Evaluation of different transfection methodologies to achieve efficient expression of the NS1 dengue protein in HepG2 cells

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HepG2, a human hepatocarcinoma cell line, has been used as a model to study infection by several pathogens including dengue virus. However, this cell line is notoriously difficult to be transfected with plasmid DNAs by traditional methods, which is a limitation for some studies involving heterologous gene expression. In the present work, we analyzed different protocols for transfection of HepG2 with the plasmid pcENS1, which encodes the dengue NS1 protein, in order to evaluate the best methodology for achieving high cell viability and transfection efficiency. We analyzed two transfection approaches using lipid-based methods (Lipofectamine and FuGENE 6) or electroporation by nucleofection. Expression of the recombinant NS1 protein was evaluated by immunofluorescence and flow cytometry. Transfection with either of the two lipid-based methods led to very low number of HepG2 cells expressing NS1 (3.9% and 6.8% with Lipofectamine and FuGene, respectively) and high cell death rates. On the other hand, the efficiency of cell transfection was remarkable higher with nucleofection when compared to these other methods, achieving 63% of cells expressing NS1 protein and more than 60% of viability in the optimized condition.

Key words: HepG2, Transfection, Nucleofection, Lipid-based methods, NS1, dengue

INTRODUCTION

High efficiency of DNA delivery and heterologous protein expression are critical factors for molecular and cell biology studies, as for example functional analyses of proteins, their localization and trafficking. In order to introduce DNA into mammalian cell, several methods of in vitro transfection are available, such as cationic liposomal reagents (Balazs and Godbey 2011), solid nanomaterials (Unciti-Broceta et al. 2012; Dréan et al. 2017), electroporation (Brunner et al. 2002) and nucleofection (Kraus et al. 2010). These techniques aim to overcome the barriers that exist within cells for introduction of a heterologous DNA, such as plasma and nuclear membranes, as well as to allow DNA escape from endosomes. Moreover, the DNA has a polyanionic characteristic and high molecular weight, which hampers passing through cell membranes (Luo and Saltzman 2000). In addition, transfection methods may be cytotoxic with variable rates.

Cationic liposomes are widely used to facilitate DNA transfection because they complex to DNA, neutralizing its charge (Susa, et al. 2008). Alternatively, electroporation destabilizes cell membranes with high intensity electrical pulses, and is considered a simple and efficient technique for introduction of exogenous genes.
Evaluation of different transfection methodologies to achieve efficient expression of the NS1 dengue protein in HepG2 cells • Costa SM et al.

Nucleofection, a modified electroporation technique, is performed with transfection solutions specific for each cell, consisting of a combination of modular protein complexes, which allows membrane association, translocation, endosomal release and the transport of the DNA to the nucleus (Hamm et al. 2002; Nakayama et al. 2007; Schimdt et al. 2008).

Some cell lines are known to be difficult to transfect, such as neuronal lines or primary cells (Hamm et al. 2002; Morris and Labhasetwar 2015). HepG2, a liver hepatocellular carcinoma cell line, is also particularly refractory to transfection. This cell line has been used for studies with dengue virus as an in vitro model for infection of hepatic cells.

Dengue is considered one of the most important human arbovirus diseases due to its morbidity and mortality in tropical and subtropical regions of the world (Bhatt et al. 2013). The disease has different manifestations and hepatic dysfunction is frequent. Liver damage was observed in histopathological analysis of dengue fatal cases (Basílio-de-Oliveira et al. 2005; Green and Rothman 2006) and several studies have been performed in order to investigate the effect of dengue virus (DENV) infection in hepatic cells, including HepG2 (El-Bacha et al. 2007; Silva et al. 2011; Thepparit et al. 2013). Transfection of these cells with plasmids encoding DENV genes is an attractive approach for mapping the isolated effects of the expression of such genes. In this regard, the nonstructural glycoprotein 1 gene (NS1) from DENV is an interesting target. The NS1 protein is essential for the viability of DENV and some reports indicate that it can be used as a protective antigen for vaccine development (Costa et al. 2007; Costa et al. 2006; Li et al. 2012), while others suggest its involvement in viral pathogenesis, including hepatic damages (Muller and Young 2013). Therefore, we decided to investigate the effect of DENV NS1 expression in HepG2 cells as an attempt to increase knowledge of its role in virus infection. However, the success of these in vitro studies requires a large number of cells expressing the recombinant protein, which depends on an efficient transfection method.

Hence, in the present work we evaluated different methods for transfection of HepG2, aiming both high cell viability and transfection efficiency. We tested two lipid based methods, a cationic liposome, Lipofectamine (Invitrogen), and a nonliposomal formulation, FuGENE® 6 (Roche), as well as the nucleofection approach using Amaxa Nucleofector™ kit V (Lonza). Cells were transfected with the mammalian expression plasmid pcENS1, which was previously constructed by our group and encodes the dengue virus 2 NS1 gene (S.M. Costa et al. 2007). Levels of transfection were evaluated by indirect immunofluorescence and flow cytometry, while cell viability was quantified using trypan blue staining. We observed that transfection by nucleofection leads to significant higher number of cells expressing the NS1 compared to the lipid-based methodologies. We believe this study may help several other investigations that use HepG2, as well as other cells that are not easily permissive to DNA transfection.
MATERIAL AND METHODS

Cell culture

HepG2 cells were purchased from ATCC (USA) in passage 84 and were cultivated in Dulbecco's modified Eagle's medium (DMEM) (SIGMA, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, USA). Cells were maintained at 37°C under humid atmosphere with 5% CO₂. Cells were used between 86 and 89 cell passages and at 70-80% confluency.

Plasmids

The recombinant plasmid pcENS1 was previously constructed in our laboratory (Costa, Freire, and Alves 2006), based on the pcDNA3 mammalian expression vector (Invitrogen). It contains a 63 nucleotide sequence that encodes 21 amino acids from the C-terminal portion of the DENV2 envelope (E) protein and the full length DENV2 NS1 gene. The pcDNA3 vector was used as a negative control.

Transfection

Three different methodologies for transfection of the HepG2 cells were evaluated. We tested two lipid-based methods: Lipofectamine (Invitrogen), a cationic liposome previously used by our group for transfection of other mammalian cells (Costa et al. 2007; Costa et al. 2011; Azevedo et al. 2011), and FuGENE 6 (Roche, Switzerland), a nonliposomal formulation. Alternatively, we used the Amaxa Nucleofector kit V (Amaxa, Germany) for transfection by electroporation.

Transfection using Lipofectamine

For detection of the recombinant protein NS1 by immunofluorescence, 5x10⁴ HepG2 cells were seeded on a slide containing eight chambers (Lab-Tek, NUNC, Denmark) at 37°C in 5% CO₂. After 24 hours, cells were transfected with either 0.4 μg or 0.8 μg of DNA, complexed with 2 μl or 4μl of Lipofectamine, respectively, maintaining the proportion of 1:5 [DNA (μg) : Lipofectamine (μl)]. The DNA:Lipofectamine complexes were diluted in 200 μl of OptiMEM medium (Invitrogen), and incubated with cells for 5 hours at 37°C in 5% CO₂. OptiMEM medium was replaced by DMEM with 10% FBS, according to manufacturer's recommendations. (Table 1). On the next day, cells were analyzed using indirect immunofluorescence in a fluorescence microscope. Viability was evaluated with 0.04% trypan blue staining and expressed as a percentage of total cells.

For analysis by flow cytometry, 1 x 10⁶ cells were plated on 25 cm² flasks and incubated in a humidified atmosphere with 5% CO₂ at 37°C for 24 hours. Cells were transfected with 8 μg of DNA and 40 μl of Lipofectamine (proportion of 1:5) in 2 ml of OptiMEM medium. Five hours later, OptiMEM medium was replaced by DMEM with 10% FBS. Cells were cultivated for 24 hours, followed by analysis by flow cytometry.

Transfection using FuGENE® 6

Cells (5x10⁴) were seeded 24 or 48 hours prior to transfection on Lab-Tek slides with eight chambers. Transfections were performed testing several conditions: FuGENE (μl):DNA (μg) ratio (3:1, 3:2 or 6:1); distinct culture media (DMEM with 10% FBS, serum-free DMEM or OptiMEM); different incubation times for DNA:FuGENE complex formation (45-120 minutes); and two final volume of DNA-FuGENE complex (10 or 20 μl) (Table 1). Totally, we tested 23 different conditions. The FuGENE reagent was diluted in 80% ethanol and all incubations were made in glass, since plastic material can affect the efficiency of transfection.

Transfection was performed for 5 hours. After this, the medium was changed to DMEM with 10% FBS and cells were maintained for 24 hours, followed by immunofluorescence analysis using optical microscope. Alternatively, FuGENE-DNA were diluted in DMEM medium with 10% FBS and cells were incubated with the complexes for 24 hours. Cell viability was evaluated using 0.04% trypan blue and expressed as a percentage of total cells.

For analysis by flow cytometry, 1x10⁶ cells were seeded on 25 cm² bottles and maintained in 5% CO₂ at 37°C. After 24 hours, 30 μl of FuGENE were incubated with 5 μg of DNA in DMEM for 120 minutes at room temperature. Subsequently, cells were washed and incubated with the FuGENE-DNA complex in DMEM with 10% FBS for 24 hours.

Transfection by Nucleofection

Four days before nucleofection, HepG2 cells were seeded on 75 cm² bottles, according to the Nucleofector™ kit V manufacturer's recommendations. Cells were removed from bottles with the aid of cell scrapes and suspended in DMEM with 10% FBS. In order to choose
the best condition of nucleofection, we used three different cell quantities: 1x10⁶, 2.5x10⁶ and 5x10⁶ cells/cuvette (Table 2). Plasmids (5 or 10 μg of DNA) were combined with 100 μl of nucleofection solution (Nucleofector Solution was mixed with Supplement Solution according to Amaxa recommendation), followed by vortex or not. Cells were centrifuged for 5 minutes at 1500 rpm, suspended in nucleofection solution and transferred to kit cuvettes. Electric shock was performed using the Nucleofector 6 equipment (Amaxa) with T-28 program.

After the electric shock, cells received 500 μl of DMEM with 10% FBS and were immediately transferred to microcentrifuge tubes containing another 500 μl of DMEM with 10% FBS. A total of 1x10⁵ cells were seeded on Lab-Tek slides in DMEM with 10% FBS for analysis by immunofluorescence. Cell viability was assessed 30 minutes after the electric shock, using 0.04% trypan blue and expressed as a percentage of total cells. The remaining cells were seeded on 75 cm² flask (2.5 and 5x10⁶ cells) or 25 cm² flask (1x10⁶ cells) for quantification of NS1 expression by flow cytometry. Cells were incubated in humid atmosphere with 5% CO₂ at 37°C for 24 hours.

**Indirect immunofluorescence analysis**

Twenty four hours following transfection, cell monolayers were washed with 0.1 M phosphate buffer pH 7.4 (PB), fixed with 4% paraformaldehyde in PB for 15 minutes, permeabilized with 0.6% of saponin in PB for 10 minutes and blocked with 1% bovine serum albumin (BSA) for 15 minutes. The whole process was conducted at room temperature. For detection of the recombinant NS1 protein, cells were incubated with a DENV2 hyperimmune mouse ascitic fluid (ATCC) for 1 hour at 37°C. Cells were washed and incubated with anti-mouse fluorescein-conjugated goat IgG (FITC, Southern Biotechnology, USA) in the same conditions. Slides were assembled with Vectashield mounting medium (Vector Laboratories, USA) and cells were visualized in fluorescence microscope Nikon H550S. Positive fluorescent cells were estimated as a percentage of total cells. The best condition for each transfection methodology, detected by the immunofluorescence analysis, was used for further quantification by flow cytometry.

**Flow cytometry analysis**

Expression of the recombinant NS1 protein was determined by flow cytometry, 24 hours after transfection of HepG2 with the plasmid pcENS1. Cells were harvested from the bottles with the aid of cell scrapi es in 3 ml of CMF solution (8g/L of NaCl; 0.4g/L of KCl; 0.1g/L of Na₂SO₄; 0.39g/L of Na₂HPO₄.12H₂O; 0.15g/L of KH₂PO₄; 1.1g/L of glucose; 0.0025g/L of phenol red, pH 7.4), centrifuged at 1500 rpm for 5 min and suspended in PBS. Approximately 10⁶ cells/well were fixed in 4% paraformaldehyde for 20 minutes and permeabilized with 0.05% saponin for 30 minutes. Subsequently, cells were incubated with DENV2 hyperimmune mouse ascitic fluid for 1 hour at 37°C, washed in PBS, followed by incubation with anti-mouse FITC- conjugated goat IgG for 30 minutes. Cells were suspended in paraformaldehyde. Samples were read in the flow cytometer Accuri and analyzed offline with Accuri software.

| Plate time (hours) | Medium                     | Complex time (minutes) | Ratio FuGENE:DNA | Final volume (μl) |
|-------------------|----------------------------|------------------------|------------------|------------------|
| 24 or 48          | OptiMEM, DMEM              | 45 or 90               | 3:1 or 3:2       | 10 or 20         |
|                   | or DMEM                    | 120                    | 6:1              |                  |

Table 1 Different conditions used for transfections with FuGENE 6

Conditions used to transfect cells in Lab Tek using FuGENE 6.
RESULTS

Transfection using Lipofectamine

Initially, cells were transfected with pcENS1 plasmid or the control vector pcDNA3 at two different conditions (Table 1), in order to determine optimal concentrations of Lipofectamine and DNA. These variations were chosen based on a standard laboratory protocol for other mammalian cells (Costa et al. 2007; Costa et al. 2011; Azevedo et al. 2011). As expected, the NS1 protein was detected by immunofluorescence only in HepG2 cells transfected with pcENS1 (Fig. 1A - D). The analysis by immunofluorescence and flow cytometry indicated that 0.8 μg of DNA led to a higher number of cells expressing NS1 compared to 0.4 μg of DNA. However, only 3.9% of cells expressed NS1 (Fig. 2B), thus revealing an unsatisfactory transfection rate. Furthermore, only 23% or 28% viable cells were recovered 24 hours post transfection with pcDNA3 or pcENS1, respectively (Table 3), indicating a high toxicity of Lipofectamine in the HepG2 cells in the used conditions. In addition, Lipofectamine induced formation of aggregates and debris (data not shown), also suggestive of a toxic effect of this liposome.

Transfection using FuGENE 6

At the time we conducted our experiments, FuGENE was indicated as a good reagent for transfection of HepG2 cells. Therefore, in order to determine the optimal transfection protocol with FuGENE to yield the greatest efficiency and the lowest cell mortality, HepG2 cells were transfected using 23 different conditions varying plating times, culture media, as well as distinct ratios of FuGENE : DNA complex and incubation times (Table 1). In all tested conditions, cells transfected with pcENS1 plasmid were positively stained by immunofluorescence assay using the DENV2 hyperimmune ascitic fluid (Fig 1E-F), while cells transfected with the control vector pcDNA3 did not react with these antibodies (Fig 1G-H). The highest transfection efficiency was obtained using DMEM medium with 10% FBS, incubation of the complex FuGENE:DNA for 120 minutes at a ratio of 6:1 and performed 24 hours after cell plating (Fig 1E-F).

Subsequently, flow cytometry analysis was used for quantification of cells expressing the NS1 protein in this optimized transfection condition. As observed in figure 2C, approximately 7% of the cells expressed NS1. Concerning cell viability, only 35% or 37% of cells survived after transfection with the pcENS1 or the control pcDNA3, respectively (Table 3). However, cells that survived transfection presented similar morphology to the non-transfected cells, suggesting that the FuGENE is less toxic to HepG2 than Lipofectamine.

Transfection by nucleofection

Since transfections based on the above lipids generated low numbers of cells expressing the NS1 protein, we tested the nucleofection, an electroporation-based method. In order to determine optimal conditions for nucleofection of HepG2, we submitted different quantity of cell to shock. We used the standard protocol of 1x10⁶ cell with 5g of plasmid, as well as 2.5x10⁶ or 5x10⁶ cells/cuvette with the same amount of DNA. Cells were pulsed with the program T-28, recommended for HepG2 by the manufacturer, which leads to the highest transfection efficiency impairing cell viability. The indirect immunofluorescence assay revealed a large number of HepG2 expressing the NS1 protein in all tested conditions (Table 2 and Fig. 3C - D). The analysis by flow cytometry 24 hours after nucleofection showed 63% (1x10⁶ cells), 36.8% (2.5x10⁶ cells) and 40.2% (5x10⁶ cells) of HepG2 stained with the DENV2 hyperimmune ascitic fluid (Fig. 4). Hence, the best result, concerning the number of cells expressing NS1, was obtained using the lowest HepG2 cell density (1x10⁶) for the same mass of DNA. The transfection efficiency using such condition was confirmed by two additional independent experiments with more than 50% of NS1 positive cells (data not shown).

Cell viability analysis revealed that the cell number influenced survival after shock. Approximately 50%, 80% and 52% of HepG2 survived, when we used 1x10⁶, 2.5x10⁶ and 5x10⁶ cells, respectively. Thus, although 1x10⁶ cells/cuvette was more effective for delivering the plasmid DNA into HepG2, this condition generated higher cell

| Plate time (hours) | Medium | Cell Quantity | DNA Quantity (μg) |
|-------------------|--------|---------------|-------------------|
| 96                | DMEM   | 1x10⁶         | 5                 |
|                   |        | 2.5x10⁶       | 5 or 10           |
|                   |        | 5x10⁶         | 5                 |

Conditions used to transfect cells by nucleofection.

Table 2 Different conditions used for transfections by electroporation with Nucleofector
death compared to other cell concentration. Likewise, transfection with the control vector pcDNA3 led to similar survival rate (approximately 60% with $2.5 \times 10^6$ cells).

Based on these results, nucleofection was by far the most efficient technique for transfection of HepG2 cells. We next performed another set of experiments by increasing the quantity of DNA in order to raise the number of viable cells expressing the NS1 protein. We used $2.5 \times 10^6$ cells/cuvette and the double amount of DNA that is recommended by the manufacturer. (Amaxa). Furthermore, the DNA was mixed with the nucleofector solution with the aid of a vortex before the addition of cells. In this new condition, the number of transfected cells increased to 42.4% (data not shown), with 68% survival (Table 3).

We observed some modifications in HepG2 morphology after nucleofection, such as the presence of aggregates, cell extensions and increase of cell size. These changes were observed in cells transfected either with pcENS1 or with the control plasmid pcDNA3 (data not shown).

**DISCUSSION**

The success of many in vitro studies depends, to an extent, on transfection efficiency. Morphological analyses as well as proteomics experiments, for

| Method        | Conditions                                      | % Viability<sup>a</sup> | pcENS1 | pcDNA3 |
|---------------|------------------------------------------------|-------------------------|--------|--------|
| Lipofectamine | 0.8μg DNA - 4μL reagent                        | 28                      | 23     |        |
| FuGENE 6      | 6 μL FuGENE:1μg DNA, in 20μL (120 min)         | 35                      | 37     |        |
| Nucleofection | 5μg DNA - $1 \times 10^6$                       | 51                      | 64     |        |
|               | 5μg DNA - $2.5 \times 10^6$                    | 79                      | 61     |        |
|               | 5μg DNA - $5 \times 10^6$                      | 52                      | 52     |        |
|               | 10μg DNA - $2.5 \times 10^6$                   | 68                      | 53     |        |

Living cells were evaluated with trypan blue staining.

<sup>a</sup> Media of three independent experiments.
Fig 2. Detection of NS1 by flow cytometry in HepG2 cells transfected with Lipofectamine or FuGENE6. HepG2 cell suspensions were transfected with pcENS1 or pcDNA3 (control) plasmids and further labeled with DENV2 hyperimmune mouse ascitic fluid and anti-mouse FITC-conjugated goat IgG, in order to determine the presence of intracellular NS1 by flow cytometry technique. (A) Ungated forward x side light scattering (FSC x SSC) flow cytometry dot plot exhibiting a region (R1) containing cells considered for the analysis. Flow cytometry dot plots gated in R1 showing the expression of NS1 (P3 region) in cells transfected with Lipofectamine (B) or FuGene6 (C). P3 Region was defined based on pcDNA3 control samples.

Fig 3. HepG2 cells expressing the NS1 protein after nucleofection. Cells were transfected with plasmids pcDNA3 (A, B) or pcENS1 (C, D) by electroporation using the Nucleofector. Cells were permeabilized, fixed and treated with DENV2 hyperimmune mouse ascitic fluid and anti-mouse FITC-conjugated goat IgG. Phase contrast (B, D); immunofluorescence (A, C). Scale bars = 50µm.

Fig 4. Production of NS1, detected by flow cytometry, in HepG2 cells transfected with pcENS1 by nucleofection. Suspensions with different HepG2 cell numbers were transfected with pcENS1 or pcDNA3 plasmids and further labeled with DENV2 hyperimmune mouse ascitic fluid and anti-mouse FITC-conjugated goat IgG in order to determine the presence of intracellular NS1 by flow cytometry. (A) FSC x SSC flow cytometry dot plot showing cells transfected with the control plasmid pcDNA3, in which a region (P3) was defined to measure the NS1-expressing cells. (B) Flow cytometry dot plots of HepG2 cells using different cell number (1.0 x 10⁶, 2.5 x 10⁶ or 5.0 x 10⁶ cells/cuvette) for transfection by nucleofection. Percentages of NS1-expressing cells are exhibited in the P3 region.
example, require a large number of cells expressing the recombinant protein for comparative purposes with non-transfected cells. Permanent transfection is not always possible, since the expression of the recombinant protein leads to an energetic cost to the cell and often deletion of part of the plasmid used in the transfection or its methylation occurs, leading to loss of expression of the protein of interest. Furthermore, some cell lines, such as HepG2, are somewhat refractory to transfection.

Therefore, in the present study we compared lipid- and electroporation-based techniques for the introduction of the DENV NS1 gene into the hepatocarcinoma HepG2 cell line. In order to obtain a large number of cells expressing the NS1 recombinant protein, HepG2 were transfected using the liposome or nonliposome formulations, Lipofectamine and FuGENE 6, respectively, or by nucleofection. These techniques were chosen based on other studies indicating their efficiency with various cell types, including hepatocytes (Gao et al. 2012; Xie et al. 2012; Magin et al. 2013).

Initially, we tested transfection of HepG2 cells with Lipofectamine, which is a widely used liposome and routinely employed by our group in transfection of other mammalian cell lines (Costa et al. 2007; Costa et al. 2011; Azevedo et al. 2011). Conditions used with Lipofectamine for HepG2 cells in the present work were those recommended by the manufacturer and successfully employed for other cell lines (Azevedo et al. 2011; Costa et al. 2007, 2011; Namvar et al. 2016). We tested two concentration of DNA:lipofectamine complex in contact with the cells. However, transfections performed with Lipofectamine generated unsatisfactory results with the HepG2 cells regardless of the amount of DNA:lipofectamine complex tested. Furthermore, we observed changes in cell morphology after transfection with Lipofectamine, such as the presence of cellular aggregates and debris, suggesting a toxic effect of this liposome similar to those previously described (Gao et al. 2012).

FuGENE revealed to be more efficient than the Lipofectamine, although the proportion of cell producing NS1 was still very low and unsatisfactory. Such result was not expected since this lipid-based method is indicated by the manufacturer for HepG2 cells. Besides, we tested 23 different transfection conditions using FuGENE and none of them led to satisfactory results. However, cells that survived transfection presented similar morphology compared to non-transfected cells, suggesting that the FuGENE is less toxic to HepG2 than Lipofectamine. Overall, transfection of HepG2 cells performed with both lipids, Lipofectamine and FuGENE, did not generate satisfactory results. Rates of transfection were lower than the commonly observed in our laboratory for other mammal cell lines (Costa et al. 2007; Costa et al. 2011). Moreover, cell viability analysis revealed that both lipid-based methods were cytotoxic to HepG2 cell line, including FuGENE, which was recommended by ATCC for HepG2 transfection.

Some authors have shown that HepG2 cells can be transfected using lipids (Bose et al. 2015). In fact, we observed an improved transfection efficiency using lipofectamine when the HepG2 cells were obtained after a high culture passage number. However, our studies revealed that an elevated number of HepG2 culture passages can influence cell features, including cell susceptibility to DENV infection (data not shown). Besides, other reports with HepG2 also have shown cellular metabolic changes after different culture passages (Ruiz-Aracama et al. 2011; King et al. 2012). Therefore, in the present study we have performed the experiments with HepG2 cells always using the same range of culture passages (from 86 to 89, which represents few replications after purchase from ATCC) in other to maintain the reproducibility of biological replicates for future analysis.

On the other hand, we observed that transfection of HepG2 cells by electroporation with Nucleofector device was an efficient methodology. The quantification by flow cytometry showed that this method led to a high number of transfected cells. Furthermore, the viability of cells submitted to nucleofection was the highest among the tested methodologies. Such results agree with transfection efficiency studies using other cell lines, in which this type of electroporation was the most effective methodology (Siemen et al. 2005; Motoyama et al. 2009). Some reports have shown that the nucleofection can affect cell ability to differentiate and to respond to chemical stimuli (Kraus et al. 2010; Lorenz et al. 2004). In our study, we observed modifications in HepG2 morphology, such as the presence of aggregates, cell extensions and increase of cell size. These changes were observed in cells transfected either with pcENS1 or with the control plasmid pcDNA3, suggesting that they were a consequence of the nucleofection itself and not because of the expression of a heterologous protein.

In conclusion, the results in the present work indicated that nucleofection was the best method to transfect HepG2 cells in comparison to two lipid-based approaches.
Although the basic mechanism of electroporation by nucleofection is not completely understood, it is the fastest and simplest method to load cells with DNA. It is generally assumed that the electrical pulse that is applied to cells transiently destabilizes the cell membrane and delivers DNA into the nucleus. Furthermore, because nucleic acids are delivered directly into the cell nucleus, cell division is not required for substrate incorporation into it and, therefore, the delay between nucleofection and the heterologous gene expression is reduced (Han et al. 2008). We believe our study will contribute to the field of transfection of mammalian cells, in particular to those highly refractory cells. In our hands, nucleofection was superior to usual assays using lipid-methods. This may have implications, for instance, in the investigations of the effect of heterologous gene expression in the abundance and expression of cellular proteins by proteomics studies. In this regard, we have recently published one study of the effect of NS1 in the expression of cellular genes of HepG2, in which we identified several proteins that can be modulated by the presence of NS1 (Rabelo et al. 2017). Such study would not be possible without the present work for achieving high transfection efficiency of the HepG2 cells.

ACKNOWLEDGEMENTS

We are grateful for the technical assistance of Service of Production and Image Treatment/IOC/Fiocruz. We also thank the Laboratory of Immunofarmacology for using the Nucleofector equipment. This work was supported by PAPES-FIOCRUZ, CNPq and FAPERJ grants.

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