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The Effect of Lipid Antioxidant α-Tocopherol on Cell Viability and Electrofusion Yield of B16-F1 Cells In Vitro

Masa Kanduser[1] · Mojca Kokalj Imsirovic[1] · Marko Usaj[1]

Abstract

Induced cell fusion is a powerful method for production of hybridoma in biotechnology and cell vaccines in medical applications. Among different alternatives, physical methods have an advantage, as they do not require any additives. Among them electrofusion, an electroporation-based cell fusion method holds a great promise. Electric pulses cause cell membrane permeabilization and due to pore formation bring cell membrane into the fusogenic state. At the same time, however, they compromise cell viability. We used a train of 8×100 μs electric pulses, delivered at 1 Hz with strengths ranging from 400 to 1600 V/cm. We evaluated electrofusion efficiency by dual color microscopy. We determined cell viability, because during electroporation reactive oxygen species are generated affecting cell survival. The novelty of our study is evaluation of the effect of lipid antioxidant α-tocopherol on cell fusion yield and cell viability on mouse B16-F1 cells. Pretreatment with α-tocopherol slowed down dynamic of cell fusion shortly after electroporation. Twenty-four hours later, fusion yields between α-tocopherol treated and untreated cells were comparable. The viability of α-tocopherol pretreated cells was drastically improved. Pretreatment of cells with α-tocopherol improved whole electrofusion process by more than 60%. We believe that α-tocopherol holds great promise to become an important agent to improve cell electrofusion method.

Keywords Electrofusion · Vitamin E · In vitro

Introduction

Biological cell fusion is a natural phenomenon occurring during fertilization, diverse cellular processes, tissue development, regeneration and immune protection. Biotechnologically induced, "artificial" cell fusion is used for hybrid cell production. The most known example of hybrid production is hybridoma technology where hybrids combine innate functions of immune B-lymphocytes and cancer cells. Thus, the somatic cell fusion between B lymphocytes and myeloma cells gives rise to hybridoma capable of producing antigen specific monoclonal antibodies while growing in vitro (Tomita and Tsumoto 2011). Alternatively, cell hybrids between dendritic and cancer cells are used for therapeutic cancer cell vaccination. Such hybrids contain the elements required for presenting tumor antigens to host immune system and consequently elicit effective immune response to tumors (Ahmed and Bae 2014; Dannull et al. 2015; Gong et al. 2008; Pinho et al. 2016; Sukhorukov et al. 2006). Among different methods used for artificial cell fusion, application of electric pulses has a great potential. Electroporated membrane is able to fuse if it is in close contact with another membrane in the same state. This kind of cell fusion is termed electrofusion. Electroporation is a driving force leading cell membrane transition to so-called fusogenic state (Kanduser and Usaj 2014; Sukhorukov et al. 2006; Teissie et al. 1982; Tomita and Tsumoto 2011; Zimmermann 1982). From this prospective, it is interesting to note that fusion pore formation is observed also in biological cell fusion processes such as exocytosis, tissue development or regeneration (Chernomordik and Kozlov 2008; Irie et al. 2017; Kreutzberger et al. 2017). The dynamics of lipid bilayer fusion is multistep process involving several lipid intermediates. The starting point is initial flat membrane, which through the fusion stalk formation, circular

✉ Masa Kanduser
masa.kanduser@fe.uni-lj.si; masakanduser@gmail.com

Mojca Kokalj Imsirovic
mojca@mithraeum.si

Marko Usaj
marko.usaj@gmail.com; marko.usaj@fe.uni-lj.si

[1] Faculty of Electrical Engineering, University of Ljubljana, Trzaska 25, 1000 Ljubljana, Slovenia

hemifusion diaphragm and fusion pore formation/expansion results in cytoplasm mixing (Chernomordik and Kozlov 2003; Kozlovsky et al. 2002; Kozlovsky and Kozlov 2002).

From the perspective of biotechnological and biomedical applications, it is important to obtain viable hybrids. Therefore, electric pulse parameters used for electrofusion need to be adjusted in order to obtain fusogenic membrane and at the same time preserve cell viability. In our previous work, we reported various factors that need to be taken into account to obtain high fusion yield (Rems et al. 2013; Ušaj et al. 2010; Usaj et al. 2013; Ušaj and Kandušer 2015). The parameters such as hypotonic media, amplitude of electric pulses that are required for high fusion yield compromise cell viability (Ušaj et al. 2010; Usaj et al. 2013). Electroporation takes place at the cell membrane level having as a consequence transient hydrophilic pore formation in the lipid bilayer (Neumann et al. 1982). Increasing number of reports indicate that reactive oxygen species (ROS) are produced at cell membrane level during electroporation (Bonnafous et al. 1999; Gabriel and Teissie 1994; Maccarrone et al. 1995; Markelc et al. 2012). Electrofusion and electrogene transfer require viable cell. Scavenging ROS produced during electroporation with antioxidant tempol can improve electrogene transfer to mice myoblasts (Markelc et al. 2012); therefore, we expect that antioxidants can also improve cell electrofusion. For quick implementation in biomedical applications, the selection of potential antioxidant used in cell fusion protocols can be based on natural substances localized on the cell membrane. Vitamin E is an important naturally occurring antioxidant (Halliwell 2011). The most common form of vitamin E α-tocopherol has a potent lipophilic antioxidant properties and has been associated with the cell membrane repair after mechanical injury in wounded myocytes (Howard et al. 2011). So far, only one paper has been dealing with the effect of α-tocopherol on electrofusion. The selected cells were plant protoplast. Preincubation of protoplasts with antioxidants ascorbate or α-tocopherol for half an hour to an hour before application of electric pulses reduced the lipid degradation and formation of lipid free radicals during electrofusion and α-tocopherol additionally increased fusion yield (Biedinger et al. 1991). The other papers investigated the effect of α-tocopherol on cell fusion of avian erythrocytes and exocytotic granules, when α-tocopherol was added to a suspension of hen erythrocytes and incubated for up to 4 h the cells spontaneously fused (Ahkong et al. 1973). Wang and Ping, however, reported that α-tocopherol has at least two roles in cell membrane. On one hand, it induces phase separation causing membrane destabilization and promote membrane fusion, while on the other it stabilizes cell membrane by interaction of its chromanol group with phospholipid head groups and thus prevents membrane fusion (Wang and Ping 1999). The latter was found for synexin aggregated chromaffin granules where addition of α-tocopherol prevented their fusion (Creutz 1981). Similarly, the Ca^{2+}-induced fusion of large unilamellar vesicles of phosphatidylserine (Aranda et al. 1996) was inhibited by α-tocopherol.

The aim of our study was to determine the role of membrane-incorporated lipophilic antioxidant α-tocopherol on cell fusion and viability after electric pulse application. To the best of our knowledge, this is the first report on the effect of α-tocopherol on mammalian cell electrofusion. We first determined optimal electric field strength with high electrofusion yield and moderately or severely affected cell viability. Then, we evaluated the effect of α-tocopherol on cell fusion and viability. The dynamic of cell fusion yield was determined by monitoring the percentage of fused cell half an hour, an hour and 20 min and 24 h after electric pulse application. The results demonstrate effective preservation of cell viability on the expense of slower initial dynamic of cell fusion in cells pretreated by α-tocopherol. Nevertheless, 24 h after the electric pulse application the fusion yields of surviving cells were comparable to untreated cells. Considering both parameters (i.e., fusion yield and cell viability), we improved electrofusion process by more than 60%. The reported results shed new light on the role of α-tocopherol in cell electrofusion and preservation of cell viability in mammalian cells. This result provides new insights and can improve biotechnological and medical applications based on cell electrofusion.

Materials and Methods

Chemicals, Cell Culture Media

Dulbecco's minimal essential medium (DMEM), fetal bovine serum (FBS), L-glutamine, sucrose, dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), magnesium chloride ($MgCl_2$), acetic acid, crystal violet (CV), trypsin and EDTA were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Germany). Antibiotics (crystacillin and gentamicin) were purchased from Pliva (Pliva d.o.o, Croatia). CMFDA and CMRA cell trackers were purchased from molecular probes (Invitrogen, USA).

Cell Culture and Electrofusion Buffers

Mouse melanoma (B16-F1) cells were cultured in humidified atmosphere at 37 °C and 5% CO_2 in the DMEM supplemented with 10% FBS, antibiotics (crystacillin, gentamicin) and L-glutamine. Cells were grown in 25 cm^2 culture flask until they reached 70–80% confluence. On the day of the experiment, cell suspension was prepared by 0.25% trypsin/EDTA solution. Trypsin solution was then removed, and culture media was added. Cells were gently rinsed from the

bottom with plastic pipette, and homogenous cell suspension was prepared.

Iso- and hypo-tonic potassium phosphate buffer—KPB (10 mM K_2HPO_4/KH_2PO_4, 1 mM $MgCl_2$) with 250 or 75 mM sucrose corresponding to osmolarities 262 and 93 mOsm (mOsmol/kg), respectively, was used in the experiments. The conductivity of both buffers was 1.67 mS/cm and pH 7.2.

Cell Preparation and Labeling

Cells were labeled with 7 μM green (CMFDA) or red (CMRA) cell trackers. Two 25 cm^2 culture flasks with cells attached at the bottom were washed and labeled: one flask with green CMFDA (excitation/emission = 492 nm/517 nm) and the other with red CMRA (excitation/emission = 548 nm/576 nm) dye. Both solutions were prepared in bicarbonate-free Krebs-Hepes buffer (Ušaj and Kandušer 2015). Cells were stained for 45 min at 37 °C. Afterward cells were washed with culture media and maintained at 37 °C for another hour. For experiments, we prepared homogenous cell suspension of green and red cells in proportion 1:1.

Electrofusion Protocol

Close cell–cell contacts were established by a modified adherence method, MAM (Ušaj and Kandušer 2015). We established a monolayer of spherical cells in a close contact by placing 40 μl drop of cells in concentration 2×10^6 cells/ml in each well of 24-well plate (TPP, Switzerland). Cells were then incubated in 5% CO_2 at 37 °C for 20 min to allow them to slightly attach to the surface and to each other, while maintaining round shape. Before electroporation, cells were washed with isotonic buffer and then 350 μl of hypo-tonic buffer was added. Two minutes later, when cells reach their maximal volume (Ušaj et al. 2009) we applied a train of 8×100 μs electric pulses (1 Hz) and electric field strengths between 400 and 1600 V/cm. We used two parallel wire electrodes (Pt/Ir = 90/10) with five mm gap. For control treatment, no pulses were applied. After pulse delivery, the cells were left undisturbed for 10 min for cell fusion to take place. Then fusion media was removed and 1 ml of complete cell culture media was added. Fusion yields were determined 30 min, 1 h and 20 min and 24 h after electroporation by dual-color fluorescence microscopy as described in details previously (Ušaj and Kandušer 2015).

Determination of Electrofusion Yield of Surviving Cells

The fusion yield was determined in three channel images of CMFDA (492 nm, HQ535/30 m), CMRA (548 nm,

D605/55 m, Chroma, USA) and bright field. Images were acquired by inverted microscope AxioVert 200 (Zeiss, Germany) under × 20 objective magnification. Three images (bright field, green, red fluorescence) were acquired from five randomly chosen fields for each sample using cooled CCD video camera VisiCam 1280 (Visitron, Germany) and PC software MetaMorph 7.7.5.0. (Molecular Devices, USA). Three channel images were created from each image triplet (bright field, green and red fluorescence) in image processing software ImageJ (NIH Image, USA). The cells were manually counted, and the fusion yield was calculated as percentage of double labeled cells as described in details previously (Ušaj and Kandušer 2015).

Determination of Viability

The viability drop caused by electric pulse application was analyzed 24 h after electrofusion experiments by means of a modified crystal violet (CV) viability assay (Ušaj et al. 2009). Culture medium was removed from 24-well plate, and cells were washed with the isotonic buffer (1 ml per well). Cells were stained for 30 min with 200 μl 0.10% CV solution per well. Dye was carefully removed, and cells were washed three times with the iso-tonic buffer and lysed with 1 ml of 10% acetic acid per well. The absorption of lysate was measured with a microplate reader Infinite M200 (Tecan, Switzerland) at 595 nm wavelength controlled with PC software Magellan (Tecan, Switzerland) at maximum 1 h after the dying procedure. The viability of treated cells was defined as a ration between treated and untreated cells (Ušaj et al. 2009).

Electrofusion with α-Tocopherol

We determined the concentration of α-tocopherol, the time and the way of incubation. We tested concentrations of α-tocopherol in the range between 0.02 and 0.7 mM on cell survival and electrofusion. Based on those results, 0.02 mM α-tocopherol was selected for treatment before and after electric pulse application. For those experiments, we added α-tocopherol 2 min before, immediately after and 10 min after electroporation (i.e., 12 min after the cells were exposed to hypotonic buffer). In separate experiments, we determined the effect of preincubation with α-tocopherol. In those experiments, cells were growing in the medium enriched with 0.05, 1, 2 and 3 mM α-tocopherol for different periods and we determined the treatment effect on cell viability. In 24-well plate, we plated 1×10^5 cells/ml 1×10^4 cells/ml and 1×10^3 cells/ml per well for 24, 48 and 72 h cell culture. Therefore, cells were incubated for various lengths of time at different concentrations of α-tocopherol in complete cell culture medium. The viability of treated cells was

analyzed by a modified crystal violet viability assay (Usaj et al. 2009).

For further electrofusion experiments, we incubated cells for 48 h in 0.05 mM α-tocopherol added in complete cell culture media. On the day of electrofusion, cells were stained with fluorescent dyes and prepared as described above. We used the electric field strengths of 1000 V/cm and 1600 V/cm. After delivery of pulses, cells were left undisturbed for 10 min. After that, fusion media was removed and complete cell culture media was added. Images for further processing and determination of the fusion yield were taken 30 min and 24 h after electric pulse application. Cell viability of treated cells was analyzed 24 h after electrofusion.

The experiments were performed as independent repetitions at different dates. Each experiment was done with different cell culture sample. The mean values were calculated, and the results are presented as a mean ± standard deviation. The differences between treatments were determined statistically by paired T test. To determine the optimum electric field strength for electrofusion, we calculated Euclidean distance from theoretical optimum (50% fused cells 100% viability) and calculated the distance to obtained results as described in "Discussion" section.

Results

First, we evaluated the dynamic of cell fusion and viability. We determined the optimal strength of the electric field that provided high fusion yield of surviving cells. Then, we evaluated the concentration and way of incubation with lipid-soluble antioxidant α-tocopherol. Finally, we determined the effect of α-tocopherol on the dynamic of cell fusion and viability.

The Optimal Strength of the Electric Field for Electrofusion

We determined the impact of electric field strengths from 400 V/cm to 1600 V/cm on cell fusion yield at three different time intervals; 30 min, 1 h and 20 min and 24 h after electric pulse application and on viability 24 h after electroporation (Fig. 1).

The percentage of fusion yield increased with increasing electric field strength and reached saturation at 1000 V/cm. At this strength, the percentage of fused cells was $44 \pm 2\%$, $36 \pm 4\%$ and $45 \pm 6\%$ recorded at 30 min, 1 h and 20 min and 24 h after electric pulse application. At the electric field strength, where the cell fusion yield reached saturation (1000 V/cm) cell viability was 60–70%. The percentage of surviving cells decreased at higher electric field strengths reaching only 35% at 1600 V/cm (Fig. 1).
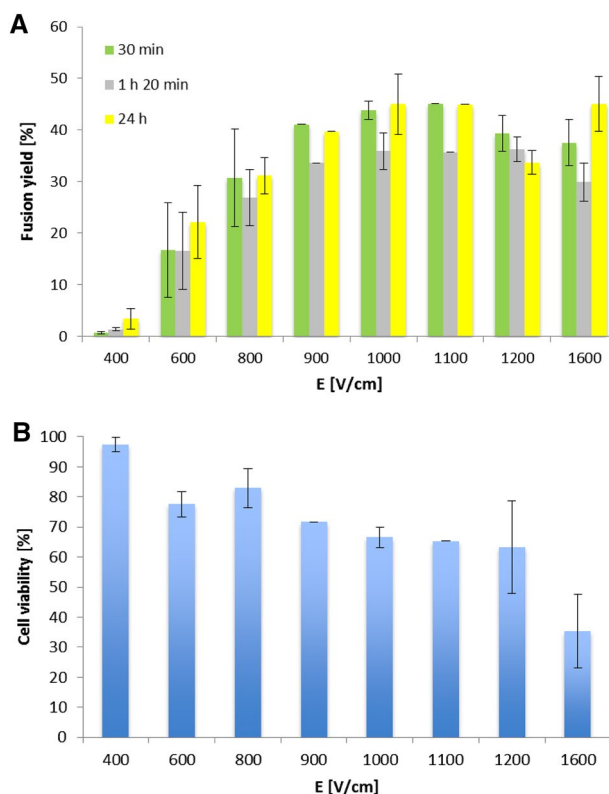

Fig. 1 Effect of electric field strength on **a** fusion yield and **b** cell viability at 8×100 μs pulses delivered at 1 Hz frequency. Cell fusion was determined 30 min, 1 h and 20 min and 24 h after electric pulse application by dual color staining with CMRA/CMFDA fluorescent dyes. Cell viability was detected spectrophotometrically 24 h after electric pulse application by crystal violet assay. The values represent three independent repetition of the experiment ± standard deviation

Thus, we determined the optimal strength of the electric field with the highest proportion of fused and viable cells at 1000 V/cm and used it in further experiments with antioxidant α-tocopherol. To test the effect of α-tocopherol on cell survival, we choose also 1600 V/cm due to low cell viability.

Effect of α-Tocopherol on Cell Viability and Electrofusion

We examined different treatment protocols and added α-tocopherol before and after electric pulse application. Cell fusion was detected at two intervals; shortly after pulse application at 30 min and 24 h after experiment.

The first question was when in electrofusion protocol we should add α-tocopherol to detect the effect on cell fusion and viability. We examined different possibilities of adding antioxidant before or after electric pulse application. For electrofusion, we use hypotonic buffer; therefore, we tested three alternatives with this buffer. The first one was addition of α-tocopherol two minutes before electric pulse application, i.e., the α-tocopherol was included in hypotonic

electrofusion buffer recipe. The second alternative was addition of the α-tocopherol immediately after electric pulse application, and the third alternative was addition of α-tocopherol 10 min after electric pulse application when the electrofusion buffer was exchanged with complete cell culture media. The electrofusion efficiency was detected thirty minutes after electric pulse application (Fig. 2). The addition of 0.02 mM α-tocopherol in complete cell culture media was toxic to the cells if added 10 min after electric pulse application (i.e., 12 min after cells were placed in electrofusion buffer) as can be seen in images acquired 30 min after electric pulse application (Fig. 2d). The viability of the cells exposed to an 8×100 µs pulses (1 Hz) of 1000 V/cm recorded 24 h after treatment was: 72% for control cells (i.e., no addition of α-tocopherol), 75% for α-tocopherol added before electric pulse application and no cell survived the addition of α-tocopherol 10 min after electric pulse application.

To determine the effect of α-tocopherol on the cell viability, we compared the survival of the electroporated cells in electrofusion medium with or without α-tocopherol. In the concentration range between 0.02 mM and 0.7 mM, the difference in cell viability was between 0 and 7% which is in the range of experimental error.

Therefore, we decided to preincubate the cells with α-tocopherol before electrofusion to allow its integration into the cell membrane. We tested the effect of different preincubation periods (24 h, 48 h and 72 h) and concentrations

of α-tocopherol in the cell culture media on the cell growth (Table 1). The best effect was obtained with the 48 h preincubation period; therefore, we further determined the optimum concentration of α-tocopherol for this preincubation period. In the concentration range between 0.05 mM and 3 mM (Table 1), we obtained the best results at 0.05 mM α-tocopherol. It seems like this concentration (and as well 1 mM concentration) stimulates cell divisions. At those concentrations, we obtained between 30 and 40% more viable cell than in the control treatment. On the other hand, longer preincubation time and higher α-tocopherol concentrations

Table 1 Effect of α-tocopherol enriched cell culture media on cell growing

Incubation at 0.05 mM	Cell viability (%)	48 h incubation	Cell viability (%)
		0.05 mM	144
24 h	133	1 mM	133
48 h	144	2 mM	30
72 h	31	3 mM	25

On the left, the percentage of the cell viability determined at 24 h, 48 h and 72 h preincubation period of cells in complete cell culture media enriched with 0.05 mM α-tocopherol. On the right, the effect of different concentrations of α-tocopherol on cell viability after 48 h preincubation period of the cells in complete cell culture media enriched with denoted α-tocopherol concentrations

Fig. 2 Cell incubation with 0.02 mM α-tocopherol before or after electric pulse application. Cell fusion yield of surviving cells was in all cases determined 30 min after electric pulse application. **a** α-tocopherol treated cells without pulse application—control conditions, **b** α-tocopherol added to the cells in electrofusion buffer two min before electric pulse application, **c** α-tocopherol added to the cells in electrofusion buffer immediately after electric pulse application and **d** α-tocopherol added to the cells 10 min after electric pulse application in cell culture media. Three-channel images of B16-F1 cells consist of bright field, red (CMRA) and green (CMFDA) fluorescence acquired at objective magnification of ×20 fused at 8×100 µs, 1 Hz, 1000 V/cm
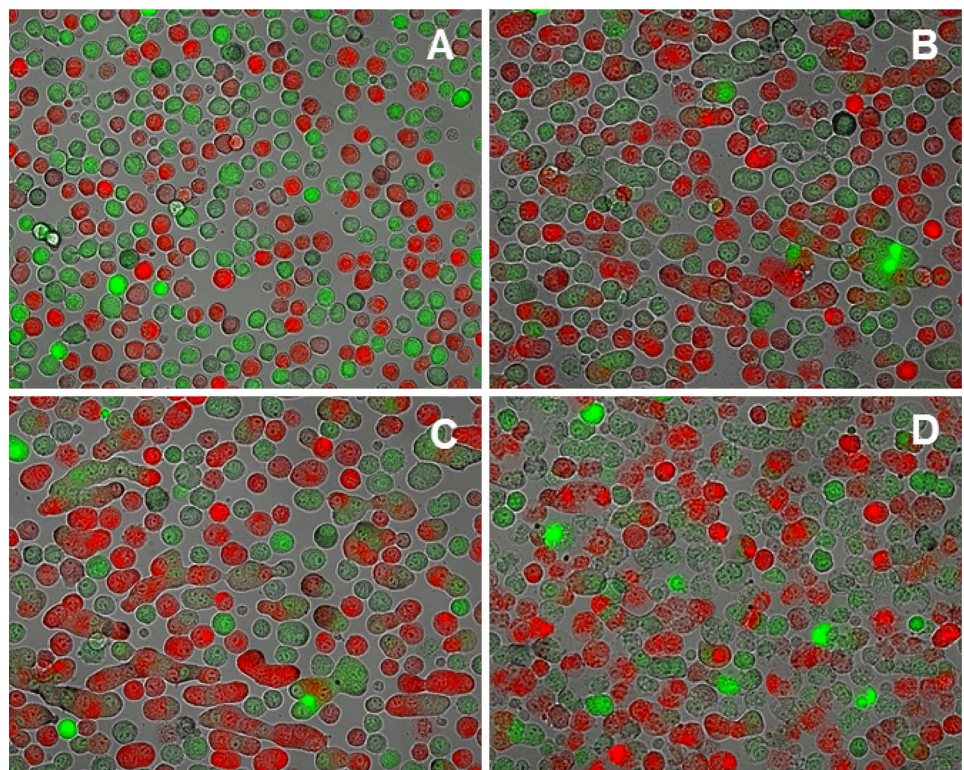
abolished cell growth in comparison with control cells (Table 1).

In further experiments, we evaluated the effectiveness of the electrofusion at two selected electric field strength: 1000 V/cm and at 1600 V/cm. Those strengths were chosen due to significantly different cell viability after electroporation (66% and 35%, respectively), but with similar yield of survived fused cells (Fig. 1). In Fig. 3 we present electrofusion yield cells determined 30 min and 24 h after electric pulse application. Thirty minutes after cell electroporation, fusion yield of cells treated with α-tocopherol reached 17% for 1000 V/cm and 26% for 1600 V/cm. Cell fusion yield determined at 30 min for 1000 V/cm was significantly lower for the cells pretreated with α-tocopherol ($P < 0.05$) in comparison with control (α-tocopherol untreated) cells. After 24 h, the values increased and reached 39% at 1000 V/cm and 41% at 1600 V/cm and were not significantly different in comparison with control. We can see, however, that the fusion yield increased with time in α-tocopherol treated cells while in untreated cells it reached saturation already 30 min after electric pulse application (Fig. 3). On the other hand, the α-tocopherol treatment significantly improved cell viability. At 1000 V/cm 93% of cells survived electrofusion process and 80% at 1600 V/cm (Fig. 3), which is statistically higher in comparison with α-tocopherol untreated cells ($P < 0.05$). The average increase in the viability of α-tocopherol pretreated electroporated cells was 26% and 45% for 1000 V/cm and 1600 V/cm, respectively. This is considerably higher from the condition where α-tocopherol was added only to the electrofusion buffer, where this difference was up to 7%.

Discussion

In our study, we evaluated the effect of α-tocopherol on cell viability and on dynamic of cell fusion yield at optimal 1000 V/cm and high 1600 V/cm electric field strengths. At these electric field strengths, cell viability is not affected by products of electrode degradation (Kotnik et al. 2001). We observed the process shortly (30 min) and 24 h after electric pulse application. The results indicate slower cell fusion process but significantly better viability of cells pretreated with α-tocopherol. Those results are important contribution for improved electrofusion needed in many biotechnological and biomedical applications, where good relation between cell fusion yield and cell viability is needed.

We have determined the optimal electric field strength with the highest ratio of fused and viable cells by plotting cell viability versus cell fusion (Fig. 4). For control (α-tocopherol untreated cells), the optimal electric field strength was 1000 V/cm (Fig. 1). As can be seen in Fig. 4, this point was the closest to the theoretical optimum (50% cell fusion yield and 100% cell viability). It is important to note that the method of the dual color fusion detection used in bulk cell electrofusion studies detects only half of all fusion events discarding cell fusion of the same color (Ušaj and Kandušer 2015).

The fusion yield was determined at three intervals. The most common intervals reported in the literature were 10 min (Ramos et al. 2002; Ušaj et al. 2010; Usaj and Kanduser 2012; Ušaj and Kandušer 2015), 2 (Rols and Teissie 1989) and 24 h (Sukhorukov et al. 2006; Usaj et al. 2013). Our results indicate that the determination of the fusion yields at different time intervals did not affect the result (Fig. 1). However, for cells pretreated with α-tocopherol, process of cell fusion was slowed down (Fig. 3). We would like to note that the observation of cell fusion dynamics in our study was limited by the methodology. We could only detect fused cells after the final step of the fusion process. Before this step can takes place cell membranes of both
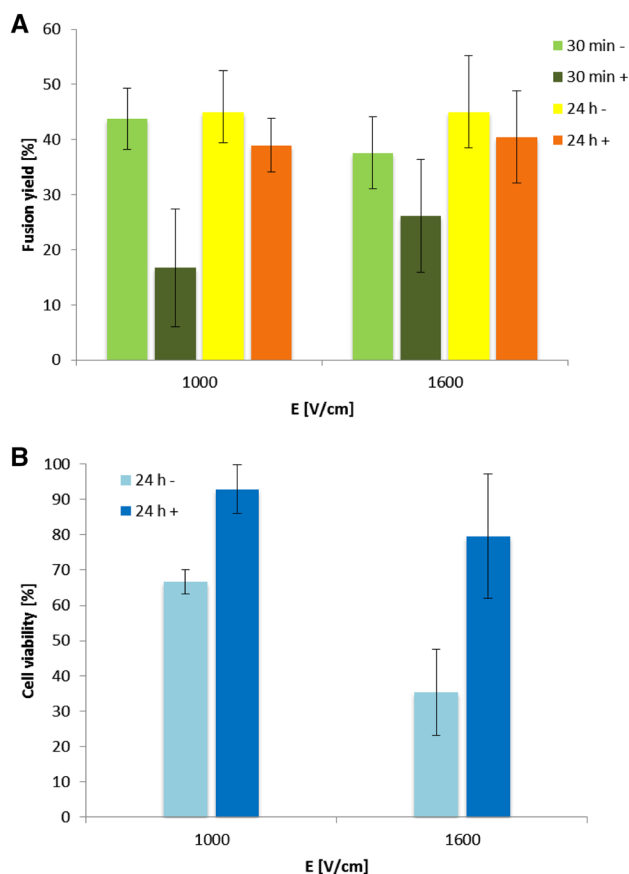

Fig. 3 Fusion yield and cell viability of B16-F1 cells incubated with 0.05 mM α-tocopherol for 48 h after application of electric pulses of 8×100 μs, 1 Hz, 1000 V/cm and 1600 V/cm. **a** Cell fusion yield determined 30 min and 24 h and **b** cell survival determined 24 h after the electric pulse application. The data are means of three independent experiments ± standard deviation. "−" cells without and "+" cells pretreated with α-tocopherol
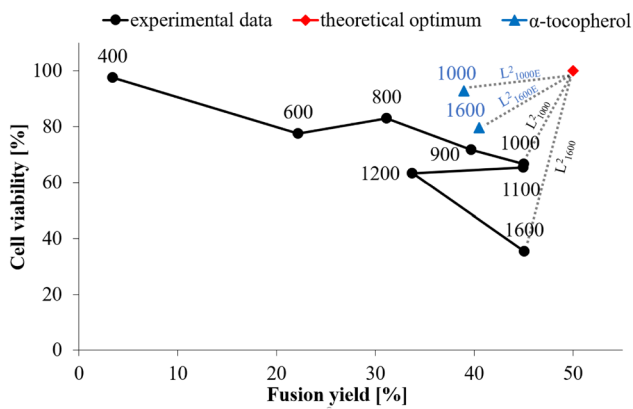
Fig. 4 Cell fusion yields versus cell viability. To determine the optimal electric field strength for cell fusion (values above data points in V/cm), we defined theoretical optimal as 50% of cell fusion yield and 100% cell viability (red rhomboid). The experimental values closest to this theoretical optimum were obtained at 1000 V/cm for α-tocopherol untreated cells. This was improved when cells were pretreated with α-tocopherol as indicated for two electric field strengths (blue triangles). Cell line B16-F1, electroporation in hypotonic buffer with 8×100 μs pulses delivered at 1 Hz. We can now calculate the distance between optimum value and each experimental data, so-called Euclidean distance (L^2, dotted lines). L^2_{1000} is the shortest distance for α-tocopherol untreated cells; thus, 1000 V/cm is indeed the optimal electric field strength for these experimental conditions. By comparing distances L^2_{1000} with L^2_{1000E} and L^2_{1600} with L^2_{1600E}, we can note considerable improvement in electrofusion method by α-tocopherol treatment

fusion partners undergo much faster processes of lipid bilayer mixing which results in lipid pore formation and its expansion (Chernomordik and Kozlov 2008; Kozlovsky et al. 2002; Kozlovsky and Kozlov 2002).

The second important objective of our work was to improve survival of cells exposed to electric pulses with the lipid antioxidant α-tocopherol. For this aim, we selected the optimal electric field strength for electrofusion (Fig. 4) and the electric field strength where cell survival was drastically reduced. It is worth to mention that cell electrofusion of B16-F1 at electric field strength of 1000 V/cm is decent both in terms of fusion yield (~40%) and cell viability (~60%). We calculated Euclidean distance to evaluate the effect α-tocopherol on fusion yield and cell viability (see also Fig. 4). The calculated distance L^2_{1000} is 33.77 for control (α-tocopherol untreated cells) and L^2_{1000E} is 13.17 for α-tocopherol pretreated cells. The result is 61% total improvement in cell electrofusion at 1000 V/cm. For 1600 V/cm, the distances L^2_{1600} and L^2_{1600E} are 64.8 and 22.5 for control and α-tocopherol pretreated cells, respectively, resulting in total of 65% improvement.

There are many factors affecting cell electrofusion. The efficiency depends on the quality of cell contacts. For example, contacts established by dielectrophoresis can be controlled by duration of dielectrophoretic signal (Usaj et al.

2013; Zimmermann 1982), while in our MAM method they are controlled with the cell plating density. In our previous papers, we reported different fusion yields; we achieved 12% of fused cells at electric field strength 1200 V/cm for 2.68×10^4 cells/cm^2 (Ušaj et al. 2010) and the fusion yields increased to 32% for 4.08×10^5 cell/cm^2 (Usaj and Kanduser 2012). In the present study, we used 4.08×10^5 cell/cm^2 and obtained similar results (Fig. 1). For anchorage dependent cell lines, we establish cell contacts before electric pulse application and maintain them for the required period of time (Usaj et al. 2013). Thus, in order to evaluate parameters affecting cell electrofusion (like here α-tocopherol) it is crucial to have the method by which one can achieve consistent cell–cell contact (in terms of quality, duration, reproducibility and applicability to other cell lines). Our MAM method seems to be ideal for such experiments (Ušaj and Kandušer 2015). However, it has some drawbacks; (i) it produces multinucleated fusion products (can be minimized by using lower cell plating density) and (ii) results in unspecific cell fusion (i.e., cell contacts are established randomly). Electrofusion of cells in suspensions aligned in "pearl chain" by dielectrophoresis is characterized by short contact durations in the range of few seconds (Abidor and Sowers 1992; Sukhorukov et al. 2006; Zimmermann 1982), and membrane fusion should be more or less completed in that time. In such cases, high electric pulse strengths can compensate this short cell–cell contact duration. To obtain specific binucleated cell hybrids, one can use microfluidic devices. Effective cell fusion of two specific cell types has been reported recently in a microfluidic device combining hydrodynamic trapping and dielectrophoretic force (Hu et al. 2013; Lu et al. 2015; Wu et al. 2017). However, our method is still of great value and the obtained results are relevant for microfluidic devices.

External factors causing stress have an important impact on cell survival. Several publications have already reported that exposure of living cells to electric pulses is stressful. Similar to other extracellular stress stimuli also electric pulse exposure results in cellular stress response, release of reactive oxygen species (ROS) and reduced cell viability (Bonnafous et al. 1999; Gabriel and Teissie 1994; Maccarrone et al. 1995; Markelc et al. 2012). Most of the ROS species generated during electroporation that takes place at the lipid bilayer level were detected on electropermeabilized sites (Bonnafous et al. 1999; Gabriel and Teissie 1994). The most potent lipophilic antioxidant, which infiltrate into the cell membrane and protects the unsaturated fatty acids from oxidative damage is α-tocopherol (Sies 1993; Wolf 1998). Our results indicate that the effect depends on the concentration range as well as on incubation schedule (Table 1). Even though electric pulse induced membrane damage differs from mechanical cell injury, the protective effect of membrane integrated α-tocopherol is similar (Howard et al. 2011;

Wang and Ping 1999). The beneficial effect of α-tocopherol on cell viability was detected only if cells were preincubated with the substance for several days before electroporation rather than just by addition in the electrofusion buffer after electroporation (Fig. 2; Table 1; Fig. 3).

However, α-tocopherol in the cell membrane does not act only as antioxidant. Our results indicate that it can temporarily reduce the fusion yield by slowing down the electrofusion process (Fig. 3) of pretreated cells. The observed effect could be attributed to membrane stabilization. The chromanol-head group of α-tocopherol binds to the head group of phospholipids. Fluorescence anisotropy and electron spin resonance measurements indicated that α-tocopherol restricts acyl chain motion within the membrane and causes an increase in order parameter and decrease in the membrane fluidity. On the other hand, α-tocopherol induces phase separation of phosphatidylethanolamines in lipid bilayers. In cell membranes, α-tocopherol in some cases promotes membrane fusion due to membrane destabilization (Wang and Ping 1999). Recent reports relate cell fusion with changes in membrane fluidity obtained by cholesterol and phosphatidylethanolamine modulation (Dawaliby et al. 2016). This information is interesting as phosphatidylethanolamine has been proposed to promote fusion pore stabilization during exocytosis (Kreutzberger et al. 2017), and its redistribution in the plasma membrane was crucial for osteoclast fusion (Dawaliby et al. 2016). α-tocopherol could interact with phosphatidylethanolamine, change cell membrane fluidity and slower cell fusion, but in our opinion the relationship between cell electrofusion and membrane fluidity is not straightforward. Taking into account that pore formation is a crucial step in biological (Chernomordik and Kozlov 2008) and electro-stimulated fusion (Zimmermann 1982), one could expect that cell membrane fluidity would affect electroporation, i.e., pore formation. We have not been able to relate cell membrane fluidity with electroporation (Kanduser et al. 2008) and with current knowledge, we cannot explain the decrease in dynamic of cell fusion yield observed in Fig. 3 with the hypothetic differences in cell membrane fluidity caused by α-tocopherol. It was shown, however, that low concentrations (2 to 10 mol %) of α-tocopherol in phosphatidylcholine lipid vesicles decreased the initial rate of Ca^{2+} induced fusion. The decrease was proportional to the amount of α-tocopherol in the lipid bilayer. This effect was attributed to the stabilization and membrane rigidification since less fluid membranes do not readily undergo cell fusion (Aranda et al. 1996). Even in the relatively simple artificial lipid bilayer the effect of α-tocopherol depends on the specific molecular species of phospholipids (Sánchez-Migallón et al. 1996). In this context, it is important to remember that cell membrane is structure that is much more

complex. The cell fusion is a multistep biological process in which electroporation of the cell membrane is only one of the steps. Besides, as discussed before antioxidant α-tocopherol plays distinct roles in the living cell (Wang and Ping 1999). If the slower cell fusion process is a consequence of physical interaction of α-tocopherol with the cell membrane, drastically improved cell viability can be attributed to its anti-oxidative effects on cell membrane lipids.

For biomedical and biotechnological applications, effectiveness of electrofusion is important. For hybrid cell vaccines and hybridoma technology, we need a method to achieve high fusion yield of functional and viable cells. Results presented in our study show that pretreatment with α-tocopherol significantly improves fraction of fused viable cells.

Conclusions

We can conclude that cells grown in the culture medium enriched with α-tocopherol tolerate stress imposed by electric pulse application much better than untreated control cells. Our results indicate that α-tocopherol must be integrated into the cell membrane to protect lipids from ROS generated during electroporation. Cell fusion process of α-tocopherol treated cells is slower compared to untreated controls at the beginning but finally the dramatic increase in cell viability and fusion yield is obtained. The reported results shed new light on the role of α-tocopherol in mammalian cell electrofusion and can improve its biotechnological and medical applications. Cell electrofusion is a complex biological phenomenon where efficient cell membrane electroporation is only one of the steps that enable its initiation. Further studies are needed to reveal complete mode of action of α-tocopherol on cell electrofusion process, which will allow us to optimize α-tocopherol treatment in order to maintain excellent cell viability without unwanted slowing down of the electrofusion process itself.

Compliance with Ethical Standards

Conflict of interest Authors declare no conflict of interests.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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