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REPORT

Enhancement of the transfection efficiency of porcine spermatogonial stem cells by far-infrared radiation-based electroporation

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Far-infrared radiation (FIR) is light of non-visible wavelengths (3–1000 μm), which are longer than those of visible light, and these wavelengths can penetrate the skin without causing damage. FIR reportedly enhances mitochondrial biogenesis (Seo *et al.* 2021), anti-inflammatory effects (Shi-Yau *et al.* 2006), wound healing (Chen *et al.* 2017), and neurite outgrowth (Wang *et al.* 2019) and suppresses the production of reactive oxygen species (Leung *et al.* 2011). In addition, FIR increases cell-membrane fluidity (Chattopadhyay *et al.* 2021; Sommer *et al.* 2008)—an important determinant of cell behavior and survival (Kandušer *et al.* 2005)—by promoting the formation of hydrogen bonds between water molecules and the oxygen atoms of the phosphate groups in the phospholipid bilayer (Sommer *et al.* 2008, 2010; Luo *et al.* 2020).

Electroporation is a means of delivering foreign DNA into the cytoplasm through pores created by the application of an electrical field to the cell membrane (Kojima *et al.* 2008; Harris and Elmer 2020). In the process of electroporating foreign DNA into cells, electroporation does not require cell culture techniques (Potter and Heller 2018), so it could be used for cells that have unknown innate characteristics. However, direct delivery of an electrical shock during electroporation can reduce cell viability (Balantič *et al.* 2021), resulting in inefficient electroporation. Therefore, improved cell viability after electroporation will contribute to enhancing resistance to electrical shock, thereby enhancing electroporation efficiency.

We optimized the electroporation conditions (buffer, voltage, Farad level, capacitance, electrical pulse number, and DNA concentration) for transfection of porcine spermatogonial stem cells (SSCs), with the aim of producing transgenic sperm (Kim *et al.* 2019). However, the electroporation-induced reduction in the viability of porcine SSCs has not been addressed. The reduced cell viability may be a result of no or slow recovery of cell-membrane fluidity (Prasanna and Panda 1997; Potter and Heller 2018). The electrical potential induced by an electrical field can induce membrane rupture or slow the membrane reorganization of porcine SSCs due to insufficient recovery of membrane fluidity (Kandušer *et al.* 2005), and electroporator cannot precisely modulate the electrical potential. Therefore, enhancement of cell-membrane fluidity by FIR could enhance the viability of porcine SSCs by accelerating the recovery of their membrane fluidity after subjecting the cells to an electrical shock during electroporation.

Here, we evaluated the effect of FIR of porcine SSCs on transfection efficiency and cell damage and optimized the electroporation conditions for porcine SSCs exposed to FIR. All experiments were conducted according to materials and methods presented in the Supplementary information.

In Experiment 1, to analyze the effect of FIR on electroporation-mediated transfection of porcine SSCs, the percentage of EGFP-positive cells (transfection efficiency), EGFP fluorescence intensity (translational level of the transfected gene), and cell viability (reduction in cell damage) were analyzed in porcine SSCs irradiated or not by FIR. Exposure of porcine SSCs to FIR before electroporation significantly increased the percentage of EGFP-positive cells (Fig. 1*A*), the intensity of EGFP fluorescence (Fig. 1*B*), and viability (Fig. 1*C*). Prolonged FIR exposure did not significantly affect the percentage of EGFP-positive cells (Supplementary Fig. 1*A*), the intensity of EGFP fluorescence

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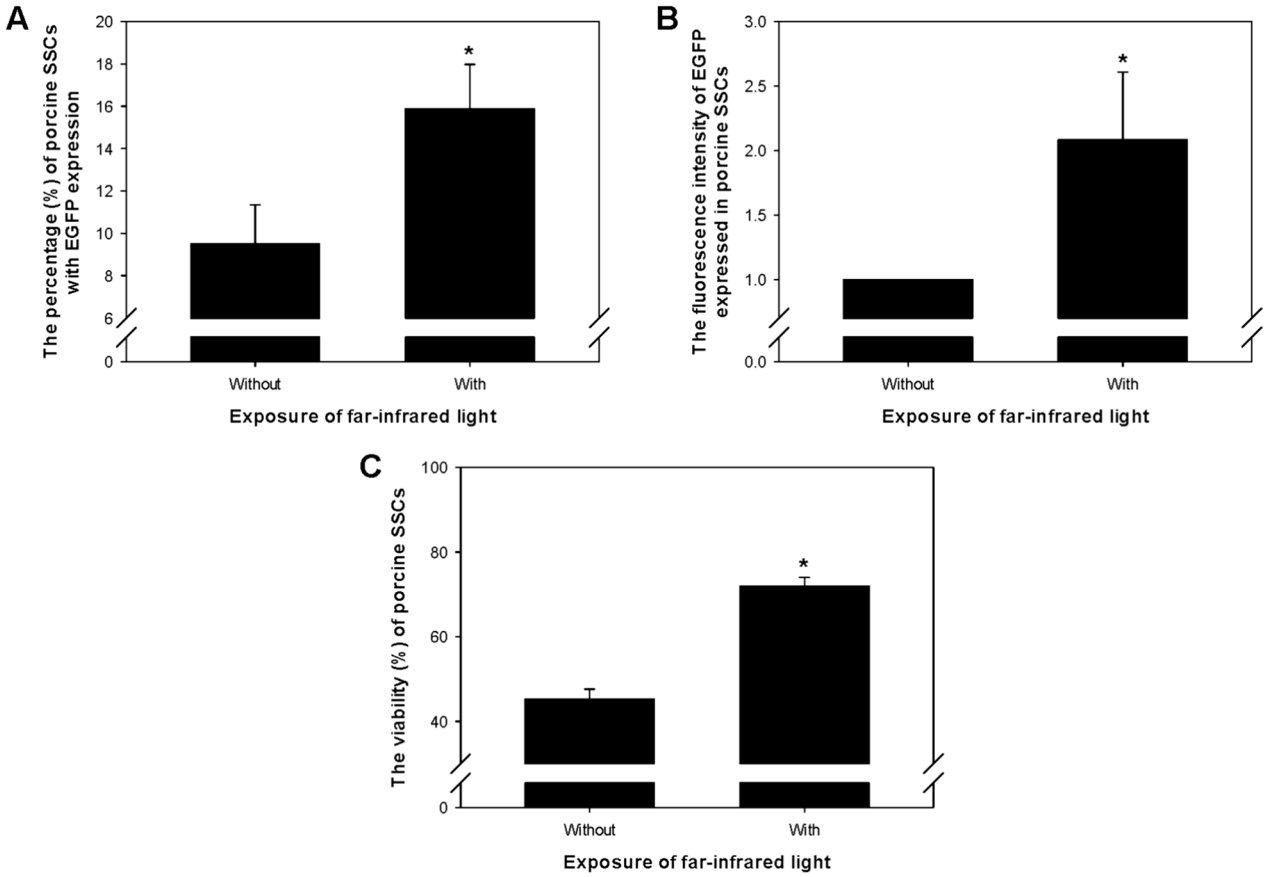

Figure 1. Effect of FIR exposure of porcine SSCs on transfection efficiency, translation of the transfected gene, and cell damage. Porcine SSCs suspended in HBSS were exposed or not exposed to FIR for 10 min and transfected with 1 μg pEGFP-N1 using a single electrical pulse from an electroporator at a capacitor setting of 500 μF and a voltage of 200 V. At 24 h post-transfection, the percentage of EGFP-expressing porcine SSCs (transfection efficiency) and the EGFP fluorescence intensity (translational level of the transfected gene) were measured by flow cytometry, and cell viability (reduction in cell damage) was analyzed by trypan blue exclusion assay. The exposure of porcine SSCs to FIR before electroporation resulted in significant increases in transfection efficiency (*A*), translation of the transfected gene (*B*), and reduction in cell damage (*C*) compared with porcine SSCs not exposed to FIR. Data are means ± standard deviation of three independent experiments. $^{*}p < 0.05$.

(Supplementary Fig. 1*B*), or viability (Supplementary Fig. 1*C*). Therefore, FIR exposure in porcine SSCs before electroporation, regardless of FIR-exposure time, improved the transfection efficiency, translation of the transfected gene, and reduction in cell damage, indicating that FIR can overcome the electrical and physical deleterious effects of electroporation.

In Experiment 2, to optimize the electroporation conditions for porcine SSCs irradiated with FIR, a single electrical pulse from an electroporator with a capacitator setting of 500 μF was used, and the cells were transfected with 1 μg pEGFP-N1 in Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY) using an electroporator at a voltage of 100, 150, 200, or 250 V. Electroporation at a voltage of 200 V resulted in the highest percentage of EGFP-positive cells (Supplementary Fig. 2*A*), and electrical pulses > 200 V significantly increased the EGFP fluorescence intensity (Supplementary Fig. 2*B*) but significantly decreased cell viability (Supplementary Fig. 2*C*). Therefore, a 200 V electrical pulse was used in subsequent experiments to introduce foreign DNA into the cytoplasm of porcine SSCs irradiated with FIR.

Next, we determined the optimal electroporation buffer for transfection of foreign DNA into irradiated porcine SSCs by electroporation using a single electrical pulse from an electroporator at a capacitor setting of 500 μF and a voltage of 200 V. The percentage of EGFP-positive cells (Supplementary Fig. 3*A*) and the intensity of EGFP fluorescence (Supplementary Fig. 3*B*) were unaffected by the electroporation buffer used. However, electroporation in porcine SSC culture medium (pSSCCM) resulted in significantly higher viability of irradiated porcine SSCs compared with electroporation in HBSS (Supplementary Fig. 3*C*). Therefore,

we used pSSCCM to introduce foreign DNA into the cytoplasm of irradiated porcine SSCs by electroporation.

We also analyzed the optimal capacitance for transfection of foreign DNA into irradiated porcine SSCs by electroporation, using a single electrical pulse from an electroporator at a voltage of 200 V and a capacitor setting of 250, 500, or 750 μF. Electroporation at a capacitor setting of 750 μF resulted in the highest percentage of EGFP-positive cells (Supplementary Fig. 4*A*) and intensity of EGFP fluorescence (Supplementary Fig. 4*B*) and significantly decreased cell viability (Supplementary Fig. 4*C*), compared with capacitor settings of 250 and 500 μF. Accordingly, a capacitor setting of 500 μF was used in subsequent experiments to introduce foreign DNA into the cytoplasm of irradiated porcine SSCs by electroporation.

We next evaluated the optimal concentration of foreign DNA for transfection into irradiated porcine SSCs by electroporation, using a single electrical pulse from an electroporator with a capacitor setting of 500 μF at a voltage of 200 V with 1, 2.5, or 5 μg pEGFP-N1. The percentage of EGFP-positive cells (Supplementary Fig. 5*A*) and the EGFP fluorescence intensity (Supplementary Fig. 5*B*) were not affected by increasing plasmid concentrations. However, transfection with 1 μg pEGFP-N1 resulted in significantly higher cell viability than 2.5 or 5 μg pEGFP-N1 (Supplementary Fig. 5*C*). These results demonstrate that 1 μg foreign DNA should be used to transfect irradiated porcine SSCs by electroporation.

Transfection of irradiated porcine SSCs with 1 μg pEGFP-N1 using one, two, or three electrical pulses from an electroporator at a capacitor setting of 500 μF and a voltage of 200 V was conducted to assess the effect of electrical pulse frequency on the transfection of foreign DNA into irradiated porcine SSCs. A non-significant numerical increase in the percentage of EGFP-positive cells (Supplementary Fig. 6*A*) and a significant increase in EGFP fluorescence intensity (Supplementary Fig. 6*B*) were noted as the electrical pulse frequency increased. However, cell viability decreased significantly with increasing electrical pulse frequencies (Supplementary Fig. 6*C*). Therefore, a single electrical pulse is optimal for introducing foreign DNA into porcine SSCs irradiated with FIR. Based on all of these results, the optimal electroporation protocol comprised 1 μg pEGFP-N1 with a single electrical pulse from an electroporator at a voltage of 200 V and a capacitor setting of 500 μF in pSSCCM.

For electroporation-mediated delivery of genes to porcine SSCs, minimizing electrical shock-induced damage is important. We used FIR because it enhances the physical strength of the cell membrane; in this study, we formulated an optimal electroporation-mediated transfection protocol for FIR-exposed porcine SSCs. The exposure of porcine SSCs to FIR before electroporation significantly enhanced all transfection-related parameters. Transfection of irradiated porcine SSCs with 1 μg pEGFP-N1 using a single electrical pulse from an electroporator with a capacitor setting of 500 μF and 200 V in pSSCCM showed the best performance. Therefore, the introduction of foreign DNA into the cytoplasm of porcine SSCs by electroporation can be enhanced by pre-exposing the cells to FIR.

FIR improves cell survival and recovery (Toyokawa *et al.* 2003; Lin *et al.* 2008; Leung *et al.* 2011; Jeong *et al.* 2017) by accelerating restoration of the plasma membrane by enhancing membrane fluidity and reducing the resealing time (Sommer *et al.* 2008; Chattopadhyay *et al.* 2021). Therefore, FIR has the potential to alleviate the cell-membrane damage induced by electrical shock during electroporation (Kandušer *et al.* 2005). This is associated with inhibition of the release of cellular components such as ATP and inorganic ions or prevention of osmotic imbalance and oxidative stress, thereby preventing apoptosis, pyroptosis, and necrosis (Chang *et al.* 2016). In this study, despite use of electrical shocks twofold stronger than the optimal level for non-irradiated porcine SSCs (Kim *et al.* 2019), FIR exposure in porcine SSCs before electroporation increased not only cell viability but also transfection efficiency (Fig. 1*A* and 1*C*). The increased survival rate of porcine SSCs may be a result of inhibition of electrical membrane permeabilization-mediated apoptosis. Furthermore, the FIR-induced enhancement of membrane integrity in porcine SSCs may enhance cell viability after electroporation, thereby increasing the transfection efficiency.

FIR promotes mitochondrial activity and biogenesis (Osellame *et al.* 2012; Chang *et al.* 2016; Seo *et al.* 2021). This enhances protein homeostasis by regulating the degradation of damaged proteins and the synthesis of new proteins (Webster *et al.* 2017; Suhm *et al.* 2018). Therefore, the FIR-induced improvement in cellular protein homeostasis could enhance cell survival by strengthening resistance to intracellular protein damage caused by electrophoresis.

The translation of proteins from transfected genes can be reduced by methylation of the promoter, which regulates the transcription of the inserted genes (Kong *et al.* 2009). In this study, porcine SSCs subjected to FIR showed heightened translation of the foreign gene (Fig. 1*B*). This phenomenon may be caused by demethylation of the promoter; indeed, irradiation at terahertz frequencies, which encompass FIR wavelengths, can induce demethylation of DNA (Cheon *et al.* 2019).

In conclusion, FIR exposure in porcine SSCs enhanced the safety and efficiency of electroporation-based delivery of genes. We formulated a protocol for electroporation-based delivery of genes to porcine SSCs irradiated with FIR. Our protocol will improve the efficiency of electroporation-based delivery of DNA to diverse types of cells.

Moreover, it will accelerate the production of transgenic sperm, a crucial step for the industrial use of transgenic animals.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s11626-024-00967-3.

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