely affected in terms of viability. The cobblestone-like morphology and doubling time of WRL 68 cells remained intact after transfection using the optimized parameters. There is no transfection-mediated toxicity was observed such as shrunken cells or other substantial morphological alteration [28].This is important so that the cells are healthy and favor protein expressions of the delivered gene.

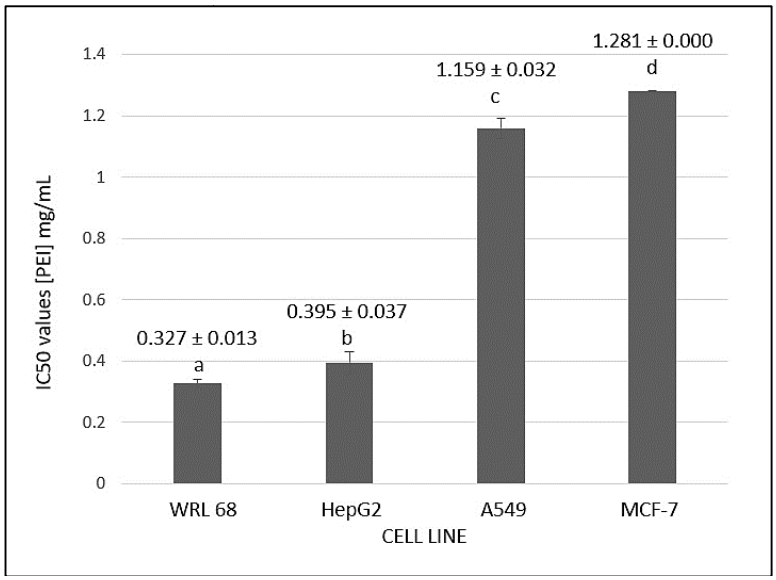

Fig. 1. IC50 values of PEI (mg/mL) on WRL-68 (normal liver), HepG2 (liver cancer), A549 (lung cancer) and MCF-7 (breast cancer) cell lines. Bars with different letter denotes significant difference between the values (P<0.05, one-way ANOVA post-hoc Tukey)

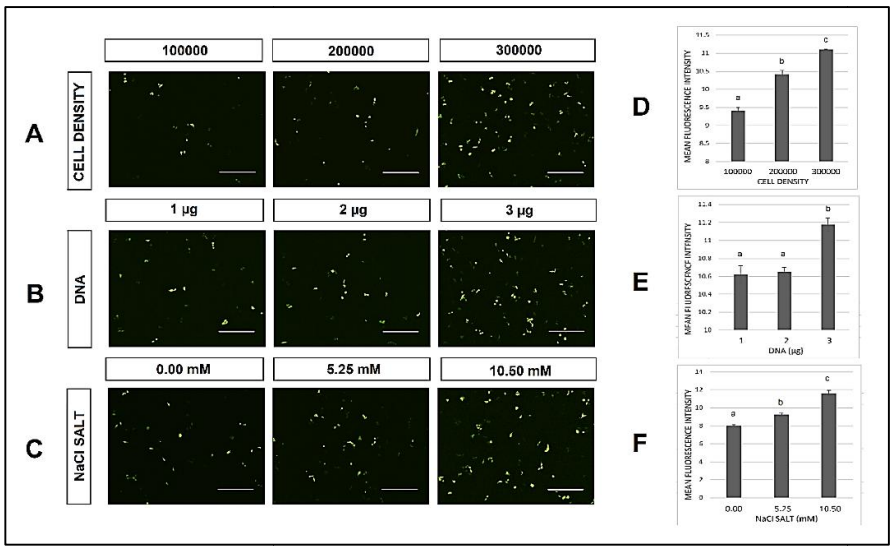

Fig. 2. Fluorescence microscopy 48 hours post-transfection of different parameters optimization (A) cell density, (B) DNA concentration, (C) NaCl salt concentration. Scale bar: 500 µm, magnification 40X. Mean fluorescence intensity of transfection parameters (D) cell density, (E) DNA concentration, (F) NaCl salt concentration. Bars with different letter denotes significant difference between the values (P<0.05, one-way ANOVA post-hoc Tukey)

Transfection efficiency of PEI was compared to a commercial transfection reagent, jet PRIME®. As illustrated in Fig. 6, jet PRIME® has resulted in higher transfection efficiency in comparison to PEI. However, transfection-mediated toxicity was observed at 24 hours post-transfection using jetPRIME®. High number of cells shrunken and detached after transfection using jet PRIME®

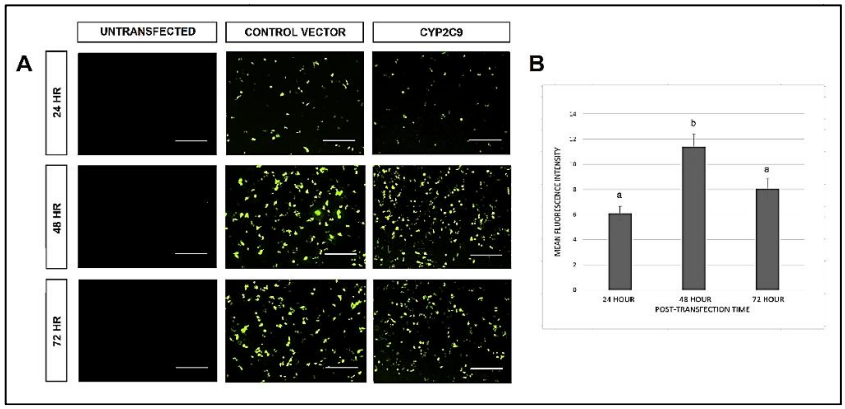

Fig. 3. (A) Fluorescence microscopy of WRL 68 cells untransfected, transfected with control vector and transfected with CYP2C9 cDNA at different time points (24, 48 and 72 hours) post-transfection. Expression of GFP allowed visualisation of transfection efficiency following different incubation periods. Scale bar: 500 µm, magnification 40X. (B) Mean fluorescence intensity of CYP2C9 transfected cells at different time points (24, 48 and 72 hours) post-transfection. Bars with different letter denotes significant difference between the values (P<0.05, one-way ANOVA post-hoc Tukey)

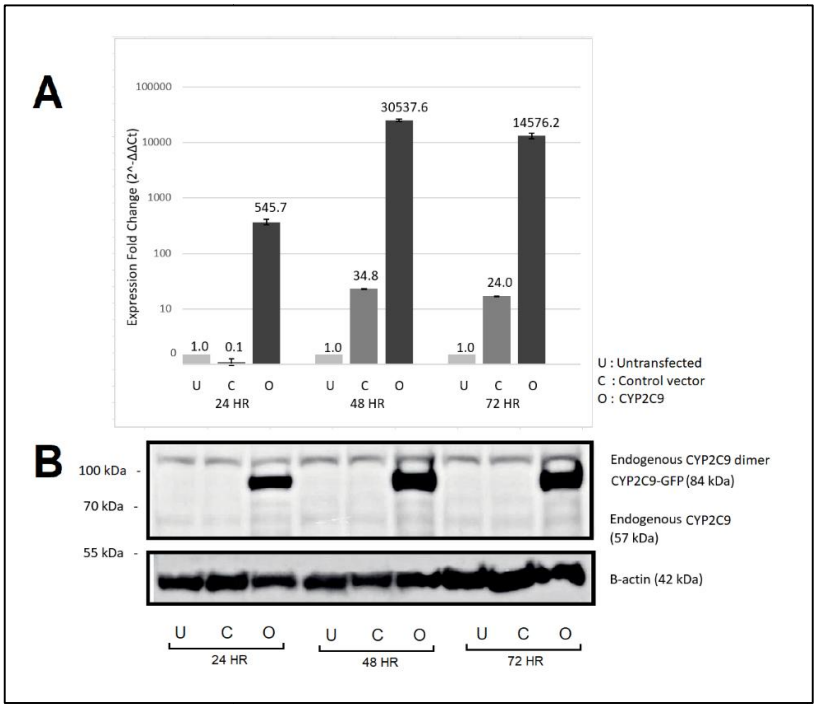

Fig. 4. (A) Real-time RT-PCR and (B) western blot analyses for endogenous and exogenous CYP2C9 expression after different post-transfection periods (24, 48 and 72 hours). Consistent with the mRNA changes, transfection time-dependently upregulated exogenous CYP2C9 protein expression and is at optimum after 48 hours. β-actin was used as loading control

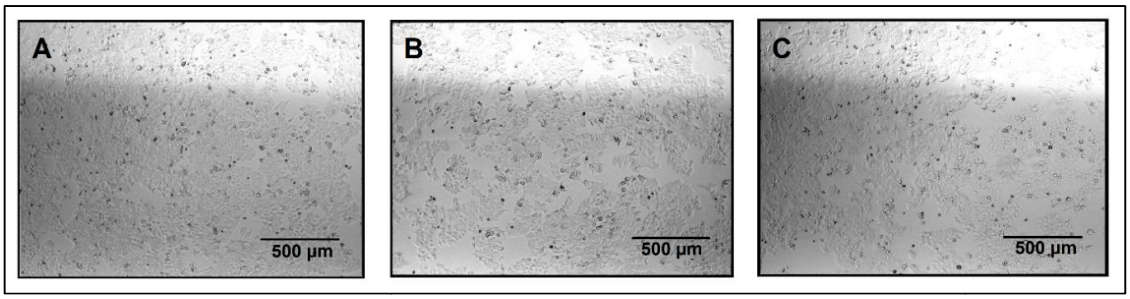

Fig. 5. Morphology of cells (B) transfected with control vector and (C) transfected with CYP2C9 cDNA 48 hours post-transfection are not different from (A) untransfected WRL-68. 40X magnification

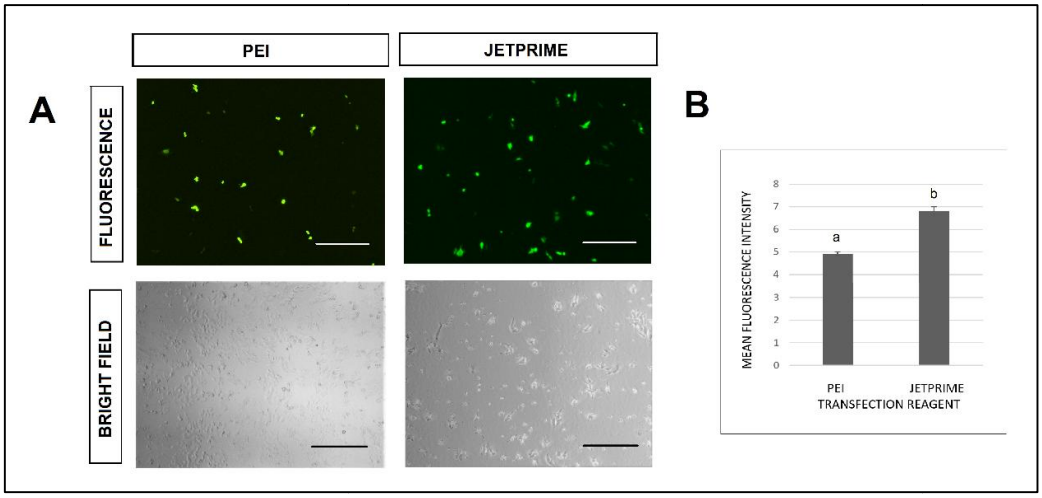

Fig. 6. (A) Fluorescence and bright field microscopy of 24 hours post-transfection using PEI and commercial jetPRIME® transfection reagent. (B) Mean fluorescence intensity of CYP2C9 transfected WRL cells using PEI and commercial jetPRIME® transfection reagent. Scale bar: 500 µm, magnification 40X. Bars with different letter denotes significant difference between the values (P<0.05, T-test)

indicating pronounced toxicity towards WRL 68 cell line as shown in the figure. Although jetPRIME® was proven to have higher transfection efficiency, the efficiency of lower cost PEI is sufficient in overexpressing CYP2C9 without significant toxicity for the purpose of further development of an *in vitro* metabolism-based cell model. The use of commercial jet PRIME® transfection reagent would be beneficial for the purpose of purification of the overexpressed enzyme or microsome preparation for downstream application such as demonstrated in previous study [3], where toxicity does not affect the cell-free enzymatic assay. In contrast, significant transfection-mediated toxicity can adversely affect experimental results of cell-based assay.

4. CONCLUSION

An efficient and inexpensive PEI-mediated transfection parameters and conditions were optimized for overexpression of CYP2C9 in WRL 68 cell host. This optimized transfection procedure requires no commercially available transfection reagent or special culture media other than DMEM, thus, it is low cost and suitable for routine or large-scale transfection experiments. Overexpression of CYP2C9 in WRL 68 cells is successfully achieved with this optimized procedure and potentially to be further developed into an in vitro model for the purpose of studying the enzyme's inhibition or metabolism.

ACKNOWLEDGEMENTS

The authors acknowledge the funder of this research, RMC UiTM, grant no. (600-IRMI/DANA KCM 5/3/LESTARI (190/2017) and grant no. 600-IRMI/FRGS 5/3/ (065/2019).

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/63507