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ORIGINAL ARTICLE

# Efficiency and cell viability implications using tip type electroporation in zebrafish sperm cells

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## Abstract

Sperm-mediated gene transfer (SMGT) has a potential use for zebrafish transgenesis. However, transfection into fish sperm cells still needs to be improved. The objective was to demonstrate the feasibility of tip type electroporation in zebrafish sperm, showing a protocol that provide high transfection efficiency, with minimal side-effects. Sperm was transfected with a Cy3-labelled DNA using tip type electroporation with voltages ranging from 500 to 1500 V. Sperm kinetics parameters were assessed using Computer Assisted Semen Analysis (CASA) and cell integrity, reactive oxygen species (ROS), mitochondrial functionality and transfection rate were evaluated by flow cytometry. The transfection rates were positively affected by tip type electroporation, reaching 64.9% ± 3.6 in the lowest voltage used (500 V) and 86.6% ± 1.9 in the highest (1500 V). The percentage of overall motile sperm in the electrotransfected samples was found to decrease with increasing field strength ($P < 0.05$). Increase in the sperm damaged plasma membrane was observed with increasing field strength ($P < 0.05$). ROS and sperm mitochondrial functionality did not present a negative response after the electroporation ($P > 0.05$). Overall results indicate that tip type electroporation enhances the internalization of exogenous DNA into zebrafish sperm cells with minimal harmful effects to sperm cells.

**Keywords** *Danio rerio* · Electroporation · Spermatozoa · Exogenous DNA · SMGT

## Introduction

Transfection of foreign genes has been explored to generate genetically modified animals by sperm-mediated gene transfer (SMGT) [1]. This technique is based on the ability of sperm cells to spontaneously bind exogenous DNA and transport it into an oocyte during fertilization [2]. However, this technique also suffers from a several drawbacks, being the most challenging the DNA bind and subsequently uptake by sperm cells. As sperm cells are considered hard-to-transfect cells, a series of studies has tried to enhance its DNA uptake [3, 4]. Besides, these studies produced different results, some actually produced transgenic animals [5] and others could not effectively generate transgenic animals [6]. These results varying according to species and the work contexts.

Classical electroporation is a non-viral electrotransfection method for DNA delivery into cells or tissues without the use of additional chemicals or viruses [7] and a fast and inexpensive approach to transfect sperm cells [8]. Despite

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increased DNA uptake by sperm cells, some problems are associated with the conventional cuvette type electroporation, such as low transfection rate, mosaic gene expression, and negative effects on cell viability [9]. Capillary electroporation (tip-type electroporation) was first described in 2008 [10] and since that, it has been consolidated as a new electrotransfection method capable to provide high transfection efficiency on a wide range of mammalian cells with high survival rates of treated cells [10, 11]. The mechanical design of tip type electroporation leads to rapid cell delivery by pipette, improving the electroporation efficiency. In addition, the design of pipette tips containing gold electrodes produced a very small electroporation reaction with more uniform electric field [10]. In this configuration, the negative effects of the conventional electroporation system, such as change in pH and generation of metal ions, harmful to the cells, are quite reduced, leading to a safer and more efficient transfection. Recently, we successful demonstrated the electrotransfection in cryopreserved bovine spermatozoa through a new protocol using the tip type electroporation [11]. However, no other studies using tip type electroporation for sperm cells have been conducted. Electroporation could be affected by several factors, on this way, the optimal conditions for tip type electroporation for each cell type must be experimentally determined [10]. In addition, interspecies and intra-species lead to a variability in transfection rate being an unsolved problem associated with electroporation [12]. Since 1999 electroporation based on cuvette chambers has been used to transform fish sperm cells [13, 14]. However, few data are available about the transfection rate for fish spermatozoa. Most studies only investigate the gene transfer efficiency, being found to be between 20–30% using conventional electroporation [12], without evaluating the transfection rate in fish sperm. Some studies have investigated the negative effects of seminal plasma DNase on sperm DNA uptake, however, no reliable data are available for optimization of electroporation for fish spermatozoa [15, 16].

Zebrafish is the most popular fish species for laboratory research being the transgenic zebrafish an experimental model for several studies in field of biomedical research, developmental biology and drug testing [17]. Zebrafish is also being considered as a possible animal model to study COVID-19 [18]. Nevertheless, laborious microinjection is still the standard method to generate transgenic zebrafish [15]. Until now, few studies been performed for zebrafish transgenesis using SMGT with controversial results [9, 13, 19]. Recently, different studies demonstrated that SMGT could be associated to genome editing tools such as CRISPR-Cas technology, increasing its potential use in the genome editing era to generate transgenic zebrafish [20–22].

The objective of this study was to demonstrate the feasibility of tip type electroporation in *Danio rerio* sperm, allowing its further use in SMGT, showing a protocol that provide high transfection efficiency, with minimal side-effects on sperm cells.

## Materials and methods

### Animals and semen collection

Management and maintenance of zebrafish were in compliance with the Zebrafish Book (www.zfin.org). Fish were obtained in a commercial establishment and kept in a closed culture system containing dechlorinated, nitrite free and aerated water at 28 ± 2 °C, pH 7.0, under photoperiod of 12 h light:12 h dark. The animals were fed ad libitum with commercial fish feed, twice daily (Tetra ColorBits). For each replicate of the experiment, 4 males with 4–6 months of age were sacrificed by sectioning the spinal cord (the accepted method when the use of anaesthesia can affect the results of sperm analysis) [23]. Through an abdominal incision and dissection, the gonads were removed and placed in a microtube containing 200 µL of Beltsville Thawing Solution—BTS at 4 ºC with pH 7.4 and an osmolality of 350 mOsm [24] obtaining a sperm pool from the different males. The experiment was performed in 3 replicates (n = 12). This study was conducted in compliance with institutional, national, or international guidelines for using animals and was approved by the Ethics Committee of the Federal University of Pelotas / RS, Brazil, (number 7836).

### Preparation of Cy-3 labelled DNA

As a marker to identify the sperm that incorporate exogenous DNA, a fluorescent-conjugated DNA was prepared by amplifying a 546 bp fragment of the pEGFP-N1 vector (Clontech Laboratories, Basingstoke, UK) with Cy-3-labelled primers as previously described by our group [25]. Briefly, PCR was performed using 35 cycles at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. A single band was confirmed by gel electrophoresis. The concentration and purity were determined by UV spectrophotometer (NanoVue Plus, GE Healthcare Life Sciences, NJ, USA). The Cy-3-labelled primers sequences were the following: 5′-ACG TAAACGGCCACAAGTTC and 5′-AGTCGTGCTGCTTCA TGTG [25, 26].

### Sperm preparation, DNA electrotransfection and experimental design

Sperm concentration was assessed from the pool of samples through sperm counting in a Neubauer chamber. For all transfection procedures exogenous DNA consisted of a 546 bp fragment labelled with Cy-3 prepared as described

above. Transfection was performed using $1 \times 10^6$ sperm cells mixed with 1 µg of a Cy3-labelled DNA were diluted in BTS in a final volume of 10µL. Tip type electroporation was conducted using the Neon® Transfection System (Invitrogen, Carlsbad, CA, USA). The experiments consisted of five electroporated groups at different voltages (500, 750, 1000, 1250 and 1500 V). Non-electroporated sperm cells (with and without DNA) were used as controls. Electroporation was carried out with different voltages as mentioned above, but always the same number of pulses (1 pulse), time constant (1 ms) and volume of the electroporated sample (10 µL). To separate sperm from DNA which has not internalized to the sperm membrane, sperms were centrifuged (5 min at $700 \times g$) and washed three times using BTS medium, followed by incubation with 1 U of DNase I (Invitrogen) for 60 min [9, 19, 25, 27]. All samples from all groups were treated with DNase I. After DNase treatment, sperm cells were washed three times with BTS medium by centrifuging samples at $700 \times g$ for 5 min [25]. After this transfection procedure, the samples were evaluated by CASA to determine cinematic parameters and flow cytometry to determine cell integrity, reactive oxygen species (ROS), mitochondrial functionality and transfection rate. This experiment was replicated three times.

## Assessment of sperm kinetics parameters using Computer Assisted Semen Analysis (CASA)

To determine kinetics parameters, 1 µL of semen sample and 4 µL of fresh water were placed on slides under coverslips and analysed using Computer-Assisted Sperm Analyses (CASA) system (AndroVision 3.5, Minitube, Germany) combined with an Axio Scope.A1® optical microscope (Zeiss, Germany). CASA software setup was adjusted according to Wilson-Leedy and Ingermann [28]. The evaluations were performed after 5 independent sperm activations in at least 500 cells for group, and for no more than 10 s after starting the sperm movement. Total motility (%), progressive motility (%), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), and straightness (STR) were accessed.

## Motility duration analysis

The duration of motility was determined based on the time recorded between activation and complete arrest of sperm progressive movement. For the activation of the spermatozoa, 1 µL of semen sample and 4 µL of fresh water were placed on slides under coverslips at room temperature, following the method described by Collares and collaborators [29].

## Flow cytometry analysis

Cell integrity, membrane fluidity, mitochondrial functionality, concentration of reactive oxygen species (ROS) and total of sperm-bound exogenous DNA were evaluated by flow cytometry (Attune® Acoustic Focusing Cytometer, Applied Biosystems, USA) as previously described [30]. To detect the sperm population, non-sperm events were eliminated from the analysis by scatter plots of FSC × SSC [31, 32] and debris were eliminated by staining cells with Hoechst 33342 at a concentration of 16.2 M (Sigma-Aldrich Co., St. Louis, MO, USA). A total of 10,000 events per sperm sample with a flow of 200 cells/s were analysed using the Cytometric Attune Software V2.1 program.

To verify the integrity of the plasma membrane (cell integrity), we used 20 M Carboxyfluorescein diacetate (CDFA) and 7.3 µM Propidium Iodide (PI) (Sigma-Aldrich Co., St. Louis, MO, USA). Cells with intact membranes were differentiated from permeable cells by their ability to exclude propidium iodide (PI) that easily penetrate dead or damaged cells. The sperm were classified as intact (CDFA + /PI−) and permeable (CDFA + /PI + ; CDFA -/PI + ; CDFA -/PI−) [11]. The rate was calculated from the number of not-damaged sperm cells with functional membrane/number of sperm cells positive for H 33342, this number was multiplied by 100 to express the percentage of spermatozoa for each category and the result were expressed as percentage of cells with intact membranes.

To determine the concentration of reactive oxygen species (ROS) concentration was determined using final concentrations of 1.0 µM and 7.3 µM for fluorescent dye 2′7-dichlorofluorescein diacetate (DCF) and PI (Sigma-Aldrich Co., St. Louis, MO, USA), respectively. The results were expressed in fluorescence intensity measured in arbitrary units, considering only the mean intensity of green fluorescence in living sperm (PI) [33].

Sperm membrane fluidity was analysed using hydrophobic Merocyanine-540 dye (M540) (Sigma-Aldrich Co., St. Louis, MO, USA) at a final concentration of 2.7 µM and YO-PRO1 marker (Invitrogen, Eugene, USA) at a final concentration of 0.1 µM. Only live sperm (YO-PRO negative) were selected and classified with high or low fluidity depending on the intensity of M540 orange fluorescence [34]. Data were expressed as the percentage of spermatozoa with higher membrane fluidity.

Mitochondrial function was assessed using the Rhodamine 123 marker (Sigma-Aldrich Co., St. Louis, MO, USA) at a final concentration of 100 nM combined with PI (7.5 µM). High or low mitochondrial function were determined depending on the intensity of Rhodamine green fluorescence [35]. Cells stained with PI were excluded from mitochondrial function analysis. The results were

expressed as the percentage of sperm cells with functional mitochondria.

The transfection rate of sperm cells after tip type electroporation was obtained by flow cytometry as previously described [25]. The efficiency of this technique was previously evaluated by confocal microscopy by Domingues et al. [25]. The intensity of orange (Cy-3-labelled exogenous DNA) and blue fluorescence (H33342) was recorded using band pass filters. The percentage of the transfected sperm was determined by the proportion of the cells emitting orange (Cy-3-labelled DNA) fluorescence out of the total number of the cells analysed.

### Statistical analysis

The normality distribution of data was evaluated by the Shapiro–Wilk test. Differences between groups on cinematic sperm parameters and flow cytometry data were analysed by Two-way ANOVA followed by Tukey's post hoc test. Transfection rate was compared by One-Way ANOVA followed by Tukey's post hoc test. A $P<0.05$ was defined as statistical significance.

## Results

### Effect of the DNA electrotransfection on sperm motion and velocity parameters

Electroporation did not activate the sperm, since in all the groups motility was initiated only after the contact with the fresh water.

The percentage of overall motile sperm in the electrotransfected samples was found to decrease with increasing field strength ($P<0.05$) when compared to the control (non-electroporated cells), as demonstrated in Fig. 1a. In addition, the presence of exogenous DNA in the electroporation process did not interfere on the total motility and progressive motility ($P>0.05$).

The reduction in overall motile sperm was less pronounced at the lowest tested voltages (500, and 750 V). Total motility in these groups were 64.8% ± 1.6 and 62.7% ± 1.9 when electroporated without exogenous DNA, and 63.6% ± 1.8 and 61.6% ± 1.6 when electroporated with exogenous DNA, respectively. Higher voltages, such as 1250 V and 1500 V, reduced cell motility to approximately 53.6% ± 1.6 and 52.7% ± 1.7, respectively ($P<0.05$), when electroporated without DNA.

The progressive motility of spermatozoa electroporated with 500 V and 750 V was not different when compared to the electroporated control with DNA ($P>0.05$), as demonstrated in Fig. 1b. Progressive motility in these groups were 59.4% ± 1.6 and 55.7% ± 1.9 when electroporated without

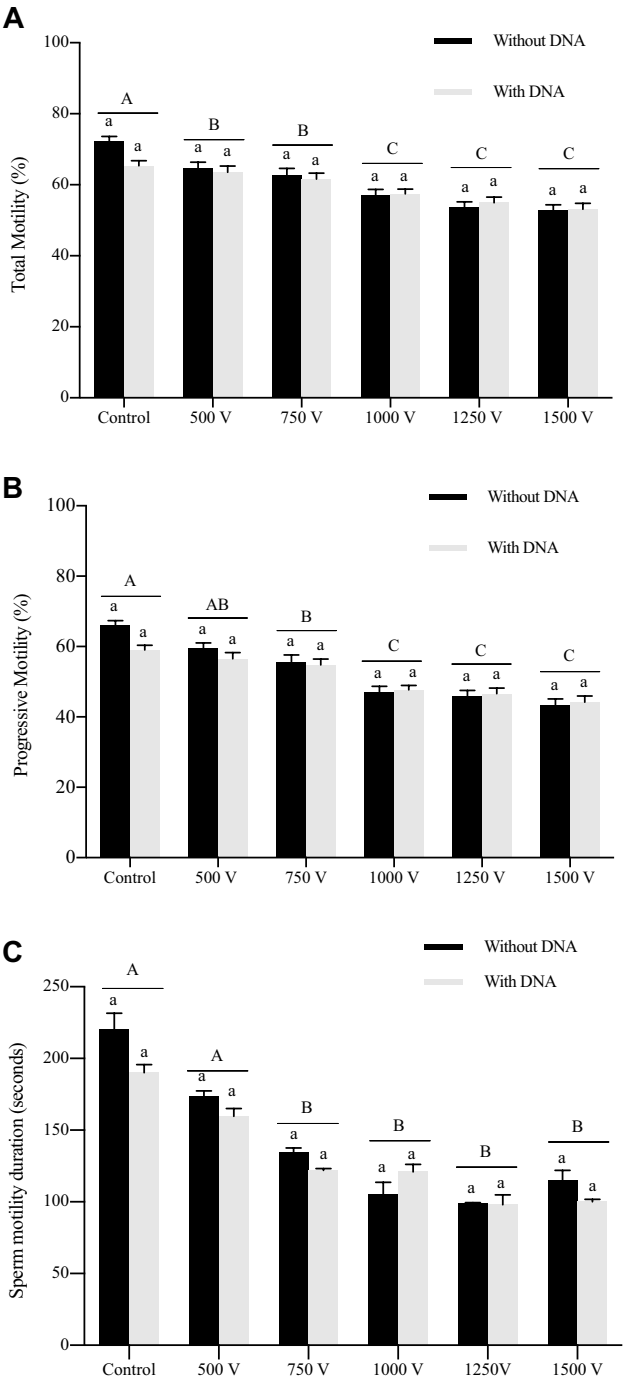


**Fig. 1** Sperm motility parameters in Zebrafish *Danio rerio* electroporated (with or without exogenous DNA). Data are expressed as mean ± SEM (N = 3). Control black bar (without exogenous DNA and not electroporated). Control gray bar (not electroporated) was transfected without electroporation. **a** Evaluation of Sperm motility by CASA (percentage of motile cells). **b** Evaluation of Progressive motility (percentage of cells with progressive motility). **c** Evaluation of Sperm motility duration (time of motility duration measured in seconds). Uppercase was used to demonstrate differences within voltages ($P<0.05$). Lowercase was used to demonstrate differences within groups treated with or without exogenous DNA ($P<0.05$)

DNA, and 56.6% ± 1.8 and 54.8% ± 1.6 when electroporated with DNA, respectively. Higher voltages also showed decreased ($P < 0.05$) progressive motility when compared to the control and to other experimental groups (Supplementary Table S1).

No significant differences were observed for curvilinear velocity (VCL) between tested voltages and control groups ($P > 0.05$, Table 1). However, higher voltages showed decreased ($P < 0.05$) straight-line velocity (VSL) and straightness (STR), when compared to other experimental groups including the control. The electroporated groups with 500 V and 750 V, in the presence of exogenous DNA, presented average path velocity (VAP) similar to non-electroporated control group ($P > 0.05$).

Electroporation with the highest voltages (1250 and 1500 V) significantly reduced ($P < 0.05$) sperm movement duration, when compared to the control group without exogenous DNA (Fig. 1c). However, in the group incubated with DNA without electroporation the sperm motility right after the initiation, decreased more drastically with elapsing time when compared with the others experimental groups ($P < 0.05$).

### Effect of DNA electrotransfection on cell integrity, ROS production and sperm mitochondrial functionality

An increase in the sperm damaged plasma membrane was observed with increasing field strength ($P < 0.05$) (Fig. 2a). On the other hand, ROS and sperm mitochondrial functionality did not present a negative response after the electroporation ($P > 0.05$) among the different voltages, as demonstrated in Fig. 2b, c.

### Assessment of transfection rate

All electroporated groups demonstrated significant higher transfection rates when compared to the control group ($P < 0.05$). The sperm autofluorescence showed a slight emission demonstrating that the fluorescence observed in the other groups belongs to exogenous DNA (Fig. 3). The transfection rate in control without electroporation was 39.4% ± 1.6. Tip type electroporation using voltages of 500, 750, 1000 and 1250 increased the transfection rate in comparison to control without electroporation ($P < 0.05$), however they did not differ among them ($P > 0.05$, Fig. 3). The highest voltage (1500 V) differ only from the controls and the 750 V group ($P < 0.05$) reaching to 86.6% ± 1.9 of sperm cells carrying exogenous DNA.

## Discussion

In this study, capillary and wire-type electroporation was used for gene transfer in zebrafish sperm. Here we look forward to improving the electroporation technique in zebrafish sperm based on capillary tip type electroporation [10]. Overall results indicate that tip type electroporation enhances the internalization of exogenous DNA into zebrafish sperm cells. We found a transfection rate around to 40% without electroporation (control) and when tip type electroporation was performed, we reach to 86% at highest voltage used. Transfection level found in control without electroporation

**Table 1** Values for velocity parameters measured by computer-assisted semen analysis

| | Exogenous DNA | VCL (μm/s) | VAP (μm/s) | VSL (μm/s) | STR (%) |
|---|---|---|---|---|---|
| Non- electroporated | – | 99.6 ± 6.1 | 91.7 ± 2.2$^{A}$ | 88.8 ± 2.2$^{A}$ | 0.93 ± 0.06$^{A}$ |
| | + | 89.5 ± 6.3 | 84.3 ± 2.2$^{A}$ | 79.4 ± 2.2$^{A}$ | 0.93 ± 0.06$^{A}$ |
| 500 V | – | 92.5 ± 7.2 | 84.5 ± 2.6$^{A}$ | 74.3 ± 2.6$^{A,B}$ | 0.92 ± 0.07$^{A}$ |
| | + | 92.5 ± 7.8 | 85.5 ± 2.8$^{A}$ | 80.1 ± 2.8$^{A,B}$ | 0.93 ± 0.08$^{A}$ |
| 750 V | – | 93.6 ± 8.8 | 84.1 ± 3.2$^{A}$ | 79.6 ± 3.2$^{A,B}$ | 0.92 ± 0.09$^{A,B}$ |
| | + | 90.0 ± 7.3 | 85.1 ± 2.6$^{A}$ | 77.2 ± 2.6$^{A,B}$ | 0.91 ± 0.07$^{A,B}$ |
| 1000 V | – | 100.4 ± 6.9 | 75.7 ± 2.5$^{B}$ | 71.8 ± 2.5$^{B}$ | 0.91 ± 0.07$^{B}$ |
| | + | 88.0 ± 5.8 | 75.4 ± 2.1$^{B}$ | 73.6 ± 2.1$^{B}$ | 0.90 ± 0.06$^{B}$ |
| 1250 V | – | 85.3 ± 7.2 | 71.7 ± 2.6$^{B}$ | 72.4 ± 2.6$^{B}$ | 0.90 ± 0.07$^{B}$ |
| | + | 81.0 ± 7.3 | 74.9 ± 2.6$^{B}$ | 69.4 ± 2.6$^{B}$ | 0.90 ± 0.07$^{B}$ |
| 1500 V | – | 86.1 ± 7.4 | 75.3 ± 2.7$^{B}$ | 74.3 ± 2.7$^{B}$ | 0.88 ± 0.08$^{C}$ |
| | + | 85.5 ± 7.6 | 73.5 ± 2.7$^{B}$ | 74.4 ± 2.7$^{B}$ | 0.87 ± 0.07$^{C}$ |

The absence or presence of exogenous DNA is represented by − and +, respectively. Sperm motion parameters from zebrafish spermatozoa presented above: *VCL* curvilinear velocity, *VAP* average path velocity, *VSL* straight-line velocity, *STR* straightness. 4 different males were used to obtain a pool of semen; each treatment was repeated three times using different pool samples (N = 3). Data are expressed as mean ± SEM. Numbers within columns with different superscripts (A, B and C) are statistically different ($P < 0.05$)

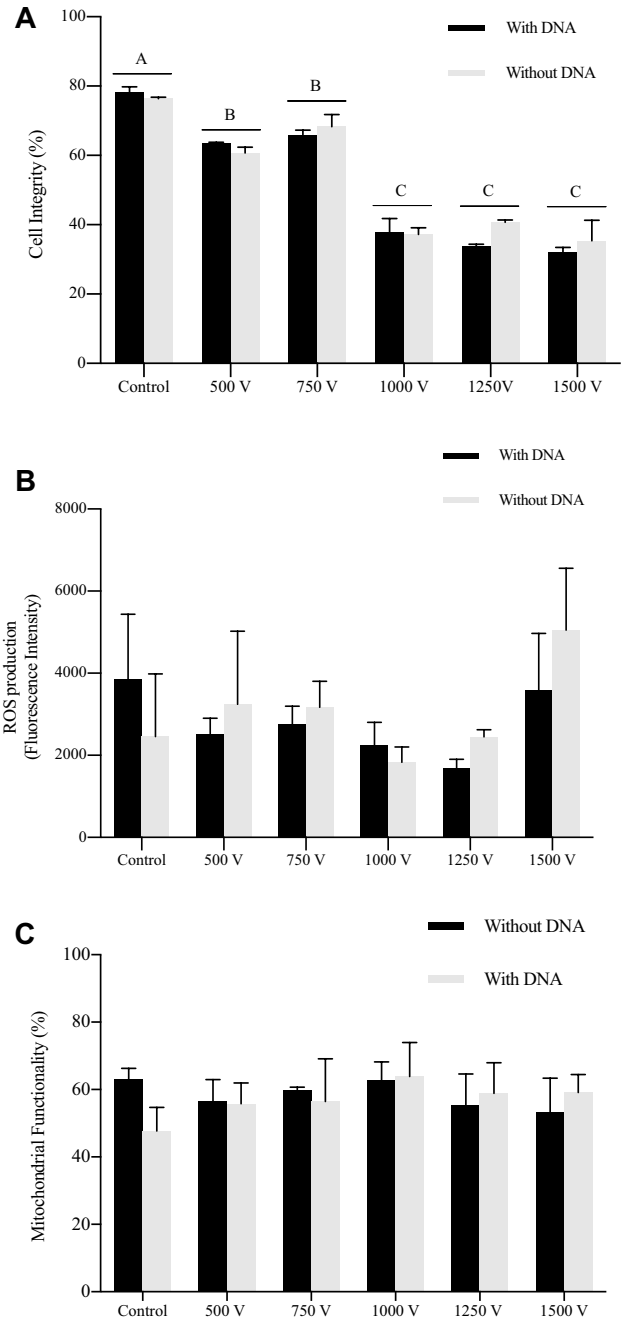


**Fig. 2** Quality parameters evaluated by flow cytometry of spermatozoa from Zebrafish *Danio rerio* electroporated (with and without DNA). Data are expressed as mean ± SEM (N = 3). Control black bar (without exogenous DNA and not electroporated). Control gray bar (not electroporated) was transfected without electroporation. **a** Evaluation of ROS production by flow cytometry (fluorescence intensity measured in arbitrary units). **b** Evaluation of cell integrity by flow cytometry (percentage of cells with intact membrane). Uppercase was used to demonstrate differences within voltages ($P < 0.05$). **c** Evaluation of sperm mitochondrial functionality by flow cytometry (percentage of sperm cells with functional mitochondria).

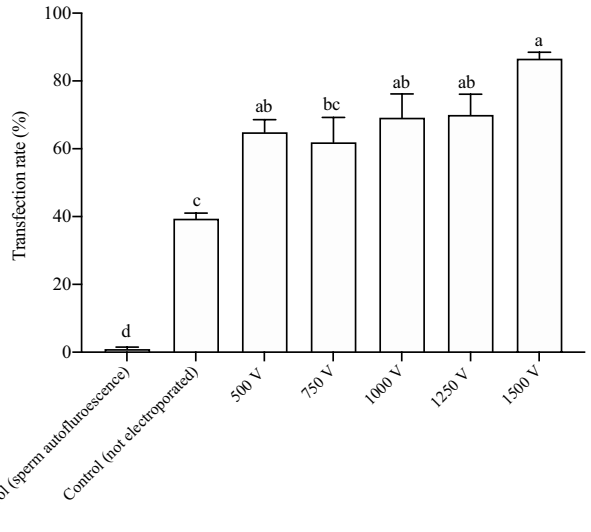


**Fig. 3** Transfection rates of zebrafish sperm cells. Percentage of cells transfected with Cy3-labelled DNA after tip type electroporation. Control of sperm autofluorescence (without exogenous DNA and not electroporated). Control (not electroporated) was transfected without electroporation. a,b,c,d Different letters among treatments indicate significant differences ($P < 0.05$), respectively. ANOVA was used for analysis. Data are expressed as mean ± SEM (N = 3)

corroborates with other previous studies. The fragment used in our study had 546 bp. We previously observed the transfection rate of 45% for cryopreserved bovine sperm on control without electroporation and using this same fragment [25]. In addition, our data are in agreement with previous studies showing that 45% of bovine sperm cells spontaneous uptake exogenous DNA, even being it an entire plasmid [36]. Lavitrano et al. shows that sperm cells take up more efficiently larger DNA molecules (7 kb) as compared with the smallest (150–750 bp) [2]. It could be explained by that fact that larger fragments had higher amount of negative charge, facilitating the interaction with sperm membrane. Moreover, we use flow cytometry to detect exogenous DNA internalized by sperm cells. Silva et al., using fluorescence in situ hybridization (FISH) shows a transfection rate following incubation around to 17% in swine sperm [37]. On the other hand, Canovas et al. detect by flow cytometry around to 29% of sperm cells carrying exogenous DNA after incubation in bovine sperm [38]. Arias et al. (2018) hypothesize that detection of exogenous DNA by flow cytometry can be 10 times more sensitive than conventional fluorescence microscopy. This fact could explain the differences among different studies that used flow cytometry to evaluate transfection rates on sperm cells.

Kim et al. tested more than 70 cell lines, obtaining transfection rates greater than 50% in hard-to-transfect cell lines such as primary and stem cells. Sperm cells are considered a hard-to-transfect cell both in mammalian and fish species.

Here, transfection rate of fish spermatozoa was positively affected for tip type electroporation, increasing the number of cells containing exogenous DNA. In the present study, voltages ranging from 750 to 1500 V demonstrated significant differences in transfection rates, while the 500 V the voltage induced minimal cell viability decrease and with the similar results regards transfection rate in comparison to higher voltages. Patil and Khoo [19] demonstrated that cuvette electroporation did not increase the number of transfected sperm, although it increased the rate of transgenic zebrafish. Blödorn et al. demonstrated that capillary tip type electroporation lead transfection rates up to 80% at 900 V; nonetheless the voltages up to 600 V reach a transfection rate of 70% with no detrimental effects observed in cryopreserved bovine cells [11].

It is well known that high voltages increase the transfection rates, however, decrease severely the cell viability and in general cell viability and transfection rates are inversely proportional. Here we demonstrate that zebrafish sperm could be transfected using tip type electroporation with minimum side effects on sperm cells using tip type electroporation. Kim et al. previously demonstrated main reasons to this improvement in transfection rate by tip type electroporation are related to design, physical geometry and electric resistance [10].

In the present study increased electric energy had a negative effect on the total motility and progressive motility of electroporated sperm. At higher voltages (1000, 1250 and 1500 V), the effects on motility were more deleterious. However, at lower voltages (500 and 750 V), the tip type electroporation approach had a slight effect on sperm motility. Patil and Khoo [19] demonstrated that cuvette electroporation negatively affects sperm motility and the results found in our study are similar to those presented by them. However, the voltage of 1500 V in cuvette electroporation reduced sperm motility to 25% in the zebrafish sperm. In our study, motility was reduced to 53%. Sin et al. observed that voltages of 625 and 1125 V in cuvette electroporation reduced the sperm motility to less than 5% in chinook salmon [39]. Blödorn and colleagues previously observed that electrotransfection resulted in a substantial detrimental effect on bovine sperm motility parameters, even at lowest voltage (500 V) was observed a reduction of almost 50% on total sperm motile count. We can attribute these results to the fact that those cells were previously cryopreserved. The capillary electroporation used in the present study, at similar voltages, increase the transfection in sperm of zebrafish with few negative effects on sperm motility. It is well known that ion concentrations (K+, Na+, and Ca2+), osmotic pressure, pH, temperature and medium dilution rate affect fish sperm motility [40]. Cuvette electroporation systems use aluminium electrodes that produce secondary chemical reactions during electric shock releasing ions [41]. Tip type electroporation use electrodes coated with gold that reduce the release of ions during transfection. In addition, the tip type system has a high electrical resistance that in turn produce fewer chemical reactions and in consequence low release of ions [10]. Also, reduced pH and temperature changes during tip type electroporation in comparison to cuvette system were reported [10]. In this sense, reduced metal and hydrogen ions release and minimal temperature changes during tip type electroporation may explain the slight deleterious effects on sperm motility and motility duration observed at lowest voltages used here.

Parameters such as progressive motility and velocity parameters are strong correlated with fertility ability in bulls. According to sperm motility results, other velocity parameters such as progressive motility and velocity of electroporated sperm cells at low voltages (500 and 750 V) were similar to controls. To the best of our knowledge, previous studies with cuvette electroporation on fish sperm has not performed robust CASA analyses such as VCL, VSL, VAP, and STR. It has been previously shown that movement variables such as VAP and VSL are strongly correlated with fertilization rates in *Anthocidaris crassispina* [42]. These results are in agreement with our previous study in bovine sperm cells using tip type electroporation [11]. In a similar way, at high voltages, there was an increased loss of integrity related to damaged plasma membrane, while at low voltages, cell integrity was slight reduced in comparison to non-electroporated spermatozoa. This is because after reaching a critical value for the electric field, the plasma membrane became irreversibly permeabilized. Consequently, this condition may decrease cell viability [43]. Cell integrity is essentially to maintain fertilization ability. Thus, taken together results of motility, progressive motility, velocity parameters and cell integrity, we suggest that tip type electroporation at low voltages could maintain fertilization rates at similar levels compared to non-electroporated zebrafish sperm, however, further studies must be conducted to elucidate if tip type electroporation can be used without interfering with fertilization rates and improving the generation of transgenic fish.

Mitochondria are essential for providing energy to flagellum and their number and function have been positively correlated with sperm motility and fertilization capacity [44]. Assessment of mitochondrial functionality by measuring mitochondrial membrane potential can be useful as a marker of fish sperm quality [45]. Some stressful procedures can affect sperm motility through two types of damage: direct damage to mitochondrial DNA or alterations to the inner or outer membrane [46]. Our experiments suggest that capillary electroporation does not affect mitochondrial function. These results corroborate with our previous results using tip type electroporation in bovine sperm, in which reduced motility was caused by electroporation conditions rather than loss of mitochondrial functionality [11].

Mitochondria are also involved with redox balance and oxidative stress in sperm. In this regard, under stress conditions, the electron transport chain has a major role in the production of a variety of reactive oxygen species (ROS) which exceeds the spermatic limited antioxidant defences. Thus, a state of oxidative stress is induced, characterized by peroxidation of sperm membrane lipids [47, 48]. Relatively little is known regarding the fatty acid composition of zebrafish spermatozoa, however, an increase in ROS has been linked to abnormal or damaged spermatozoa in other species [49, 50]. The present results demonstrated that ROS concentration were not affected by tip type electroporation, even in high voltages, suggesting that these conditions do not increase oxidative stress.

## Conclusion

In summary, zebrafish sperm was successfully transfected using tip type electroporation, with minimal loss in sperm motility, cell integrity and without effects in progressive motility, velocity parameters, mitochondrial functionality and ROS production at lower voltages, such as 500 V. This tip type electroporation provides a method with minimal harmful effects to introduce exogenous DNA into sperm cells and future studies should be conducted to assess the potential of tip type electroporation in the generation of transgenic zebrafish by SMGT.

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### Compliance with ethical standards

**Conflict of interests** The authors declare that they have no competing interests.

**Ethical Approval** The methodology used in this study was approved by the Ethics Committee of the Federal University of Pelotas / RS, Brazil, (number 7836).

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