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Post-pulse addition of trans-cyclohexane-1,2-diol improves electrotransfer mediated gene expression in mammalian cells

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ABSTRACT

Electric field mediated gene transfer is facing a problem in expression yield due to the poor transfer across the nuclear envelope. Trans-cyclohexane-1,2-diol (TCHD) was shown to significantly increase chemically mediated transfection by collapsing the permeability barrier of the nuclear pore complex. We indeed observed a significant increase in expression by electrotransfer when cells are treated post pulse by a low non toxic concentration of TCHD. This was obtained for different pulsing conditions, cell strains and plasmid constructs. An interesting improvement in cell viability can be obtained. This can significantly enhance the non-viral gene electrical delivery.

1. Introduction

The plasma membrane is a barrier that hinders the transfer of molecules such as nucleic acids into cells. One physical method known to safely improve plasmid DNA delivery into cells is electropermeabilization (or electroporation) [1,2]. The application of controlled electric pulses causes a transient permeabilization of the plasma membrane and allows non permeant molecules (such as polar macromolecules) to enter the cells [1,3–5]. When the electric pulses are delivered on a cell in presence of plasmid (pDNA), gene expression can be detected [1]. This is indeed a multistep process. With the electric field pulse, pDNA are pushed against the cell membrane and pDNA/membrane aggregates are created [6,7] and remain trapped at the membrane for several minutes [6,8]. The challenge with optimizing gene electrotransfer is to get a balance between the positive contribution of creating defects supporting the cytoplasmic transfer of plasmid DNA and the need to preserve cell viability. Once it crosses the plasma membrane, pDNA is actively transported along tubulin filaments by motor proteins through the cytoplasm to the nuclear envelope [9,10] or trapped within actin coated vesicles [11,12].

The Nuclear envelope represents the last barrier that pDNA needs to cross to gain access to the transcriptional machinery. Only a very small fraction of pDNAs introduced in the cytoplasm, whatever the method, microinjection [13] as well as electrotransfection [6], can indeed reach the nuclear compartment. Furthermore pDNA present in the cytoplasm is sensitive to degradation by nucleases during the cell cycle [14]. Therefore, there is a need for a fast transfer of pDNA to the nucleus. It has been described that DNA transfection is more efficient in dividing cells compare to non-dividing cells due to the loss of nuclear envelope integrity during cell division [15–18] suggesting that the direct entry of pDNA into the nucleus requires modifications of the nuclear envelope components. One putative target is the nuclear pore complex (NPC), a multiprotein complex known to control transport through the nuclear envelope [19–21].

Different approaches have been reported to obtain such a molecular modification. One physical approach in order to destabilized NPC is to expose nuclear envelope to controlled electric pulses. Nanosecond electric pulses (nsEPs 4–600 ns) were described as being able to permeabilize internal organelles as intracellular granules, endocytic vesicles, nuclear envelope and to induce calcium release from endoplasmic reticulum (reviewed in [22]). The use of a two sequential pulses combination was proposed to improve gene transfer. Long (ms) electrical pulses were applied first as described above to allow plasmid access to cytoplasm and were followed by nanosecond electric pulses, in order to destabilize the nuclear envelope [23]. This combination was supposed to increase the number of plasmids entering the nucleus and thus, enhance gene expression. This was described as inducing a 3.6 fold increase in GFP gene expression in cells exposed to one

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nsEP 30 min after classical electrotransfection [24]. However, such a combination can be ineffective since more recently, nsEPs were shown to have no major contribution to gene electrotransfer [25]. No effect on constitutive protein expression was detected. In a similar approach, it was first shown that the application of one 5μs pulse of several kV/cm alone or after an electrotransfection protocol affects the morphology of the nucleus [26] while it was observed that such a combination of classical electrotransfection followed by one 5 μs 5 kV/cm pulse did not increase plasmid DNA expression as compared to the classical electrotransfection protocol [27]. Electrically mediated alterations of the nuclear envelope were not enhancing gene nuclear uptake.

The Nuclear Pore Complex (NPC) is composed of 30–50 different proteins (Nucleoporins), and can be, in a simplified form, divided in three basic elements: the nuclear basket, the central core and the cytoplasmic fibrils [28]. The central channel of the NPC form an aqueous channel that allow the passive diffusion of molecules smaller than 25–40 kDa across the NPC [29]. However, the wall of the NPC is rich in phenylalanine-glycine repeats (FG-nucleoporins) that act as hydrophobic barrier blocking the transfer of macromolecules such as DNA that therefore, requires active and facilitated transport [20,21]. This active transport is mediated by specific interactions between the translocating element and transport receptor with cargo via adaptor molecules. This complex association allows a highly orchestrated, rapid and efficient nuclear transport. *In vitro* studies evaluate the transport rate to 1000 cargo molecules per seconds per NPC meaning that at least 10 transport molecules cross a given NPC simultaneously [20]. Nevertheless, this process is highly selective meaning that it is dependent on the presence of Nuclear Localization Sequences (NLS) on the translocating element. These NLS allow the association of the translocating element to importin α and β promoting its transfer into the nucleus. Thus, in order to increase nuclear import of DNA, NLS-peptides and NLS-proteins have been attached to pDNA [30–32] but with limited success. This phenomenon does not seem due to size of the DNA but more to its hydrophilic properties that tend to exclude the DNA from the nuclear pore even if associated with importin.

The amphipathic alcohol *trans*-cyclohexane-1,2-diol (TCHD) has the ability to disrupt bonds between FG-nucleoporins, that makes up the NPC barrier without affecting the integrity of the nuclear envelope. Moreover, it has been described that TCHD can transiently increase the size of NPC [33] thus modifying the permeability [21]. It was shown that gene expression mediated by a chemical method (poly- and lipoplexes) was increased when the cells were treated by a low concentration of TCHD [34]. We hypothesized that a process similar would occur along electrotransfection. After the transfer of free plasmid DNA into the cell cytoplasm by electropermeabilization, treatment with TCHD could facilitate plasmid DNA access into the nucleus and thus increase gene expression. In this study we compared transfection rate between EP alone and EP combined with TCHD treatment. We also analyzed the impact of the delay between EP and TCHD treatment as well as the TCHD incubation time on gene expression and cell viability.

2. Material and methods

2.1. Cell culture

B16F10 cells were grown as a monolayer culture on T75 flasks (Nunc, Denmark) in Dulbecco's Modified Eagle Medium with 4.5 g/l D-Glucose and L-Glutamine (DMEM; Gibco/ Life technology) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO) and the antibiotics penicillin (100 U/ml) and streptomycin (100 U/ml) (Gibco/ Life technology) at 37 °C, 5% CO_2 atmosphere in a humidified chamber until they reached 70% confluence.

Chinese Hamster Ovarian (CHO) cells (Wild-Type Toronto) were grown as a monolayer culture in Minimum Essential Eagle Medium with Earle's salts and nonessential amino acids (EMEM; Eurobio, Les Ulis, France), supplemented with 10% fetal bovine serum (GIBCO/Life Technologies, Grand Island, NY), L-glutamine (0.58 g/l, GIBCO/Life Technologies), 2.95 g/l tryptose-phosphate (Sigma-Aldrich, St. Louis, MO), BME vitamins (Sigma-Aldrich), 3.5 g/l glucose (Sigma-Aldrich) and the *anti*biotics penicillin (100 U/ml) and streptomycin (100 μg/ml, GIBCO/Life Technologies) at 37 °C, 5% CO_2 atmosphere in a humidified chamber until they reached 70% confluence. CHO cells could grow in suspension. Plated cells were trypsinized and cultured in suspension in spinner (Corning Inc, Corning, NY, USA). 0.5×10^6/ml cells were cultured in the same culture medium in hermetic closed spinner at 37 °C with a soft stirring. Every day, the cell culture was diluted 2-fold. Growing cells in suspension avoided a trypsinization step before the delivery of electric pulses. The extracellular matrix was preserved.

2.2. Plasmid DNA

pCMV-eGFP-C1 (Clontech, Mountain View, CA) a 4.7-Kb plasmid encoding GFP, was used in experiments with B16F10 cells (map in Suppl Fig. 1). This plasmid was amplified in Escherichia coli DH5α and purified with the Maxiprep DNA Purification System (Qiagen, Germany) according to the manufacturer's protocol. The purification was verified by agarose electrophoresis.

pCMV-CpGfree-tdTomato (Invivogen, Toulouse, France) a 4.4-Kb plasmid encoding Tomato, was used in experiments with CHO cells (map in Suppl Fig. 1) and directly purchased.

2.3. Gene electrotransfection protocols and TCHD treatment

B16F10 cells were trypsinized and washed twice in medium. CHO cells were collected from spinner. Cells were suspended in pulsation buffer (PB; 10 mM K_2HPO_4/KH_2PO_4, 1 mM $MgCl_2$, 250 mM sucrose [pH 7.4]) at a concentration of 5×10^6 cells/ml, and 20 μg/ml pCMV-EGFP-C1 plasmid or 20 μg/ml pCMV-CpGfree-tdTomato plasmid was added. 100 μl of cell solution was dropped between stainless steel, flat, parallel electrodes (0.4 cm gap) in contact with the bottom of a culture dish (Nunc, Denmark). Two different electrical parameters were used: (i) Low field long pulse (LF-LP) parameters that consist in 6 square-wave pulses of 600 V/cm, duration 5 ms, (ii) High field short pulse (HF-SP) parameters consist in 4 square-wave electric pulses of 1200 V/cm, duration 100 μs (Fig. 1). All pulses were applied at 1 Hz frequency, at room temperature using a pulse generator (electrocell S20; Betatech, St Orens, France). Pulse delivery was monitored on line on the touch screen. In pulses conditions, 20 μl FBS (Sigma Aldrich, St Louis, USA) were added just after pulsing in the drop, which was hanging on the dish. Cells were kept at room temperature.

In the indicated conditions, 20 μl of TCHD (Sigma Aldrich, St Louis, USA) diluted in pulsing buffer (to get a final concentration 1% w/v), or 20 μl of pulsing buffer alone were added to the cells 0 or 10 min after pulse delivery and incubated 10, 20 or 30 min at 37 °C. Cells were then transferred to 2 ml of their respective culture medium in 12 well plates (Nunc, Denmark) and incubated 24 h at 37 °C with 5% CO_2. Cells untreated with TCHD were transferred in 2 ml culture medium 5 min after electropermeabilization (Fig. 1).

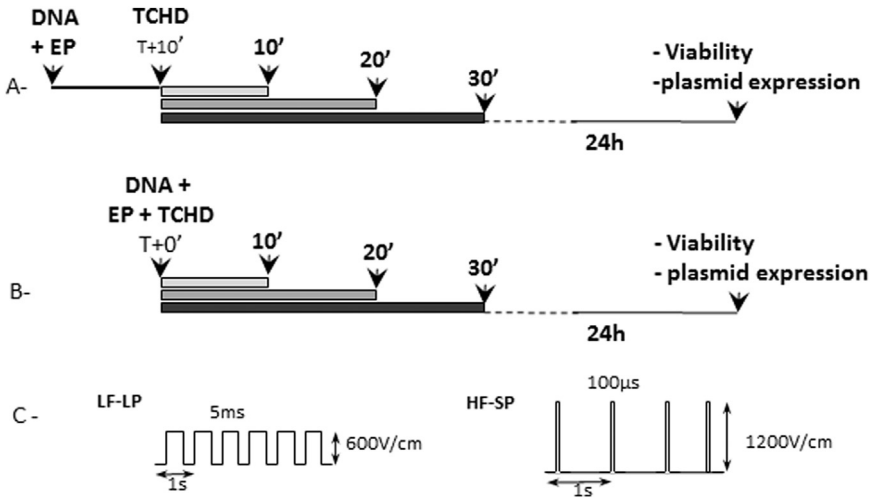

Fig. 1. Experimental design B16F10 cells were trypsinized and suspended in pulsing buffer at 5 × 106 cells/ml. 100 µl of cells (0.5 × 106 cells) were pulsed between 0.4 cm spaced flat electrodes at room temperature. 20 µl of TCHD 1% (final concentration W/v) was added 10 min (A) or just after (B) pulses and incubated 10, 20 or 30 min. At the end of incubation, 2 ml of culture medium was added and cells were cultured for 24 h at 37 °C, 5% CO2. Cells were pulsed with two different parameters (C): Low Field-Long pulses (LF-LP) that consist in 6 square wave electric pulses of 600 V/cm (240 V), duration 5 ms were applied at the frequency of 1 Hz or High Field-Short Pulses (HF-SP) that consist in 4 square wave electric pulses of 1200 V/cm (480 V), duration 100 µs applied at the frequency of 1 Hz. Plasmid expression and cell viability were analyzed 24 h after pulses.

2.4. Cell viability measurement

Cell viability was analyzed 24 h after treatment by Crystal violet staining (Merck, Darmstadt, Germany) [35]. Briefly, plated cells were washed with PBS 1 × and incubated 20 min with the 0.1% crystal violet solution. After 3 washes, a 10% acetic acid solution was added to lyse stained cells. Absorbance measurement was realized by spectrophotometric measurement at 595 nm (Novaspec II, Pharmacia biotech, Uppsala, Sweden).

2.5. Plasmid expression quantification by flow cytometry

The percentage of GFP or Tomato positive cells and the fluorescence intensity were determined by flow cytometry. Cells were trypsinized, centrifuged 5 min at 500 × *g* and transferred in 200 µl of FACS buffer (PBS 1 × plus 2.5% fetal bovin serum). Acquisition was performed on a FACScalibur cytometer (BD bioscience, San Jose, CA) and data were analyzed using FlowJo software (Tree Star, Ashland, OR). By gating on the fluorescent emission, it was possible to evaluate the percentage of fluorescent cells and their mean fluorescence intensity.

2.6. Plasmid expression observation by microscopy

For observation under the microscope of fluorescent protein expression, culture plates were placed on the stage of an inverted digitized fluorescence microscope (Leica DMIRB, Wetzlar, Germany). Cells were observed with a Leica 20X objective and the L4 block filter (Exc: BP480/40; Em: BP527/40) for GFP expression and mCherry-T filter (Exc: BP560/40; Em: BP630/75) for Tomato expression. Imaging was obtained with a Coolsnap fx camera (Roper) and the metavue software (Molecular Devices, LLC).

2.7. Statistical analysis

Statistical analysis was carried out using Prism 5 statistical version (GraphPad Software Inc., San Diego, CA). We used *t*-Test analysis. All comparisons, except when it is indicated with lines, were related to the basic EGT condition without addition of TCHD.

3. Results

3.1. Effect of TCHD on transfection rate and GFP plasmid expression under Low Field-Long pulse conditions in B16F10 cells

B16F10 cells were transfected with 20 µg/ml of pCMV-eGFP plasmid, 6 pulses of 600 V/cm, duration 5 ms, delay 1 s. It was previously described that TCHD dose over 2% highly affected cell viability even though it increased transfection rate in a chemical protocol transfer [30]. In our study, the addition of 2% TCHD (final concentration) after electropermeabilisation has drastically affected the cell viability without any increase of the transfection rate (data not shown). Therefore, TCHD 1% (final concentration w/v) was selected and was added 10 min or just after pulsing and then incubated 10, 20 or 30 min prior culture medium was added. Protein fluorescence (*i.e.* gene expression) and cell viability were analyzed 24 h after pulses (Fig. 1). In order to compare and standardize data on each cell culture, each value was related to the one obtained on the same day in the plasmid electrotransfection condition in absence of TCHD. This calculation gave the relative increase of GFP positive cells population and GFP mean fluorescence intensity due to TCHD treatments.

The electrotransfected (GFP positive cell) population was significatively increased by 1.5–2.3 fold with TCHD treatment. No difference in the positive effect was observed if TCHD was added 10 min or just after pulses (Fig. 2A and D). On the contrary, GFP mean fluorescence intensity was significantly increased with TCHD treatment 10 min after pulses (2.5 fold ± 0.8) than with TCHD treatment just after pulsing (1.3 fold ± 0.5) (Fig. 2B and D). Moreover, TCHD incubation duration (10, 20 or 30 min) did not impact the increase in GFP positive population nor the intensity of plasmid expression. Interestingly, TCHD treatment in pulsed or unpulsed conditions had no deleterious effects on cell viability (Fig. 2C). Similar relative increases were obtained with a hypoosmotic pulsation buffer previously described to give a better transfection level than isoosmotic buffer [36] (data not shown).

3.2. Transfection is controlled by pulsing conditions

It was observed that High Field-Short Pulses (HF-SP) should be more efficient in gene electrotransfection than previously used

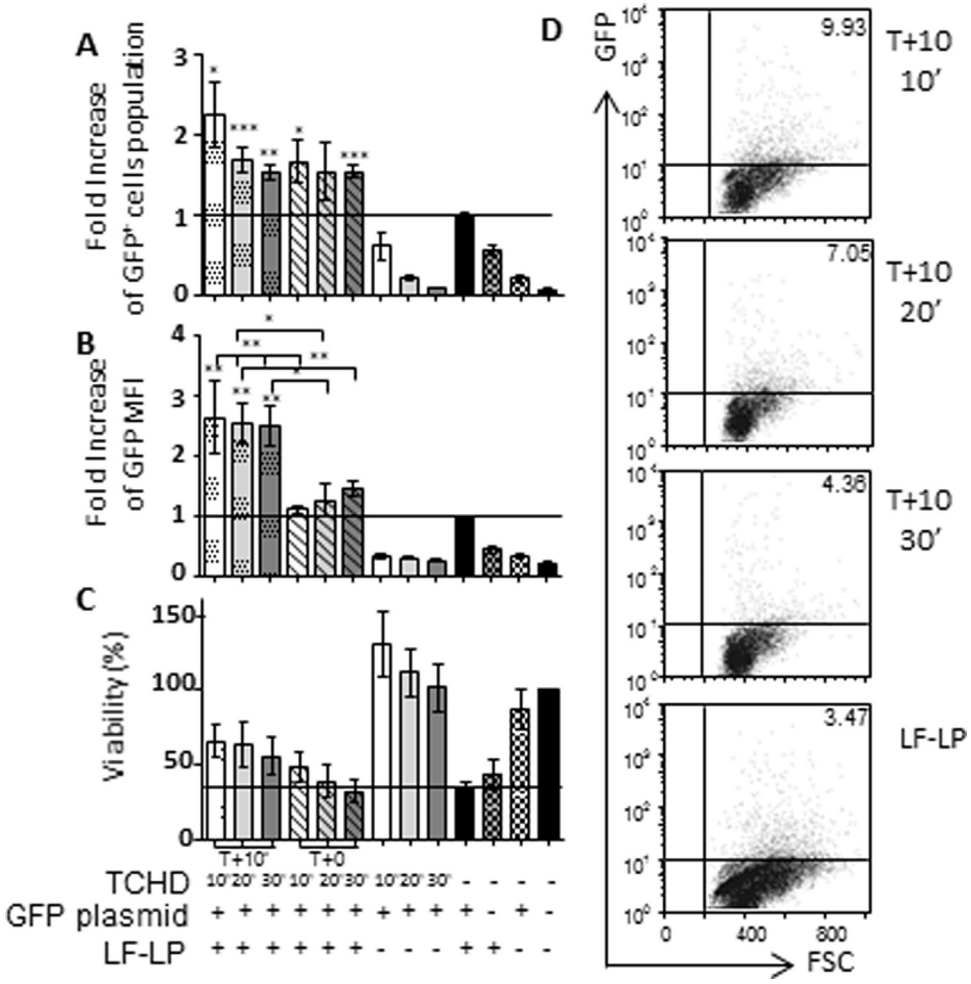

Fig. 2. TCHD increases GFP plasmid expression in B16F10 cells under LF-LP electrical parameters B16F10 cells were pulsed in the presence or not of 2 μg GFP plasmid DNA. 6 square wave electric pulses of 600 V/cm, duration 5 ms were applied at the frequency of 1 Hz at room temperature using flat electrodes spaced of 0.4 cm. 20 μl of TCHD 1% (final concentration W/v) was added 10 min or just after pulse and incubated 10, 20 or 30 min. 24 h after treatment GFP expression was analyzed by flow cytometry to determine transfected cells percentage (A) and GFP mean fluorescent intensity (MFI) (B) fold increase based on the value of the LF-LP condition in presence of plasmid (black line). Viability (C) was determined by crystal violet coloration. (D) Representative cytometry dot plots of pulsed cells treated or not with TCHD 10 min after pulse. Statistical analysis was conducted using two-tailed Mann-Withney *t*-test (n=6, 3 independent experiments). $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$.

low field long pulses (LF-LP) [37–40]. B16F10 cells were pulsed with 20 μg/ml of pCMV-eGFP plasmid under HF-SP or LF-LP conditions. The percentage of GFP positive cells rose 4 fold under HF-SP compared to LF-LP (Fig. 3A, D and E). The GFP mean fluorescence intensity rose 7.6 fold under HF-SP compared to LF-LP (Fig. 3B, D and E). Even though not statistically significant, viability of HF-SP treated cells appeared higher than the viability of LF-LP treated cells (Fig. 3C and E). This difference was significant in absence of plasmid. We checked if the positive effect of the post pulse addition of TCHD was still present under these more efficient transfecting conditions.

3.3. TCHD is efficient in increasing transfection rate under HF-SP conditions

Cells pulsed with 20 μg/ml of pCMV-eGFP plasmid under HF-SP conditions were treated with TCHD 1% (final concentration w/v), 10 min after pulses for a 10, 20 or 30 min incubation time. Relative to pulsed cells without TCHD addition post-pulse (considered as a reference) the GFP positive population was increased by about 1.5 fold by the post-pulse TCHD addition (Fig. 4A and D). GFP mean fluorescence intensity was increased by 2–2.5 fold. As with LF-LP conditions, there was no difference in transfection rate improvement if TCHD was incubated 10, 20 or 30 min. Under these parameters, cell viability was positively affected as compared with electrotransfected cells without TCHD.

3.4. TCHD increases transfection rate and plasmid expression in another cellular model

In order to determine if the effect of TCHD on transfection rate was cell and plasmid independent or only present in B16F10 cells, similar experiments were performed with a tomato-coding plasmid used to transfect CHO-WTT cells, reported to give different responses to electropermeabilisation [41]. These cells were grown in suspension during a 2 days culture in spinner, to avoid the trypsin treatment step and leave their EMC intact, then washed and were pulsed in pulsing buffer. Cells were treated under the same protocol as previously described. CHO cells were pulsed with LF-LP conditions and were treated with TCHD 1% (final concentration w/v) 10 min or just after pulses followed by a 10, 20 or 30 min incubation. Due to the spectral limitation of the cytometer and its poor sensitivity for detect Tomato expression, this decrease of sensitivity in detection of expression prevented an accurate evaluation of tomato expression and of the positive cell population (suppl. Fig. 2). This did not affect a relative comparison in the mean fluorescence intensity. Under these conditions, it was also observed that TCHD increased Tomato mean fluorescence intensity

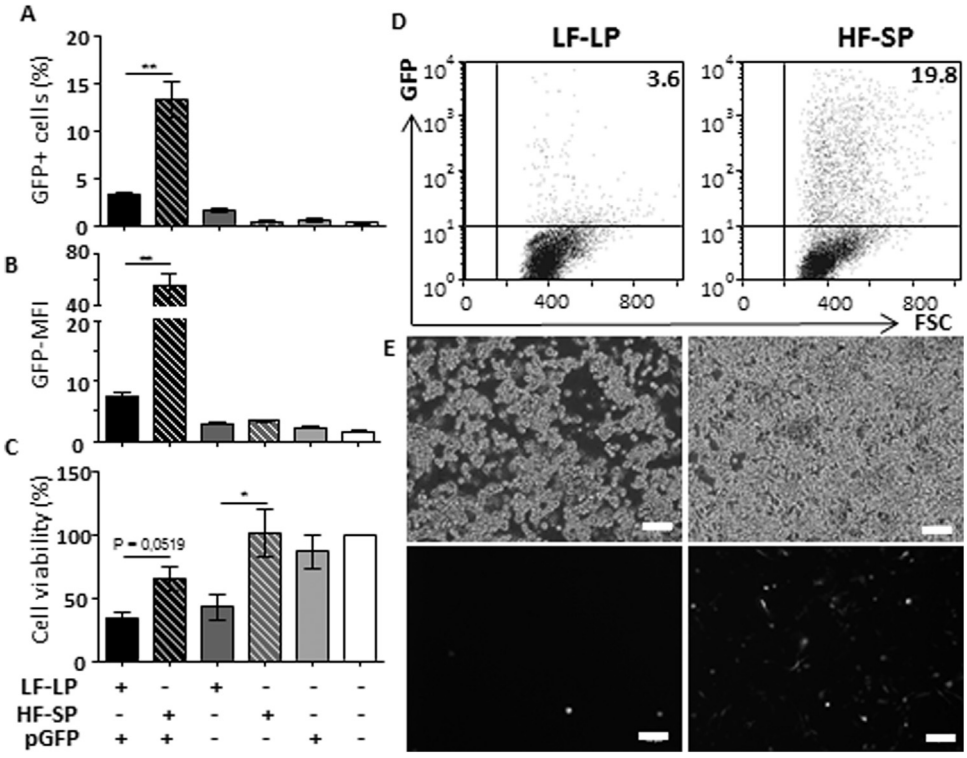

Fig. 3. High Field-Short Pulses induce a more efficient B16F10 cells transfection than Long Duration- Low Field pulses B16F10 cells were pulsed between flat electrodes spaced of 0.4 cm with LV-LP or HV-SP parameters in the presence or not of 2 μg GFP plasmid DNA. LV-LP parameters consist in 6 square wave electric pulses of 5 ms, 600 V/cm (240 V) applied at the frequency of 1 Hz. HF-SP parameters consist in 4 square wave electric pulses of 1200 V/cm (480 V), duration 100μs applied at the frequency of 1 Hz. 24 h after treatment GFP expression was analyzed by flow cytometry to determine transfected cells percentage (A) and GFP mean fluorescent intensity (B). Viability (C) was determined by cristal violet coloration. (D) Representative cytometry dot plots and (E) representative pictures in bright field (higher row) and fluorescence (lower row) images of GFP expression in LF-LP (left) and HF-SP (right) conditions. Scale bar represents 100 μm. Statistical analysis was conducted using two-tailed Mann-Withney *t*-test (n=6, 3 independent experiments). $^{*}p < 0.05$, $^{**}p < 0.005$.

(plasmid expression) 2 fold relative to the TCHD untreated cells (Fig. 5A and C). TCHD had a negative effect when added just after pulse (Fig. 5A). Moreover, it appeared that TCHD did not increase pulsed CHO cell viability (Fig. 5B).

4. Discussion

In this study, we observed that the post-pulse addition of TCHD increases electrotransfered pDNA expression. This result is confirmed with different electrical parameters, different plasmids and in different cell lines.

The increase in expression could result from an enhanced entrance of pDNA into the nucleus due to the effect of TCHD on the nuclear envelope. The percentage of plasmid expressing (positive) cells is increased whether TCHD is added just after or 10 min after pulse delivery. Nevertheless, an increase in Mean Fluorescence Intensity reflecting plasmid DNA expression is observed only when TCHD is added 10 min after pulsing. This difference between the observations can be explained by the following timing sequence: it has been described that the application of transfecting electric pulses to cells in presence of plasmid DNA induces formation within 1 s of localized pDNA/membrane aggregates in the part of the cell facing the cathode [6]. 10 min later, pDNAs have crossed the membrane and are detected in the cytoplasm [6]. Due to limits in the sensitivity of the video detection, it was never possible to detect the pDNA after its uptake into the nucleus as only a very small fraction of pDNAs can indeed reach the nuclear compartment from the cytoplasm [6]. Moreover, plasmid DNA bigger than 2000 bp does not diffuse in a free manner in the cytoplasm [42,43] but uses microtubules and dynein to reach the nucleus [9]. The association of plasmid DNA to cytoskeleton microtubules is dependent on the presence of specific sequence on the DNA as Nuclear Localization Sequences (NLS) carried by CMV or SV40 promotors [44]. pCMV-eGFP and pCMV-CpGfree-tdTomato plasmid used in this study are both controlled by pCMV promotor. The association of plasmid DNA, alone or encapsulated in clathrin vesicles, to cytoskeleton motors induces a very fast migration of plasmid DNA through the cytoplasm to the nucleus (in less than 15 min) [10]. Therefore when TCHD is added just after pulses, because it has amphipathic properties, it will affect the properties of electropermeabilized membrane [5,45–49] and impairs the transfer of plasmid DNA through the membrane. This effect could explain the difference in GFP-MFI if TCHD is added just after or 10 min after pulsing [5,45–49]. The effect of TCHD on the nuclear envelope cannot be detected.

The only exception appears with a 30 min incubation time when plasmid DNA already migrates through the cytoskeleton and is near the nucleus. TCHD addition 10 min after pulses occurs after electropermeabilized membrane already reseals [50–52] and pDNA is released in the cytoplasm. However, because TCHD has amphipathic properties, it is able to diffuse across plasma membrane rather fast. It was observed that its effect on the nuclear envelope was observed less than 1 min after its incubation with cells [34]. Due to TCHD effect, NPC opening occurs just before plasmid DNA localization near the nucleus (Figs. 2–5). This timing allows more DNA plasmid copies to enter the nucleus increasing expression, *i.e.* fluorescence intensity. Thus, it appears that TCHD post treatment addition, after plasmid DNA electrotransfer to the cytoplasm, does not increase the number of plasmid copies entering in the cells, nor the number of cells that are transfected, but, it increases the number of plasmid copies entering the nucleus.

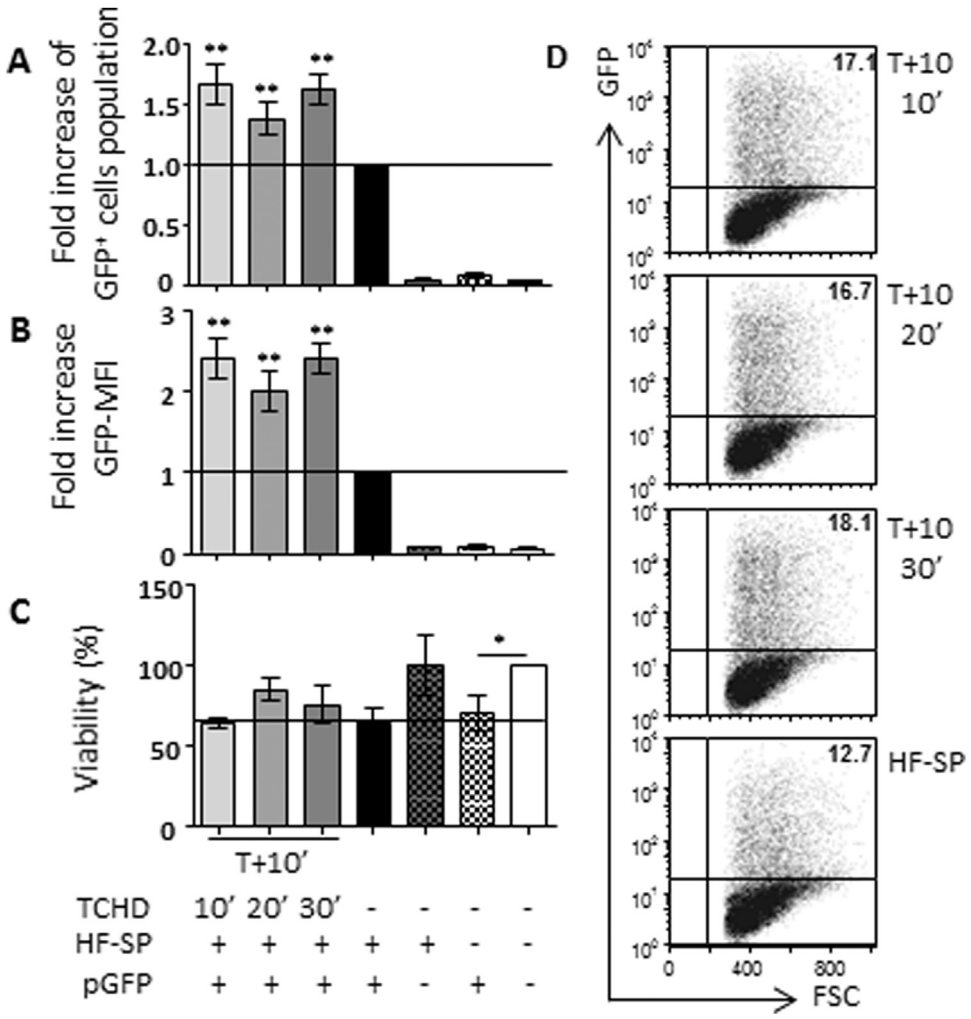

Fig. 4. TCHD increases GFP plasmid expression in B16F10 cells under HF-SP electrical parameters B16F10 cells were pulsed in the presence or not of 2 μg GFP plasmid DNA. 4 square wave electric pulses of 1200 V/cm (480 V), duration 100μs were applied at the frequency of 1 Hz at room temperature using flat electrodes spaced of 0.4 cm. 20 μl of TCHD 1% (final concentration W/v) was added 10 min after pulses and incubated 10, 20 or 30 min. 24 h after treatment GFP expression was analyzed by flow cytometry to determine transfected cells percentage (A) and GFP mean fluorescent intensity (B) fold increases using as a reference the values of the HF-SP condition in presence of plasmid (black line). Viability (C) was determined by crystal violet coloration. (D) Representative cytometry dot plots of pulsed cells treated or not with TCHD 10 min after pulse. Statistical analysis was conducted using two-tailed Mann-Withney *t*-test (n=6, 3 independent experiments). $^{**}p < 0.005$.

This results in an increase of cell fluorescence intensity in each cell and thus, to an increase in the percentage of positive cells, *i.e.* which fluorescence is now above the detection threshold set on the cytometer (*i.e.* the cell autofluorescence).

TCHD incubation time does not reveal significant differences in number of transfected cells or in plasmid expression. In experiments in which Cyanine-5 labeled plasmid DNA was microinjected in the cytoplasm of vero cells, it was shown that the nuclear Cy-5 fluorescence intensity increases during at least one hour after the injection only when TCHD was in the incubation solution [34]. In our experiment we add TCHD 10 min after pulses and let it up to a 30 min incubation meaning up to 40 min after gene electrotransfer. No significant difference was linked with the duration of this incubation period. This time is maybe too short to see significant differences. It should also be proposed that by adding TCHD, the plasmid quantity that enters the cell saturates the nuclear machinery. Because, as it was previously described [38,39], we showed that efficiency under LF-LP parameters can be improved by using HF-SP parameters, the lack of effect of TCHD incubation time on transfection can not be explained by this saturation hypothesis. Moreover these HF-SP parameters are less critical for cell survival. One open question may be the negative consequence of the long incubation (up to 40 min) in the pulsation buffer [53,54]. The induced starving should interfere with expression.

A decrease in cell survival was always observed when plasmid is added to the pulsing buffer. In Fig. 4, the survival of unpulsed B16F10 cells is decreased from 100% without plasmid (considered as a reference) to 70 ± 10% in presence of GFP plasmid. Under HF-SP parameters, cell survival decreases from 100 ± 19% in absence of plasmid to 64.5 ± 9.1% in presence of GFP plasmid. This was already observed more than 20 years ago [55]. It was reported that "the permeability of cell membranes for an indifferent dye was shown to increase noticeably if the cells were pulsed in the presence of DNA". This suggested that the pDNA interaction with the membrane was affecting its organization in a dramatic way as the uptake of macromolecules was present several minutes after the electric pulse delivery [56]. This negative effect was partly counterbalanced by the incubation with post-pulse addition of TCHD.

To conclude, this study demonstrates that transfection rate can be increased *in vitro* by the use of chemical compounds such as TCHD that act directly on the nuclear envelope and more precisely on the NPC. A previous study showed that TCHD did not prevent electrotransfection *in vivo* [57,58] Expression of a fluorescent protein is observed in post-mitotic neurons with a localized delivery of electric pulses following the injection of a TCHD-plasmid

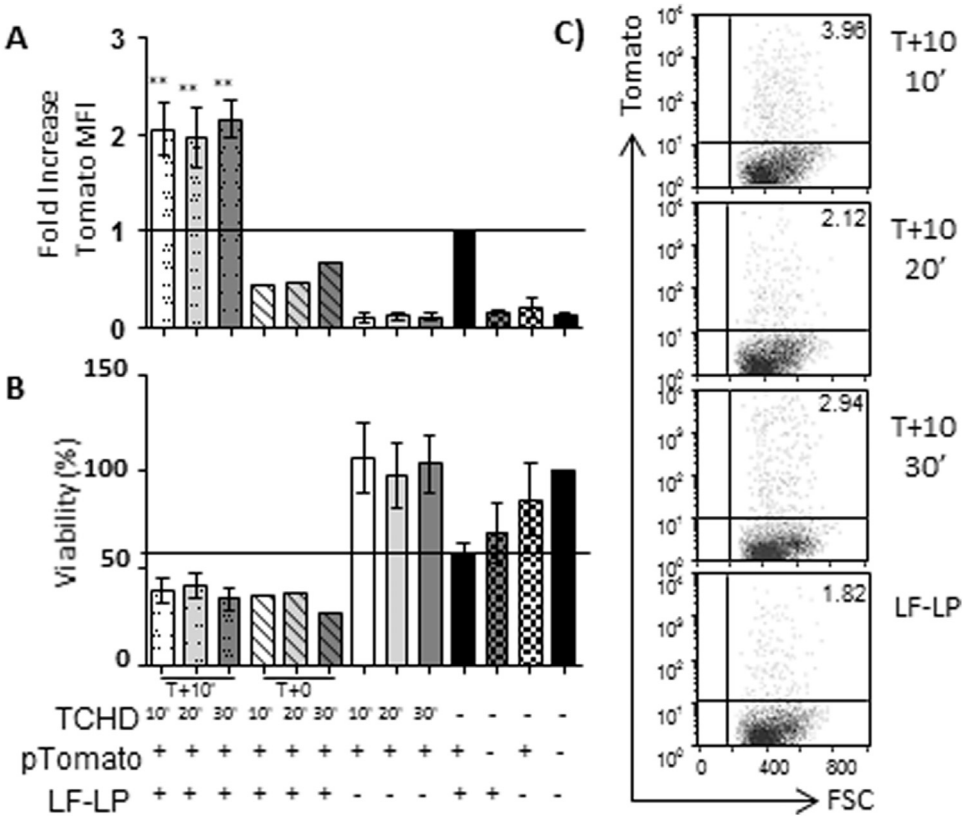

Fig. 5. TCHD increases Tomato plasmid expression in CHO cells under LF-LP electrical parameters CHO cells were pulsed in the presence or not of 2 μg tomato plasmid DNA. 6 square wave electric pulses of 600 V/cm, duration 5 ms were applied at the frequency of 1 Hz at room temperature using flat electrodes spaced of 0.4 cm. 20 μl of TCHD 1% (final concentration W/v) was added 10 min or just after pulse and incubated 10, 20 or 30 min. 24 h after treatment tomato expression was analyzed by flow cytometry to determine Tomato mean fluorescent intensity fold increase (A) based on the value of the LF-LP condition in presence of plasmid (black line). Viability (B) was determined by crystal violet coloration. (C) Representative cytometry dot plots of pulsed cells treated or not with TCHD 10 min after pulse. Statistical analysis was conducted using two-tailed Mann-Withney *t*-test (n=5, 3 independent experiments). $^{**}p < 0.005$.

mix in the brain. Such a protocol did not give any details on the local concentration in TCHD. Furthermore this work did not discuss the specific electrical and chemical parameters that could be required to optimize transfection in ventricular versus cortical neurons while our current work emphasized on the efficacy of TCHD use on electrotransfection efficiency comparing strictly identical electrical conditions. Nevertheless, these results amplify the promising effect of TCHD use *in vivo*.

5. Conclusions

To conclude, this study demonstrates that transfection rate can be increased *in vitro* by the use of chemical compounds such as TCHD that act directly on the nuclear envelope and more precisely on the NPC. It is crucial to remind that TCHD is a metabolite of the solvent cyclohexanone, which was found as a contaminant of intravenous dextrose and the parenteral feeding solution, and was also leached into the infusion fluids from the administration set. Because TCHD is FDA approved it can be considered to be used for *in vivo* studies in order to increase naked DNA transfection. By this way it can be considered to decrease plasmid quantity in order to decrease negative side effects of electro-gene-transfer in clinical studies.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.07.012.

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