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Transferência gênica mediada por espermatozoides através de eletroporação capilar em zebrafish *Danio rerio*

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Lembre, não existem erros. Apenas lições”

(Cherie Carter Scott)

Resumo

DANELUZ, Larissa Oliveira.**Transferência gênica mediada por espermatozoides através de eletroporação capilar em zebrafish *Danio rerio*.** 2019. 55 f. Dissertação (Mestrado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A transferência gênica mediada por espermatozoides (SMGT) é uma biotecnologia promissora para transgênese em espécies aquáticas, baseada na capacidade natural dos espermatozoides serem portadores espontâneos de DNA exógeno. Porém, ainda possui alguns entraves, o que torna necessário implementar estratégias para otimizar a técnica da SMGT. Métodos físicos, como a eletroporação, têm sido utilizados para melhorar a eficiência de absorção de DNA nos espermatozoides, mas ainda a eletroporação convencional apresenta alguns efeitos negativos sobre a viabilidade celular. Assim, sistemas alternativos de eletroporação, como a eletroporação capilar podem ser explorados para a transfecção de células espermáticas, a qual tem demonstrado eficácia muito alta, com alta taxa de transfecção, sendo rápida, reprodutível e possuindo baixo índice de perda celular. O objetivo deste estudo foi validar um método de eletroporação capilar eficiente para incorporação de DNA em espermatozoides de Zebrafish *Danio rerio*. A eletroporação capilar foi realizada em dois grupos distintos (com e sem DNA exógeno) usando cinco diferentes voltagens: 500V, 750V, 1000V, 1250V e 1500V. Parâmetros cinéticos foram determinados usando análise computadorizada de sêmen, enquanto a integridade da membrana, fluidez da membrana, funcionalidade mitocondrial, índice de fragmentação de DNA, concentração de espécies reativas de oxigênio (ROS), peroxidação lipídica na membrana plasmática (LPO) e absorção de DNA foram avaliadas por citometria de fluxo. Os resultados revelaram que todos os grupos eletroporados demonstraram percentagens significativamente maiores de espermatozóides transfectados quando comparados ao grupo controle ($P < 0,05$). Grupos eletroporados em altas voltagens (1000V, 1250V e 1500V) demonstraram efeitos negativos na integridade da membrana celular, quando comparados a outros grupos e controle. As maiores tensões (1250V e 1500V) reduziram 50% da duração de motilidade, quando comparadas ao grupo controle sem DNA. A eletroporação com maiores voltagens, como 1000V, 1250V e 1500V, levou a um aumento de 45% na concentração de espécies reativas de oxigênio nas células espermáticas ($P < 0,05$), enquanto as menores voltagens (500V e 750V) não diferiram do grupo controle ($P > 0,05$). A fluidez da membrana e os danos no DNA aumentaram com o aumento da voltagem. A funcionalidade mitocondrial espermática foi a única, entre os parâmetros não cinéticos, apesar das diferentes tensões, que não apresentou resposta negativa após a eletroporação ($P > 0,05$). Sob a recomendação do uso de tensões de até 750 V, com otimização de 25% de taxa de transfecção, este método representa uma alternativa segura e eficiente para a eletroporação de espermatozóides de Zebrafish *Danio rerio*.

Palavras-chave: Eletroporação capilar, SMGT, célula espermática, DNA, transgênese.

Abstract

DANELUZ, Larissa Oliveira. **Sperm-mediated gene transfer by capillary-type electroporation in zebrafish *Danio rerio*** 2019. 55 f. Dissertação (Mestrado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Sperm-mediated gene transfer (SMGT) is a promising biotechnology for transgenesis in aquatic species, based on the natural ability of spermatozoa to be spontaneous carriers of exogenous DNA. However, it still has some obstacles, which makes it necessary to implement strategies to optimize the SMGT technique. Physical methods, such as electroporation, have been used to improve the efficiency of DNA uptake in spermatozoa, but even conventional electroporation has some negative effects on cell viability. Thus, alternative electroporation systems such as capillary electroporation can be exploited for transfection of sperm cells, which has shown very high efficacy, with high transfection rate, being fast, reproducible and possessing a low rate of cellular loss. The objective of this study was to validate an efficient capillary electroporation method for DNA incorporation in Zebrafish spermatozoa Danio rerio. Capillary electroporation was performed in two distinct groups (with and without exogenous DNA) using five different voltages: 500V, 750V, 1000V, 1250V and 1500V. Kinetic parameters were determined using computerized semen analysis, while membrane integrity, membrane fluidity, mitochondrial functionality, DNA fragmentation index, reactive oxygen species (ROS) concentration, lipid peroxidation at the plasma membrane (LPO) and absorption of DNA were evaluated by flow cytometry. The results showed that all electroporated groups showed significantly higher percentages of transfected spermatozoa when compared to the control group ($P < 0.05$). Electroporated groups at high voltages (1000V, 1250V and 1500V) demonstrated negative effects on cell membrane integrity when compared to other groups and control. Higher voltages (1250V and 1500V) reduced the motility duration by 50% when compared to the control group without DNA. Electroporation with higher voltages, such as 1000V, 1250V and 1500V, led to a 45% increase in the concentration of reactive oxygen species in sperm cells ($P < 0.05$), while the lowest voltages (500V and 750V) did not differ from the control group ($P > 0.05$). Membrane fluidity and DNA damage increased with increasing voltage. The spermatic mitochondrial functionality was the only one, among the non-kinetic parameters, despite the different stresses, which did not show a negative response after electroporation ($P > 0.05$). Under the recommendation of using voltages of up to 750 V with 25% transfection rate optimization, this method represents a safe and efficient alternative for the electroporation of Zebrafish spermatozoa Danio rerio.

Keywords: capillary-type electroporation, SMGT, sperm cell, DNA, transgenesis.

Lista de Abreviaturas e Símbolos

ANOVA	Análise de variância
DNA	Ácido desoxirribonucléico
EGFP	Proteína verde fluorescente
GH	Hormônio do crescimento
mRNA	RNA mensageiro
PCR	Reação em cadeia da polimerase
RNA	Ácido ribonucleico
SMGT	Transferência gênica mediada por espermatozoides
ROS	Espécies reativas de oxigênio
LPO	Lipoperoxidação lipídica
FAO	Organização das Nações Unidas para a Alimentação e a Agricultura

Sumário

1. INTRODUÇÃO GERAL

A transferência de genes – transgênese - é uma área especial da biologia molecular que se tornou promissora para o melhoramento genético de espécies utilizada na aqüicultura, envolvendo a alteração do genoma de um organismo multicelular através da inserção, modificação ou deleção de um gene com o objetivo de modificar características fenotípicas de interesse específico (HOUDEBINE, 2002; CARTER, 2004). Atualmente, existem uma variedade de técnicas para produzir peixes transgênicos, as quais têm sido desenvolvidas com objetivo de aumentar a eficiência de integração dos transgenes no genoma receptor, produzir transgênicos puros na primeira geração e em grande quantidade.

Dentre os modelos experimentais utilizados, o zebrafish (Danio rerio), um peixe tropical de água doce, vem sendo inserido na comunidade científica devido as suas inúmeras vantagens, como elevadas taxas de reprodução, baixo custo, embriões transparentes (o que possibilita a visualização da expressão de genes repórteres em linhagens transgênicas), além de outros atributos importantes da biologia desta espécie (BEN-MOSHE; FOULKES; GOTHILF, 2014 ; DAI et al., 2014).

Sabe-se que espermatozoides de diversas espécies têm capacidade espontânea de absorver moléculas de DNA exógeno e internalizá-las no núcleo (ANZAR et al., 2006). A técnica que utiliza esta capacidade natural com o intuito de gerar animais transgênicos, de forma rápida e barata, é conhecida como Transferência Gênica Mediada por Espermatozoides (SMGT, Sperm-Mediated Gene Transfer) (SMITH; SPADAFORA, 2005).

A transferência gênica mediada por espermatozoides (SMGT) é uma biotecnologia baseada na capacidade natural dos espermatozoides de serem portadores espontâneos de material genético exógeno. Quando a fertilização ocorre, se esse material genético é transferido para o ovo, o genoma embrionário resultante é modificado e um indivíduo transgênico é gerado (LAVITRANO et al., 1989). Outro fato importante a ser considerado na SMGT é a quantidade de DNA exógeno incubado com espermatozoides e o tempo de contato, o que pode influenciar na taxa de internalização, pois o método convencional é baseado apenas na incubação de DNA com espermatozoides, os quais poderiam reagir contra o DNA exógeno pela ativação de endonucleases (MAIONE et al., 1997).

Portanto, todas essas questões dificultaram o rápido progresso da pesquisa em peixes transgênicos pela SMGT (RASAL et al., 2016), tornando necessária a implementação de estratégias para otimizar a técnica, a fim de aumentar a consistência e eficiência de internalização pelas células. Dentre essas estratégias, destaca-se a eletroporação, a qual consiste na aplicação de pulsos elétricos curtos que geram permeabilidade reversível da membrana celular, facilitando a entrada de macromoléculas exógenas na célula (CHEN; POWERS, 1990).

A SMGT auxiliada pela eletroporação é uma técnica que pode aumentar a eficiência de absorção de DNA pelo espermatozoide e, em seguida, utilizá-lo para fertilizar ovos para produzir animais transgênicos (PRAMOD et al., 2016). Essa abordagem é considerada uma alternativa para a rápida transferência de genes, a partir do uso de breves pulsos elétricos para transitoriamente permear a membrana plasmática e transferir os ácidos nucléicos para as células (DE VRY et al., 2010). Mas apesar dos bons efeitos sobre a absorção do DNA, a eletroporação convencional apresenta alguns problemas que diminuem o interesse por essa metodologia, como os efeitos negativos sobre a viabilidade celular e baixa taxa de transfecção (KIM et al., 2008). Assim, sistemas alternativos de eletroporação devem ser explorados para a transfecção de espermatozoides.

A eletroporação capilar foi descrita pela primeira vez em 2008, onde o método foi eficaz em uma ampla gama de células de mamíferos, incluindo linhagens celulares, células primárias e células-tronco. Em comparação com o método convencional, a eletroporação capilar tem eficácia muito alta, onde a taxa de transfecção tem aumentado significativamente em comparação com a eletroporação convencional e também fornece uma condição mais saudável para as células eletroporadas (KIM et al., 2008). Além disso, é rápido, reprodutível e possui baixo índice de perda celular, além de manter alta viabilidade celular (BREES; FRANSEN, 2014).

Como nenhum outro estudo relatou o uso de eletroporação capilar em espermatozoides de peixes, bem como estudos adicionais sobre essa temática são necessários e há grande potencial à frente, o objetivo deste estudo foi desenvolver um método de eletroporação capilar eficiente para incorporação de DNA nos espermatozoides de *Danio rerio* minimizando os efeitos prejudiciais para posterior uso em SMGT.

2. REVISÃO BIBLIOGRÁFICA

2.1 Zebrafish (*Danio Rerio*) como modelo biológico

O Zebrafish é um pequeno teleósteo (3-4 cm) da família *Cyprinidae*, sendo uma espécie bastante conhecida pelo seu uso como peixe ornamental. É um peixe tropical de água doce e originário da Ásia Central, encontrado principalmente na Índia (ENGESZER et al., 2007). O pioneiro a estudar esta espécie foi George Streisinger que, no final da década de 60, aplicou as técnicas de análise mutacional para estudar seu desenvolvimento embrionário (GRUNWALD; EISEN, 2002).

Embora os animais vertebrados utilizados mais comumente nas pesquisas biológicas sejam os roedores, perfazendo 95% dos estudos experimentais (SILVEIRA; SCHNEIDER; HAMMES, 2012), o zebrafish é um modelo que vem sendo utilizado com sucesso considerável no cenário acadêmico/ científico, sendo considerado um importante biomodelo em diversas áreas da ciência, tais como: transgênese molecular, genética, genômica estrutural e molecular, desenvolvimento embrionário, teratologia, comportamento animal, toxicologia e neurociência (SILVEIRA; SCHNEIDER; HAMMES, 2012; ARUNACHALAM et al., 2013).

2.2 Transgênese Animal

A história da transgenia é marcada por marcos históricos, como o desenvolvimento de transdiferenciação celular, DNA recombinante, modificação genética de células alvo e, finalmente, a geração de organismos geneticamente modificados (TONELLI et al., 2017).

A partir do ano de 1982, quando deu-se início a comercialização de insulina recombinante produzida em bactérias por meio de engenharia genética e, também, quando foi gerado pela primeira vez um animal transgênico através de microinjeção pronuclear em embriões zigóticos de um fragmento de DNA contendo o promotor da metalotioneína e o gene do hormônio do crescimento (GH) de ratos, a transgênese animal começava a ganhar legítima atenção e enfoque (PALMITER et al., 1982). E desde então, animais transgênicos são desenvolvidos a fim de obter melhoramento genético (MILAZZOTTO et al., 2010), produção de proteínas recombinantes (LILLICO

et al., 2007) e também, modelos animais para diversas doenças, principalmente para o câncer (WALRATH et al., 2010).

A transgenia melhorou o conhecimento do mundo acerca dos processos biológicos e moleculares em numerosos organismos. Além das vantagens teóricas em aumentar nossa compreensão dos mecanismos fisiológicos, a transgenia animal possui uma gama de aplicações em vários setores comerciais no mundo. O aprimoramento ou remoção de características particulares pode otimizar a produtividade pecuária, por exemplo (WHEELER; BLECK; DONOVAN, 2001).

Além disso, os animais transgênicos são considerados biorreatores importantes, que podem produzem substâncias farmacêuticas e outros produtos de interesse para a saúde humana e animal (MACLEAN; LAIGHT, 2000)

2.3 Técnicas para geração de peixes transgênicos

Nas últimas três décadas, vários métodos foram desenvolvidos para transferir genes exógenos aos animais. Inúmeras técnicas estão atualmente disponíveis para a geração de modelos animais geneticamente modificados para biomedicina e pesquisa básica. Em peixes, uma variedade de métodos de entrega de genes inclui microinjeção de DNA exógeno nos núcleos de oócitos (OZATO et al., 1986; TSAI et al., 1995), eletroporação de ovos e sêmen (INOUE et al., 1990; POWERS et al., 1990), infecção retroviral (LIN et al., 1994), métodos mediados por lipossomas (LU et al., 2002) e bombardeamento de partículas (YAZAWA et al., 2005).

Pesquisas envolvendo transgênese em espécies aquáticas tiveram avanços significativos nas últimas décadas, gerando organismos com grande importância comercial e científica. Em 2000, a Tilápia do Nilo transgênica foi criada com o gene repórter lacZ (RAHMAN et al., 2000). Também foram produzidos peixes transgênicos resistentes a bactérias, com enfoque em utilizá-los como indicadores ambientais de poluição (SARMASIK et al., 2002). Em termos de contribuições para a pesquisa básica científica, peixes transgênicos foram usados como modelos para estudar a integração de genes exógenos no genoma receptor (HE et al., 2013). Peixes transgênicos foram desenvolvidos com índices aumentados de crescimento corporal (RAHMAN et al., 1998), tolerância ao frio (WANG et al., 1995) e resistência a doenças (SARMASIK et al., 2002). Além disso, peixes transgênicos foram especificamente criados para atuar

como bioindicadores (GONG et al., 2003; ZENG et al., 2005) e biorreatores (POHAJDAK et al., 2004; HRYTSENKO et al., 2011).

No passado, a microinjeção de DNA plasmidial em embriões representava o estado da arte em gerar peixe transgênico (COLLARES et al., 2010). No entanto, esta abordagem sofre de desvantagens significativas, apesar de sua proeminência geral e aplicação frequente em peixes desde os anos 80 (MCLEAN et al., 1987; GUYOMARD et al., 1989), como a distribuição em mosaico do transgene injetado, integração tardia de transgenes em altos números de cópias e baixas taxas de transgenia, tornando a estratégia de produção de linhas transgênicas uma árdua tarefa (SOROLDONI et al. 2009). Além disso, outra dificuldade que a microinjeção também demonstra, é que como o córion é duro em algumas espécies aquáticas e os ovos são opacos, isso dificulta a inserção de micropipetas de vidro (SIN et al., 2000). Assim, esta técnica de microinjeção em ovos de peixe é complicada, lenta, demorada, inadequada para a transferência de genes em massa e requer uma grande quantidade de habilidades e equipamentos relativamente caros. Além disso, microinjeções causam danos mecânicos aos óvulos fertilizados. Todos esses problemas impediram o rápido progresso na pesquisa transgênica em peixes.

Portanto, a fim de evitar adversidades, o uso de espermatozoides como vetores para entrega de genes e consequentemente para a produção de animais transgênicos tem sido estudado como método alternativo, uma vez que os espermatozoides de praticamente todos os animais são capazes de absorver material genético externo, tais como ratos (LAVITRANO et al., 1989), coelhos (BRACKETT et al., 1971), galinhas (SQUIRES; DRAKE, 1993), peixes (PATIL; KHOO, 1996), touros (GAGNE; POTHIER; SIRARD, 1991) , aves (ROTTMANN et al., 1992) e sapos (HABROVA et al., 1996). Das técnicas mencionadas, a SMGT é forte candidata a tornar-se o método com melhor custo-benefício para a geração de animais transgênicos, o que culminará no aumento significativo da aplicação dos animais transgênicos na produção comercial e na pesquisa biomédica (KANG et al., 2008; CAMPOS et al., 2011).

2.4 Transferência gênica mediada por espermatozoides (SMGT)

Em espécies aquáticas, existem algumas vantagens inegáveis na aplicação da SMGT. Primeiro, porque esta técnica é considerada uma transferência genética

em massa, fornecendo uma abordagem poderosa para estudar a regulação gênica *in vivo* e para obter variedades transgênicas de animais com características genéticas especiais. Em segundo lugar, esta técnica supera alguns problemas dos sistemas convencionais de transferência de genes para peixes, devido às características do ovo, tais como opacidade, viscosidade, flutuabilidade, pronúcleos invisíveis e córion resistente. Em terceiro lugar, os espermatozoides de peixes são fáceis de manusear porque a água é suficiente para ativá-los, não sendo necessários meios especiais para isso. Em quarto lugar, os espermatozoides de animais aquáticos podem ser mantidos por longos períodos em temperaturas mais baixas sem grandes danos celulares (TSAI, 2000).

No entanto, muitos entraves ainda estão prejudicando o sucesso da SMGT nas pesquisas científicas, como por exemplo, a dúvida se o embrião incorpora o DNA exógeno que foi acoplado na membrana celular espermática ou se incorpora a macromolécula internalizada no compartimento intracelular dos espermatozoides (CAVALCANTI et al., 2016). Outro fato importante a ser considerado na SMGT é a quantidade de DNA exógeno incubado com espermatozoides e o tempo de contato, o que pode influenciar negativamente na taxa de internalização, pois o método convencional é baseado apenas na incubação de DNA com espermatozoides, e por ação de DNases, poderiam reagir contra o DNA exógeno (MAIONE et al., 1997).

Outro principal problema da SMGT, está relacionado com a baixa incorporação de DNA pelos espermatozoides e, por ser uma etapa crítica, leva a uma diminuição da motilidade da célula e assim, sucessivamente a capacidade fertilizante (FEITOSA et al., 2009). Sendo assim, os procedimentos para SMGT necessitam de melhorias e novas estratégias precisam ser pensadas, pois essas dificuldades técnicas diminuem o progresso da pesquisa em peixes transgênicos pela SMGT (RASAL et al., 2016), tornando necessária a implementação de novas metodologias para otimizar a técnica em questão, a fim de melhorar a consistência e eficiência da captação do DNA pela célula espermática.

2.5 Eletroporação e a transferência gênica mediada por espermatozoides

O uso da SMGT na transgênese pode permitir a produção de proteínas e de produtos farmacêuticos de interesse comercial (PURSEL et al., 2004). Neste contexto,

um dos métodos que pode ser empregado para aumentar a eficiência e produtividade da SMGT é a eletroporação para entrega do DNA exógeno à célula espermática.

Portanto, essa técnica pode ser aplicada com sucesso quando os espermatozoides são eletroporados. Isto pode ser explicado por dois fatores: Primeiro, a eletroporação facilita a entrada do DNA exógeno para as células espermáticas devido à abertura de pequenos poros transitórios na membrana celular, induzidos pelo campo elétrico. Segundo, essa metodologia não exige que o DNA exógeno fique em contato por muito tempo com o sêmen. Assim, isso evita que o DNA seja degradado por endonucleases que podem estar presentes no meio.

A eletroporação foi pensada como um sistema de entrega de genes eletricamente mediado, proposto em 1982 (NEUMANN et al., 1982; ZIMMERMANN, 1982), que se tornou uma das ferramentas de transfecção mais poderosas e eficazes. Essa metodologia utiliza energia elétrica na forma de pulsos elétricos para formar numerosos poros na membrana celular e gerar um potencial dependente de voltagem no nível da membrana. Quando a diferença de potencial de tensão passa para um nível crítico, os componentes da membrana são rearranjados e permitem a entrada de macromoléculas (KNIGHT; SCRUTTON, 1986).

A eficiência do processo depende do comprimento do pulso (milissegundos), da intensidade de campo (volts / centímetro) e da força iônica do meio (TSONG, 1983). Por exemplo, tensões mais altas tendem a aumentar a eficiência da SMGT e, consequentemente, a absorção de DNA, no entanto, também causam mais efeitos deletérios sobre os parâmetros de qualidade seminais (PRAMOD; KUMAR; MITRA, 2016).

Apesar dos bons efeitos sobre a absorção do DNA, a eletroporação convencional apresenta algumas dificuldades que diminuem o interesse por essa metodologia, como os efeitos negativos sobre a viabilidade celular e baixa taxa de transfecção (KIM et al., 2008). Assim, modernos sistemas de eletroporação devem ser explorados para a transfecção de espermatozoides.

Descrita pela primeira vez em 2008, a eletroporação capilar foi eficaz em uma ampla gama de células de mamíferos, incluindo linhagens celulares, células primárias e células-tronco (KIM et al., 2008). Assim, vários outros estudos foram publicados utilizando o método de eletroporação capilar para transfecção de culturas celulares primárias (SCHUMANN et al., 2015), células tumorais (SONG et al., 2010), células endoteliais (HSU; MENG, 2010) células de o sistema nervoso (PARK et al., 2009) e

células-tronco (GUNDRY et al., 2016), entre outros. Diferente da eletroporação convencional, a eletroporação do tipo capilar usa ponteiras de pipetas consumíveis especializadas, contendo eletrodos de ouro como uma câmara de eletroporação, o que reduz os efeitos prejudiciais dos métodos de eletroporação, melhorando as taxas de transfecção. Em comparação com o método convencional, a eletroporação capilar tem maior eficácia, onde a taxa de transfecção é otimizada significativamente em comparação com a eletroporação convencional e também fornece uma condição menos prejudicial para as células eletroporadas (KIM et al., 2008). Além disso, é rápido, reprodutível e possui baixo índice de perda celular, além de manter alta viabilidade celular (BREES; FRANSEN, 2014).

3 OBJETIVOS

3.1 Objetivo Geral

Validar um método eficiente de eletroporação capilar para incorporação de DNA em espermatozoides do peixe Zebrafish *Danio rerio*.

3.2.Objetivos Específicos

- Avaliar o efeito da eletroporação capilar nos parâmetros cinéticos das células espermáticas de Zebrafish *Danio rerio;*
- Avaliar o efeito da eletroporação capilar nos parâmetros de viabilidade celular espermáticas de Zebrafish *Danio rerio;*
- Determinar parâmetros de voltagem, número de pulsos e tempo mais eficientes para a eletroporação capilar em espermatozoides de Zebrafish *Danio rerio.*

4 CAPÍTULO

4.1 Manuscrito 1 – Sperm-mediated gene transfer by capillary-type electroporation in zebrafish *Danio rerio*

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Sperm-mediated gene transfer by capillary-type electroporation in zebrafish *Danio rerio*

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Abstract

Sperm-mediated gene transfer (SMGT) is a promising biotechnology for transgenesis in aquatic species, based on the natural ability of spermatozoa to be spontaneous carriers of exogenous DNA. However, it still has some obstacles, which makes it necessary to implement strategies to optimize the SMGT technique. Physical methods, such as electroporation, have been used to improve the efficiency of DNA uptake in spermatozoa, but even conventional electroporation has some negative effects on cell viability. Thus, alternative electroporation systems such as capillary electroporation can be exploited for transfection of sperm cells, which has shown very high efficacy, with high transfection rate, being fast, reproducible and possessing a low rate of cellular loss. The objective of this study was to validate an efficient capillary electroporation method for DNA incorporation in Zebrafish spermatozoa Danio rerio. Capillary electroporation was performed in two distinct groups (with and without exogenous DNA) using five different voltages: 500V, 750V, 1000V, 1250V and 1500V. Kinetic parameters were determined using computerized semen analysis, while membrane integrity, membrane fluidity, mitochondrial functionality, DNA fragmentation index, reactive oxygen species (ROS) concentration, lipid peroxidation at the plasma membrane (LPO) and absorption of DNA were evaluated by flow cytometry. The results showed that all electroporated groups showed significantly higher percentages of transfected spermatozoa when compared to the control group ($P < 0.05$). Electroporated groups at high voltages (1000V, 1250V and 1500V) demonstrated negative effects on cell membrane integrity when compared to other groups and control. Higher voltages (1250V and 1500V) reduced the motility duration by 50% when compared to the control group without DNA. Electroporation with higher voltages, such as 1000V, 1250V and 1500V, led to a 45% increase in the concentration of reactive oxygen species in sperm cells ($P < 0.05$), while the lowest voltages (500V and 750V) did not differ from the control group ($P > 0.05$). Membrane fluidity and DNA damage increased with increasing voltage. The spermatic mitochondrial functionality was the only one, among the non-kinetic parameters, despite the different stresses, which did not show a negative response after electroporation ($P > 0.05$). Under the recommendation of using voltages of up to 750 V with 25% transfection rate optimization, this method represents a safe and efficient alternative for the electroporation of Zebrafish spermatozoa Danio rerio.

Keywords: capillary-type electroporation, SMGT, sperm cell, DNA, transgenesis.

1. Introduction

In the last three decades, several methods have been developed to transfer foreign genes to animals. Numerous techniques are currently available for the generation of genetically engineered animal models for biomedicine and basic research. In fish, a range of gene-delivery methods include microinjection of foreign DNA into the oocyte nuclei [1,2], electroporation of eggs and semen [3,4], retroviral infection [5], lipossome-mediated methods [6] and particle bombardment [7].

In the last decade, the microinjection of plasmidial DNA into early embryos was the most used technique to generate transgenic fish [8]. However, this technique presents significant disadvantages, making the generation of transgenic species a laborious task [9]. The difficulties of microinjection include the tough and opaque chorion, which hinders insertion of glass micropipettes. Thus, this microinjection technique in fish eggs is complicated, slow, time-consuming, requires a great deal of skills and relatively expensive equipment, and unsuitable for mass gene transfer. Also, microinjection cause mechanical damage to the fertilized eggs. All these issues prevented rapid progress in transgenic research in fish.

Therefore, in order to avoid adversity, the use of spermatozoa as DNA vector for gene delivery and consequently production of transgenic animals has been studied as an alternative method, since the spermatozoa of practically all animals are able to take up exogenous DNA macromolecules or RNA, such as mouse [10], rabbit [11], chicken [12], fish [13], cattle [14], avian [15] and frog [16]. A number of studies have reported the use of spermatozoa as an exogenous DNA vector for the generation of transgenic fish [2,8,17].

Sperm-mediated gene transfer (SMGT) is a biotechnology based on the natural ability of spermatozoa to be spontaneous carriers of exogenous DNA. When fertilization occurs, if this DNA is transferred to the egg, the resulting embryonic genome is modified and a transgenic individual is generated [10].

In aquatic species, there are some undeniable advantages in applying SMGT. First, because this technique is considered a gene transfer in mass, providing a powerful approach to studying gene regulation in vivo and to obtaining transgenic varieties of animals having special genetic traits. Secondly, this technique overcomes some problems of conventional gene transfer systems for fish, due to egg characteristics such as opacity, viscosity, buoyancy, invisible pronuclei and resistant chorion. Third, fish sperm are easy to handle because water is enough to activate them, no special means required for this. Fourth, sperm from aquatic animals can be maintained for long periods in lower temperatures without major cellular damage [18].

However, many mechanisms involved in SMGT are not yet fully understood, for example, if the embryo incorporate of the exogenous DNA that was attached to the sperm membrane or

if incorporate the macromolecule internalized in the intracellular compartment of spermatozoa [19]. Another important fact to be considered in the SMGT is the amount of exogenous DNA incubated with spermatozoa and the time of contact, which may influence in the rate of internalization, because the conventional method is based solely on the incubation of DNA with sperm cells, wich could be react against exogenous DNA by activating endonucleases [20].

Therefore, all these issues hindered the rapid progress of research on transgenic fish by SMGT [21], making it necessary to implement other strategies to optimize the SMGT technique, in order to improve the DNA uptake consistency and efficiency of sperm cells.

SMGT – aided electroporation its a valuable and inexpensive technique that can improve the efficiency of DNA uptake by the sperm and then, use it to fertilize the eggs to produce transgenic animals [22]. This approach is considered an alternative for rapid gene transfer, starting from the use of brief electrical pulses to transiently permeate the plasma membrane and transfer the nucleic acids to the cells [23].

Electroporation was thought of as an electrically mediated gene delivery system, proposed in 1982 [24,25], which has become one of the most powerful and effective transfection tools. This methodology uses electrical energy in the form of electric pulses to form numerous pores in the cell membrane and generate a voltage-dependent potential at the membrane level. When the voltage potential difference passes to a critical level, the membrane components are rearranged and allow the entry of macromolecules [26]. The efficiency of the process depends on the length of the pulse (milliseconds), the field strength (volts / centimeter) and the ionic strength of the medium [27]. For example, higher tensions tend to increase the efficiency of SMGT and hence DNA uptake, however, it also causes more deleterious effects on seminal parameters [22]. Despite the good effects on DNA absorption, conventional electroporation has some problems that diminish interest in this methodology, such as negative effects on cell viability and low transfection rate [28]. Thus, alternative electroporation systems should be explored for sperm transfection.

Capillary electroporation was first described in 2008, where the method was effective in a wide range of mammalian cells, including cell lines, primary cells and stem cells [28]. Hence, several other studies have been published using the capillary electroporation method for transfection of primary cell cultures [29], tumor cells [30], endothelial cells [31], cells of the nervous system [32] and stem cells [33], among others. Different of conventional electroporation, capillary-type electroporation uses specialized consumable pipette tips containing gold electrodes as an electroporation chamber, which reduces the harmful effects of electroporation methods, improving transfection rates. Compared with the conventional method, capillary electroporation has very high efficacy, where transfection rate has been greatly increased compared to conventional cuvette electroporation and also provides a

healthier condition for electroporated cells [28]. In addition, it is fast, reproducible and has low index of cell loss, in addition to maintaining high cell viability [34].

As no other study reported the use of capillary and wire-type electroporation in fish spermatozoa and additional studies on the subject are required and there is great potential ahead, the objective of this study was to develop an efficient capillary electroporation method for DNA incorporation in sperm cells of the *Danio rerio* fish with minimal detrimental effects for later use in SMGT.

2. Materials and Methods

2.1 Experimental Animals

Management and maintenance of zebrafish were in compliance with the Zebrafish Book (www.zfin.org). The fish were obtained in a commercial establishment and kept in a closed culture system containing dechlorinated and aerated water at 28 ± 2 ° C, pH 7.0, and nitrite free, under photoperiod of 12 h light: 12 h dark. The animals were fed ad libitum with commercial fish feed twice daily (Tetra ColorBits). After three weeks of acclimation, the animals were sacrificed by sectioning the spinal cord, an accepted method since the use of anesthetics may affect the results of sperm analysis. The methodology used in this study was approved by the Ethics Committee of the Federal University of Pelotas / RS, Brazil, under number 7836.

The gonads were removed from 12 adult fish of reproductive age, aged 4 to 6 months, through abdominal incision dissection, to obtain four pools of cells from three animals (four replicates and n=12). The pair of testicles from each pool was stored in the extender Beltsville Thawing Solution – BTS; (Minitüb, Germany) at 4ºC with pH 7.4 and an osmolality of 350 mOsm [35].

2.2 Sperm preparation, electroporation and experimental design

After collection, sperm samples concentrations were adjusted to 1 x 10^6 cells in 10 µL. The electroporation of the samples was performed using 1µg / µL of the labeled DNA. Sperm electroporation was performed in two distinct groups (with and without DNA) using five different voltages: 500 V, 750 V, 1000 V, 1250 V and 1500 V. Non-electroporated spermatozoa (with and without DNA) were used as controls. The electroporation was performed with different voltages, but always the same number of pulses (1 pulse), time constant (1 ms) and volume of the electroporated sample (10 µL). All electroporation experiments were performed using a capillary and wire-type electrode, Neon Transfection System (Invitrogen, Carlsbad, CA, USA). To avoid external interference created by spontaneous adherence of DNA to the cell surface, sperm samples were incubated with 1 U of DNase I for 30 minutes and then, washed three

times with 1X BTS medium by centrifuging samples at 700 ×g for 5 minutes [36]. The experiment procedure had four replicates.

2.3 Preparation of Cy-3 labelled DNA

Fluorescence-conjugated exogenous 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 [36]. 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). Samples were stored at –20°C until further use. The Cy-3-labelled primers sequences are the following: 5'-ACGTAAACGGCCACAAGTTC and 5'-AGTCGTGCTGCTTCATGTG [37].

2.4 Assessment of sperm kinetics parameters using computer-aided semen analysis (CASA)

To estimate kinetics parameters, 1 µL of diluted semen and 4 µL of Milli-Q water were placed on slides under coverslips at 4ºC and analyzed using computer-assisted sperm analyses (CASA) system (AndroVision 3.5, Minitube, Germany) combined with an Axio Scope.A1® optical microscope (Zeiss, Germany).

For evaluations, 5 activations per sample were prepared and 1-2 fields per activation were analyzed up to 10 s. Each sample was investigated to include at least 500 cells. Sperm motion parameters evaluated were: total motility (%), progressive motility (%), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), and straightness (STR). The other parameters analyzed by CASA system, such as the curvilinear trajectory (LIN), amplitude of lateral head displacement (ALH) and beat cross-frequency (BCF), are in the supplementary materials.

Additionally, the time or duration of motility after sperm activation was determined based on the time of complete arrest of the progressive movement of the spermatozoa following the method described by [38].

2.5 Flow cytometry analysis

To perform the flow-cytometry analysis, the Attune® Acoustic Focusing Flow Cytometer (Applied Biosystems, USA) was used. To detect the sperm population, non-sperm events were eliminated from analysis by scatter plots of FSC × SSC [39,40] and debris was eliminated by

staining of the cells with Hoechst 33342 at a concentration of 16.2 M (Sigma-Aldrich Co., St. Louis, MO, USA), except for those samples used to measure the DNA fragmentation index.

Membrane integrity, membrane fluidity, mitochondrial functionality, DNA fragmentation index, concentration of reactive oxygen species (ROS), plasma membrane lipid peroxidation (LPO) and DNA uptake were evaluated by flow cytometry (Attune® Acoustic Focusing Cytometer, Applied Biosystems, USA) as previously described [41]. A total of 10,000 events per sperm sample with a flow of 200 cells/s were analyzed using the Cytometric Attune Software V2.1 program.

The evaluation of membrane fluidity, plasma membrane lipid peroxidation and DNA fragmentation index by flow cytometry assays, were performed only in the electroporated groups without exogenous DNA, and it was not possible to perform on electroporated spermatozoa in the presence of exogenous Cy3-labelled DNA, because of the overlapping fluorescence emission wavelengths of Cy3 dye (emission around 568nm) and others dyes fluorescence emission around this value.

2.5.1 Membrane integrity

To verify the integrity of the plasma membrane, we used 20.0 µM Carboxyfluorescein diacetate (DCF), which fluoresces green, and 7.3 µM Propidium Iodide (PI), which fluoresces red (Sigma-Aldrich Co., St. Louis, MO, USA). Live cells with intact membranes were distinguished from non-viable cells by their ability to exclude propidium iodide (PI) that easily penetrate dead or damaged cells. The sperm were classified as non-injured (DCF + /PI-) and injured (DCF + /PI+; DCF-/PI+; DCF-/PI-) [42,43]. The results are expressed as percentage of cells with intact membranes

2.5.2 Concentration of reactive oxygen species (ROS)

ROS concentration was determined using the fluorescent dye 2’7-dichlorofluorescein diacetate at a final concentration of 1.0 µM, which emits green fluorescence when oxidized by intracellular ROS, and PI (7.3 µM final concentration). As our reported measurement, we used the median intensity of green fluorescence of the living sperm (PI-) only [44].

2.5.3 Sperm membrane fluidity

Sperm membrane fluidity was analyzed using hydrophobic Merocyanine-540 dye (M540) at a final concentration of 2.7 µM (Sigma-Aldrich Co., St. Louis, MO, USA) and YO-PRO1 marker, which fluoresces green, at a final concentration of 0.1 µM (Invitrogen, Eugene, OR,

USA). Only live sperm (YO-PRO negative) were selected and classified into high fluidity cells (high M540 concentration) and low fluidity cells (low M540 concentration). Sperm cells with higher membrane fluidity fluoresce intense orange, while those with lower membrane fluidity display weak orange fluorescence [43]. Data were expressed as the percentage of spermatozoa with higher membrane fluidity.

2.5.4 Mitochondrial functionality

Mitochondrial function was assessed using the Rhodamine 123 marker (final concentration of 100 nM) combined with PI (7.5 µM). Spermatozoa with high mitochondrial function show intense green fluorescence and those with low mitochondrial function have weak fluorescence [45]. PI was used to identify spermatozoa with damaged cell membrane with red fluorescence. Cells stained with PI were excluded from mitochondrial function analysis. The results were expressed as the percentage of functional mitochondria in the cells.

2.5.5 DNA fragmentation index

Sperm DNA integrity was evaluated using the acridine orange method previously described [35] where the metachromatic colorant acridine orange emits green fluorescence in reaction to double-stranded DNA and an orange or red fluorescence in reaction to single-stranded DNA, identifying a break in the DNA. For this evaluation, 10 µL of semen were mixed with 5 µL TNE (0.01 M Tris-HCl; 0.15 M NaCl; 0.001 M EDTA; pH 7.2). After a 30 seconds incubation, 10 µL of Triton solution 1x were added, followed by another 30 seconds incubation, and finally, 5 µL acridine orange (Sigma-Aldrich Co., St. Louis, MO, USA) were added (2 mg/mL in deionized H_2O). This reaction was then incubated for 5 min. The rate of DNA integrity was determined by the proportion of sperm emitting green fluorescence compared with the total number of spermatozoa analyzed (green, red or orange). The results of the flow cytometry are expressed as the percentage of sperm with fragmented DNA [46].

2.5.6 Plasma membrane lipid peroxidation (LPO)

In order to evaluate plasma membrane lipid peroxidation (LPO), the sperm were loaded with 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C_{11}-BODIPY), a lipophilic membrane probe that changes irreversibly its fluorescence from red to green upon oxidation.

2.5 Assessment of DNA uptake by electroporated sperm cells

Data on DNA uptake by electroporated sperm cells were obtained by flow cytometry as previously described [36]. The intensity of orange (Cy-3-labelled 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 analyzed.

2.6 Statistical analysis

Descriptive statistics were calculated based on the mean and standard error mean calculated for each of the analyzed variables. Differences between groups on kinetic sperm parameters and flow cytometry data were analyzed by two-way ANOVA followed by Tukey's post hoc test. Pearson's correlation and linear regression was used to test voltage associated parameters. A $p < 0.05$ was defined as statistical significance.

3. Results

3.1 Effects of electroporation on seminal parameters

Electroporation had no observable effect on the ability of the sperm to be activated, since sperm motility was initiated in sperm samples from all experimental groups right after electroporation when placed in contact with fresh water.

3.1.1 Effect of electroporation on kinetic parameters

The percentage of motile sperm in the electroporated samples was found to decrease with increasing field strength ($P<0.05$) when compared to the control (non-electroporated cells), as demonstrated in Figure 1A. In addition, the presence of DNA in the electroporation process did not interfere on the total motility and progressive motility ($P>0.05$). Total motility of the control groups, without and with DNA, was 72.2% ± 1.36 and 65.2% ± 1.4, respectively.

The reduction in sperm total motility was less pronounced at the lowest tested voltages (500 V, and 750 V). Total motility in these groups were 64.7%± 1.61 and 62.6%± 1.99 when electroporated without DNA, and 63.5%± 1.75 and 61.6%± 1.64 when electroporated with DNA, respectively. Higher voltages, as 1250 V and 1500 V, reduced cell motility to approximately 53.6%± 1.63 and 52.7%± 1.71, respectively ($P<0.05$).

The progressive motility of spermatozoa electroporated with 500V and 750V was statistically equal when compared to the electroporated control with DNA ($P>0.05$), as demonstrated in Figure 1B. Progressive motility in these groups were 59.4%± 1.84 and 55.7%±

2.27 when electroporated without DNA, and 56.5%± 2.0 and 54.8%± 1.87 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.

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

Electroporation with the highest voltages (1250 V and 1500V) significantly reduced ($P< 0.05$) motility period, when compared to the control group without DNA (Figure 2). 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 ($P<0.05$).

According to correlation analysis for kinetic parameters, as demonstrated in Figure 8A, 8B e 8C, the voltage increase has a strong negative linear relationship with the parameters of total, progressive motility and the time elapsed between the activation and the full arrest of spermatozoa ($r = -0.9924$, $P<0.0001$; $r = -0.9486$, $P<0.0001$; $r = -0.8872$, $P<0.0001$, respectively), which means that higher voltages tend to be related to decreased sperm motility, as shown in Figure 1A and 1B.

3.1.2 Effect of electroporation on ROS production, cell disruption, sperm membrane fluidity and mitochondrial functionality

The electroporation with higher voltages, as 1000V, 1250V and 1500V lead to an increase in the concentration of reactive oxygen species in zebrafish sperm cells ($P<0.05$), while the lowest voltages were not different to the control group ($P > 0.05$), such as demonstrated in Figure 3A.

An increase in the sperm membrane rupture rate was observed with increasing field strength ($P <0.05$) (Figure 3B). Membrane fluidity increased along with the increase of the voltage. Membrane fluidity in control group was approximately 23.94% ± 0.80 and was lower from all electroporated groups ($P <0.05$). The procedure of electroporation of the spermatozoa generated greater membrane fluidity (Figure 3C).

In addition, sperm mitochondrial functionality was the only, among the non-kinetics parameters, despite of the different voltages, that did not present a negative response after the electroporation ($P>0.05$), as demonstrated in Figure 3D.

690 3.1.3 Effect of electroporation on peroxidation of membrane lipids and DNA
691 fragmentation
692
693 The table 2 depicts the finding that electroporated sperm with highs voltages undergo
694 greater lipid peroxidation compared to sperm in fresh semen or electroporated with low
695 voltages.
696 A significant increase in DNA damage in spermatozoa was observed according to
697 increasing of voltages above 1000V ($P<0.05$). The fragmented DNA index in these groups
698 were 2.29% ± 1.1 in 1000V, 5.34% ± 1.9 in 1250V and 6.16% ± 3.1 in 1500V, as demonstrated
699 in Table 2. No significant differences were observed between other tested voltages (500 V and
700 750V) and the control group ($P>0.05$).
701
702 3.1.4 Assessment of DNA uptake by electroporated sperm cells
703
704 Overall the results indicate that electroporation enhances the association between zebrafish
705 sperm and exogenous DNA.
706 DNA-uptake efficiency of sperm cells was positively affected for capillary electroporation,
707 increasing the amount of DNA internalized by electroporated cells, as shown in Figure 4. All
708 electroporated groups demonstrated significant higher percentages of transfected sperm cells
709 when compared to the control group ($P<0.05$). The percentage of transfected DNA in control
710 group was 39.43%± 1.63. Electroporation efficiency ranged from 64.93%± 3.65 in lowest
711 voltage to 86.62%± 1.88 in highest voltage.
712 Based on correlation analysis results, the voltage applied in the electroporation process and
713 the amount of DNA internalized by spermatozoa have a significant ($P<0.05$) strong linear
714 relationship ($r = 0.8169$), which means that higher voltages are related to higher amounts of
715 DNA internalization in zebrafish spermatozoa, as shown in Figure 5D.
716
717 **4. Discussion**
718
719 This is the first study that demonstrated the effects of capillary and wire-type electroporation
720 on the cellular components of fish sperm and kinetics parameters. The results of this study
721 indicate that the efficiency of introducing foreign DNA into sperm can be enhanced if the sperm
722 is initially electroporated via capillary type electroporation at voltages up to 750V.
723 To make the cells permeable to exogenous DNA, we used a protocol of capillary type
724 electroporation, which involved exposing the cells to one single and brief pulse (1 ms) of
725 electric shock in different voltages: (500V, 750V, 900V, 1000V, 1250V and 1500V). We found

the higher detrimental effects only at the highest voltages (1000V, 1250V and 1500V). It means that all the other electroporated groups demonstrated higher percentages of transfected sperm cells in comparison to the control groups, without or with minimal detrimental effects. So, it was possible to make the cells capable to take DNA without subjecting them to undue stress.

The application of an electric pulse to a cell suspension induces polarization of the membrane components and develops a voltage potential across the membrane. This procedure is called electroporation. When the potential difference between the inside and outside of the cell membrane passes to a critical level, the membrane components are rearranged as pores in localized areas, and their membrane becomes transiently permeable, allowing the passage of ions and macromolecules [47] [27]. The pore size can be changed by varying the pulse length (in milliseconds), field strength (in volts/centimeter), and ionic strength of the media [27]. Therefore, it is notable that higher voltages generate larger pores in the cell membrane and thus significant and irreversible deleterious effects on the sperm cell. It is clear that the morphologic structure of the spermatozoon is extremely important for the processes of fertilization and embryo development, thus, larger membrane pores generate cell death because the cells can not reverse the disturbance caused.

Several problems happen with conventional electroporation, such as it has a relatively low cell viability and low transfection rate, there is a need for optimization of the cell-specific protocol, and it involves a sensitive experimental process. In the cuvette system, occurr bubble generation and turbulent flow during electroporation, which promote the spread of the hydroxyl and hydrogen ions from the area near the electrode to the cells. As a result, cells are more likely to be damaged. [28,48]. The capillary electroporation system is rapid, simple, and opportune in this case. Considerably reduces cell death because of its wire-type electrode, which has a small surface area and the buffer solution throughout the capillary is neutral. In this system, a capillary that integrated with the wire-type electrode acts as a substitute for a conventional cuvette as an electroporation reaction chamber. Therefore, that many of the problems of conventional electroporation, such as the change in the and generation of metal ions, are remarkably eliminated in the capillary electroporation system, because the geometry of the capillary also limits the diffusion and the mixing of the hydroxyl and hydrogen ions during the procedure. [28]. Further, the capillary type minimizes the time for which the cells are exposed to the harmful environment, which might have increase cell viability.

According to the review by Browne and collaborates [49], the sperm motility is essential for longevity in external fertilization fish because high-speed sperm can rapidly locate oocytes in open water before the end of their motility time.

Our results demonstrated that electroporation at lower voltages, such as 500 V and 750 V, significantly conserved the motility parameter when compared to cells exposed to higher field strength, although all voltages significantly reduced sperm motion parameters at some level.

763 However, the values of total motility after electroporation with low voltages indicate a hopeful
764 possibility of subsequent fertilization, considering that reproductive success may be dependent
765 on both the number of motile sperm and their velocity [50]. Similar to previous studies pointing
766 that the increased field strength had a deleterious effect on the motility of the spermatozoa
767 [2,13,51] we observed that the capillary system has the similar negative effect on sperm motility
768 on higher voltages.

769 Symonds and collaborates [52] demonstrated that the activity of salmon sperm decreased
770 from 82% to 2% as sperm were electroporated in the conventional cuvette system at voltages
771 increasing from 625 Vcm^{-1} to 1000 Vcm^{-1}. Xie and collaborates in 1992 [53], also showed that
772 the survival rate of cultured cells decreased from 100% to 40% as electroporation voltages
773 increased from 0 $kVcm^{-1}$ to 15 kV cm^{-1}.

774 According to our results, progressive motility, as well as motility and velocity parameters of
775 sperm cells electroporated at minor voltages remained almost unchanged when compared to
776 the non-electroporated controls, and this is a good trait to point out regarding the possible
777 fertilizing ability of the remaining motile cells. Motion variables as VAP and VSL for example,
778 are strongly correlated with fertilization rates in *A. Crassispina* (0.928 and 0.902, respectively)
779 [54].

780 In addition to the automated CASA analysis, this study presents analyses by flow cytometry
781 of different sperm structures that are essential to the maintenance and viability of sperm
782 fertilization capacity.

783 There is a powerfully positive correlation between DNA uptake by the sperm cells with the
784 increase of voltage on electroporation. Thus, although non electroporated sperm cells DNA
785 uptake efficiency was 39.43%, the lowest voltage tested, 500 V, had 64.93% of the cells
786 transfected. The higher percentage of transfected cells was obtained at 1500 V, where the
787 percentage of transfected cells touched 87%. Besides, no significant difference was observed
788 between 500 V and 750 V. Higher voltages tend to increase the efficiency of DNA
789 internalization by sperm cells but produce harmful effects to the sperm cell with respect to cell
790 viability.

791 Overall the results indicate that procedure of electroporation enhances association between
792 the DNA exogenous and zebrafish sperm. Studies by other researchers have also shown that
793 DNA can be internalized into sperm cells of fish [4,8,13,51,52,55–61].

794 As detected in our experiments, these results suggest that the capillary electroporation does
795 not affect mitochondrial function, and also corroborates with the idea that the reduced motility
796 is caused by electroporation conditions, rather than loss of mitochondrial functionality [62]. The
797 mitochondria are essential for provide energy to the flagellum and their number and function
798 have been positively correlated with sperm motility and fertilization capacity [63]. The

799 assessment of mitochondrial functionality, through measurement of mitochondrial membrane
800 potential, can be useful as quality marker of fish spermatozoa [64].
801 Two types of damage may be caused to the mitochondrial membrane potential during some
802 stressors procedures which may affect the spermatozoa motility: direct damage to the nuclear
803 or mitochondrial DNA or alterations to the inner or outer membrane; and indirect damage
804 provoked by the fragmentation of nuclear DNA, on which certain mitochondria proteins are not
805 coded by genome [65].
806 A significant correlation between the nuclear DNA alterations in spermatozoa and poor
807 sperm parameters or impaired reproductive efficiency is reported in both humans and animals
808 [66,67]. The maintenance of DNA integrity or maintenance of the genetic code to be
809 transmitted to the offspring, is of paramount importance as it decreases the chance of
810 mutations or death during the hatching stage of fish [68]. These data suggest that perhaps
811 because the plasma membrane was damaged, the DNA was exposed to the effects of
812 electroporation on higher voltages, such above 1000V. In addition, in study by Irvine and
813 collaborates [66] it was observed that semen with a high level of DNA damage presented low
814 motility and velocity [69,70], thus this is another fact that may perhaps explain why the
815 electroporated groups with high voltages obtained low rates of motility.
816 Another important aspect of mitochondria is their relation with redox balance and oxidative
817 stress within the spermatozoa. In this regard, under stress conditions, the electron transport
818 chain has a main role in the production of a variety of reactive oxygen species (ROS) which
819 exceeds the spermatic limited antioxidant defenses, and a state of oxidative stress is induced,
820 characterized by peroxidation of sperm membrane lipids [71,72]. Relatively little is known
821 regarding the fatty acid composition of zebrafish sperm, however, an increase in ROS has
822 been linked to abnormal or damaged spermatozoa in other species [73,74].
823 Plasma membrane is also a key component of gametes fusion (Yu et al., 2002). At the
824 biophysical level, the instability caused by the capillary electroporation process will determine
825 the higher membrane fluidity and lower plasma membrane integrity, but since at low voltages
826 the process is transient and fast, subsequent fertilization events will not be affected.
827

828 **5. Conclusion**

829

830 In this study, we confirmed that capillary electroporation system considerably can affect
831 zebrafish sperm DNA-uptake efficiency positively, as well as it can, in lower voltages (500 V
832 and 750 V) be safe to sperm membrane integrity, membrane fluidity, mitochondrial function
833 parameters, peroxidation of membrane lipids and DNA fragmentation. However, despite of
834 conserving sperm mitochondrial functionality, sperm motility is reduced after capillary
835 electroporation. Therefore, the capillary electroporation can be used to improve sperm DNA

uptake efficiency, if applied with low voltages (voltages up to 750 V). As this is a first work on the subject, it is similarly important to say that additional studies are needed to understand if this protocol can be used without interfering in the fertilization rates and if this protocol can improve the generation of transgenic fish.

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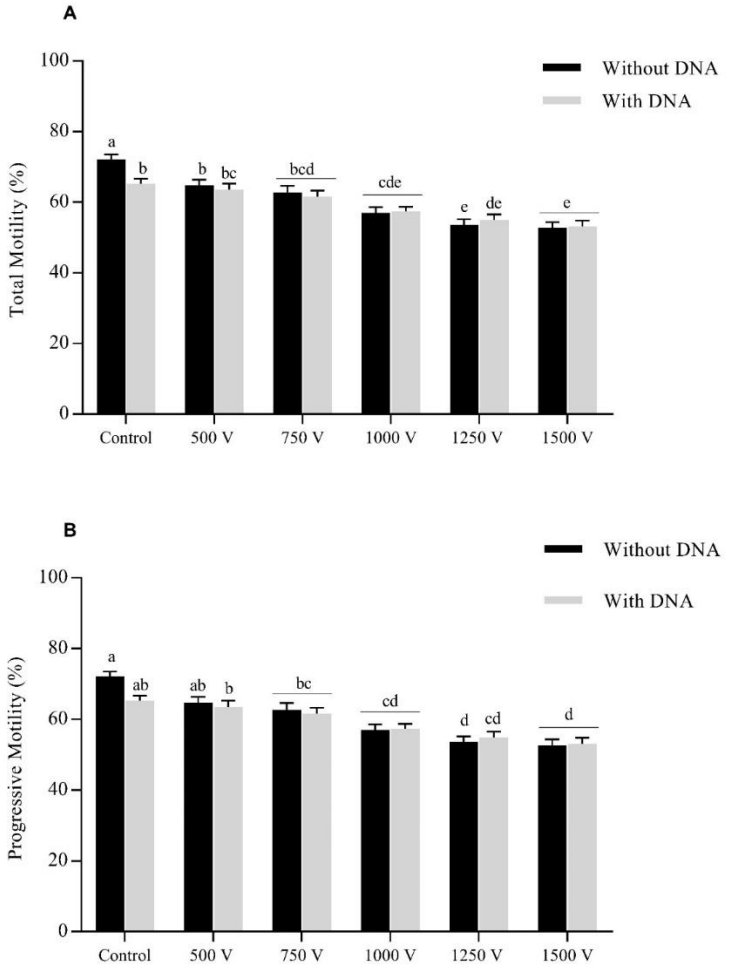
Table 1 Values for motion parameters measured by computer-assisted semen analysis

Sperm Treatments	Exogenous DNA	VCL (µm/s)	VAP (µm/s)	VSL (µm/s)	STR (%)
Non-electroporated Control	-	99.62 ± 6.07^{A}	91.65 ± 2.17^{A}	88.82 ± 2.18^{A}	0.928 ± 0.061^{AB}
	+	89.50 ± 6.25^{A}	84.31 ± 2.23^{AB}	79.40 ± 2.24^{AB}	0.934 ± 0.063^{A}
500 V	-	92.50 ± 7.15^{A}	84.49 ± 2.55^{AB}	74.30 ± 2.57^{B}	0.915 ± 0.072^{ABC}
	+	92.48 ± 7.77^{A}	85.46 ± 2.78^{AB}	80.11± 2.79^{AB}	0.930 ± 0.078^{AB}
750 V	-	93.64 ± 8.84^{A}	84.05 ± 3.16^{ABC}	79.60 ± 3.17^{AB}	0.918 ± 0.089^{ABC}
	+	90.04 ± 7.29^{A}	85.07 ± 2.61^{AB}	77.19 ± 2.62^{B}	0.912 ± 0.074^{ABC}
1000 V	-	100.36 ± 6.89^{A}	75.65 ± 2.46^{BC}	71.84 ± 2.47^{B}	0.906 ± 0.069^{ABC}
	+	87.98 ± 5.76^{A}	75.42 ± 2.06^{BC}	73.57 ± 2.07^{B}	0.898 ± 0.058^{BCD}
1250 V	-	85.31 ± 7.15^{A}	71.73 ± 2.55^{C}	72.41 ± 2.57^{B}	0.896 ± 0.072^{BCD}
	+	80.98 ± 7.29^{A}	74.88 ± 2.61^{BC}	69.40 ± 2.62^{B}	0.903 ± 0.074^{ABC}
1500 V	-	86.05 ± 7.44^{A}	75.27 ± 2.66^{BC}	74.27 ± 2.67^{B}	0.881 ± 0.075^{CD}
	+	85.48 ± 7.60^{A}	73.54 ± 2.72^{BC}	74.44 ± 2.73^{B}	0.868 ± 0.071^{D}

The absence or presence of exogenous DNA is represented by - and +, respectively. Sperm motion parameters above: curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), straightness (STR). Four pools from twelve different fish were used; each treatment was repeated three times in each pool (N = 12). Data are expressed as mean ± SEM. Numbers within columns with different superscripts differ ($P<0.05$).



1103

1104 **Fig 1** Total and progressive motility of spermatozoa from Zebrafish *Danio rerio* electroporated (with
1105 and without DNA). Data are expressed as mean ± SEM. Different letters indicate significant
1106 difference between experimental groups (P<0.05)

1107

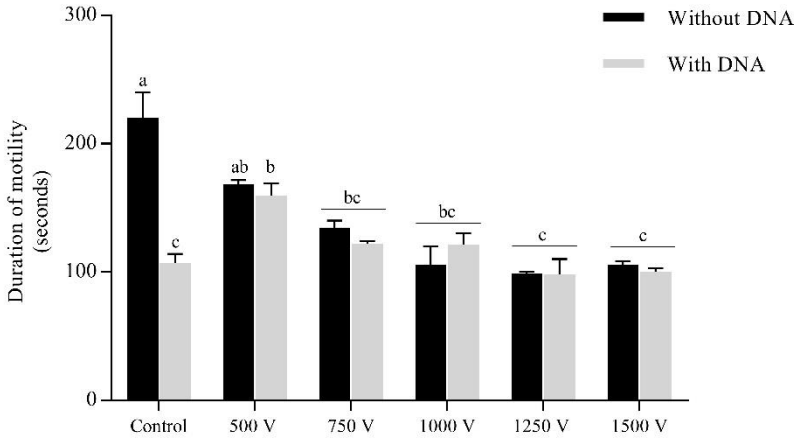


Fig 2 Motility period in Zebrafish *Danio rerio* electroporated (with and without DNA). The values are means ± SEM. The different letters represent significant differences among experimental groups ($P < 0.05$)

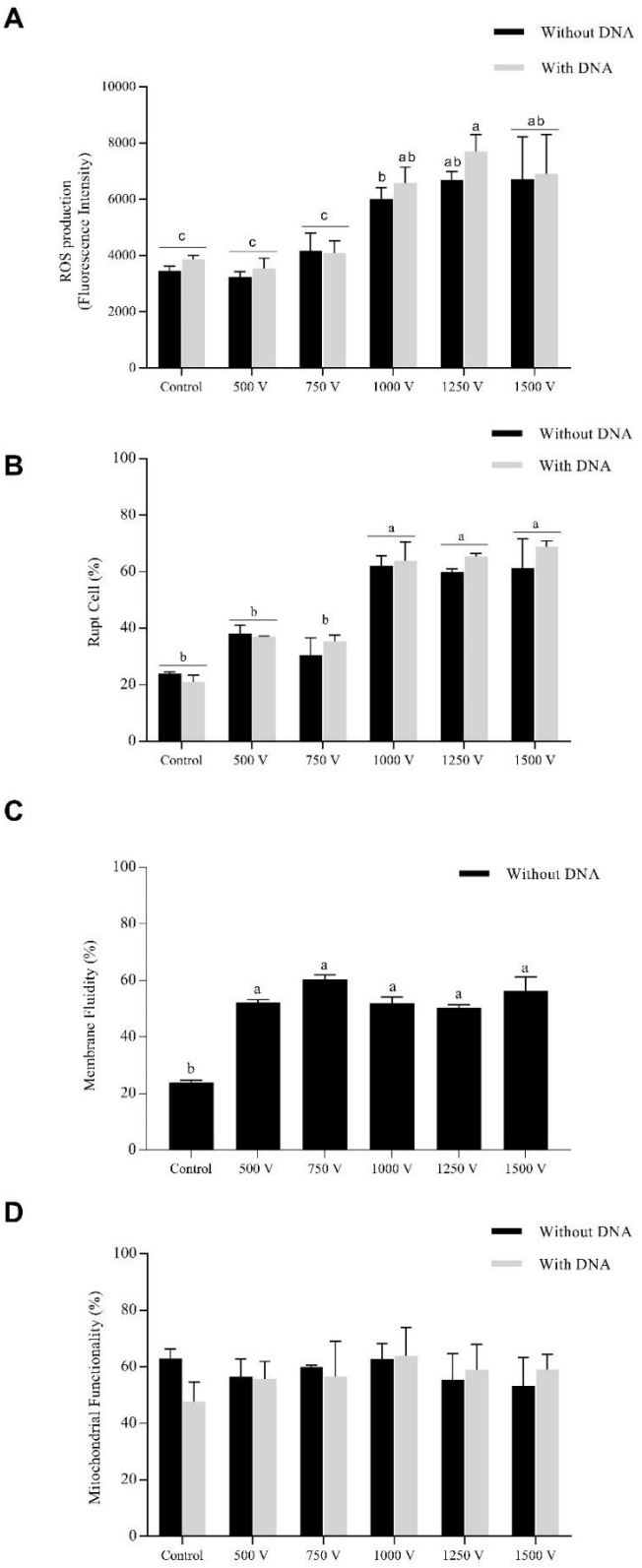


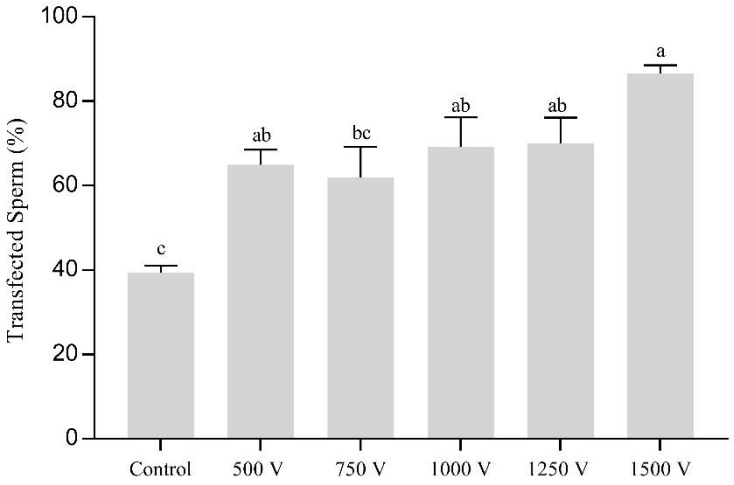
Fig 3 Quality parameters evaluated by flow cytometry of spermatozoa from *Danio rerio* electroporated (with and without DNA). A, ROS production (fluorescence intensity). B, disrupt cells (%). C, membrane fluidity (%). D, sperm mitochondrial functionality (%). Data are expressed as mean ± SEM. Different letters indicate significant difference between experimental groups ($P<0.05$)

Table 2 Values for sperm quality parameters measured by computer-assisted semen analysis

Sperm Treatments	Exogenous DNA	Fragmented DNA (%)	Lipid Peroxidation (%)
Non-electroporated Control	-	0.67 ± 0.3 ^{b}	9.0 ± 0.87^{b}
	+	-	-
500 V	-	2.08 ± 1.0^{ab}	17.4 ± 3.59^{b}
	+	-	-
750 V	-	3.28 ± 0.8^{ab}	15.5 ± 9.65^{b}
	+	-	-
1000 V	-	2.29 ± 1.1^{ab}	18.38 ± 2.45^{a}
	+	-	-
1250 V	-	5.34 ± 1.9^{a}	25.25 ± 2.92^{a}
	+	-	-
1500 V	-	6.16 ± 3.1^{a}	23.12 ± 2.42^{a}
	+	-	-

The absence or presence of exogenous DNA is represented by - and +, respectively. Four pools from twelve different fish were used; each treatment was repeated three times in each pool (N = 12). Data are expressed as mean ± SEM. Numbers within columns with different superscripts differ (P<0.05)

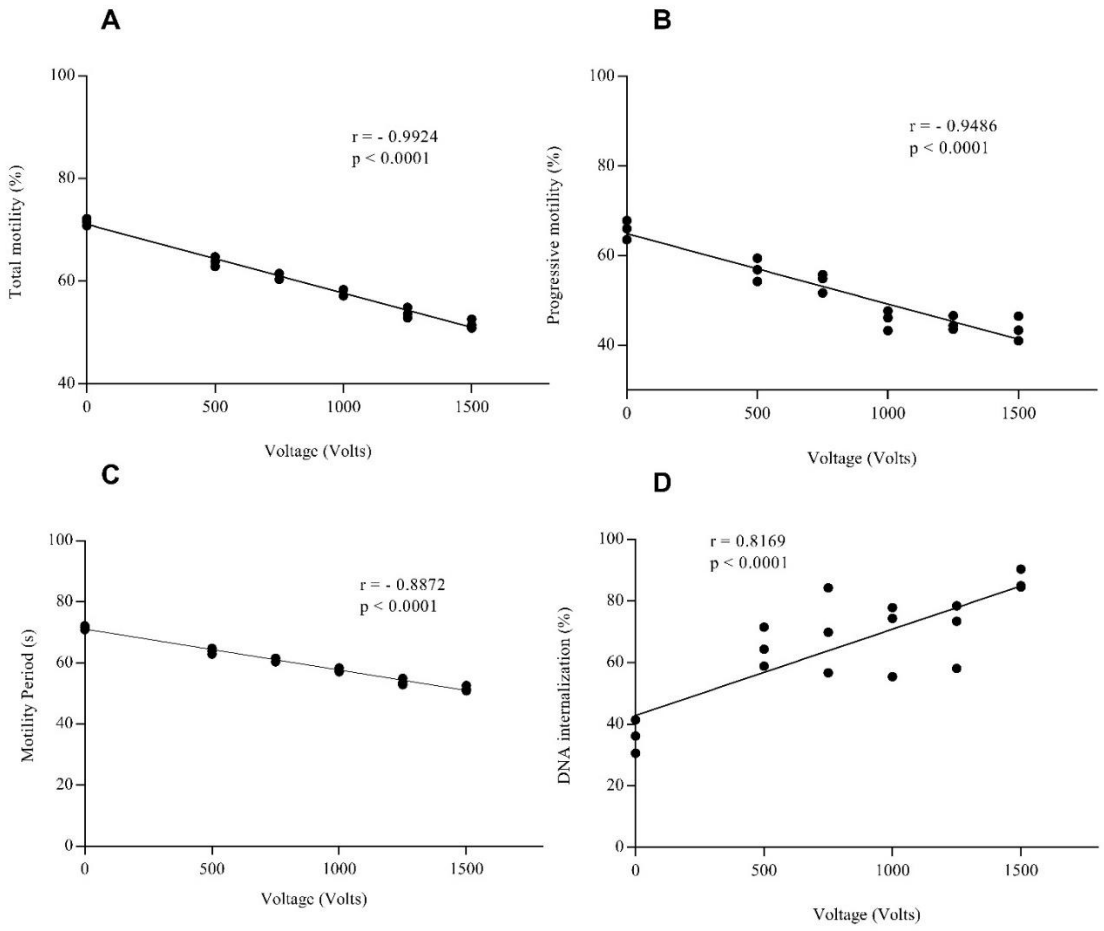
1141



1142

1143 **Fig 4** Exogenous DNA-uptake by electroporated sperm cells. Data are expressed as mean ± SEM.
1144 Different letters indicate significant difference between experimental groups (P<0.05)

1145



1146

1147 **Fig 5** Scatterplots of different sperm parameters associated with the increase of voltage. The voltage
1148 increase has a strong negative linear relationship with the parameters of (a) total motility, (b) progressive
1149 motility and (c) motility period. At the same time, the increase in voltage has a strong linear relationship
1150 with the amounts of (d) internalized DNA. Correlation coefficient r and p values are shown above

1151

5 CONCLUSÃO GERAL

O sistema de eletroporação tipo capilar pode afetar positivamente a eficiência de captação de DNA dos espermatozoides de Zebrafish *Danio rerio*, bem como, em baixas tensões (500 V e 750 V) ser seguro para integridade da membrana espermática, fluidez de membrana, parâmetros de função mitocondrial, peroxidação de lipídios de membrana e fragmentação de DNA. No entanto, apesar de conservar a funcionalidade mitocondrial espermática, a motilidade espermática é reduzida após a eletroporação capilar. Portanto, a eletroporação capilar pode ser usada para melhorar a eficiência de captação de DNA, se aplicada com baixas tensões (voltagens de até 750 V). Como este é um primeiro trabalho sobre o assunto, é igualmente importante dizer que estudos adicionais são necessários para entender se esse protocolo pode ser usado sem interferir nas taxas de fertilização e se esse protocolo pode melhorar a geração de peixes transgênicos.

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