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Generation of Calpain-3 knock-out porcine embryos by CRISPR-Cas9 electroporation and intracytoplasmic microinjection of oocytes before insemination

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ABSTRACT

Limb girdle muscular dystrophy type R1 (LGMDR1) is an autosomal recessive myopathy described in humans resulting from a deficiency of calpain-3 protein (CAPN3). This disease lacks effective treatment and an appropriate model, so the generation of KO pigs by CRISPR-Cas9 offers a way to better understand disease ethology and to develop novel therapies. Microinjection is the main method described for gene editing by CRISPR-Cas9 in porcine embryo, but electroporation, which allows handling more embryos faster and easier, has also recently been reported. The objective of the current study was to optimize porcine oocyte electroporation to maximize embryo quality and mutation rate in order to efficiently generate LGMDR1 porcine models. We found that the efficiency of generating CAPN3 KO embryos was highest with 4 electroporation pulses and double sgRNA concentration than microinjection. Direct comparison between microinjection and electroporation demonstrated similar rates of embryo development and mutation parameters. The results of our study demonstrate that oocyte electroporation, an easier and faster method than microinjection, is comparable to standard approaches, paving the way for democratization of transgenesis in pigs.

1. Introduction

The generation of animals as human disease models facilitates the development of treatments for diseases that have no cures or effective treatments to alleviate disease symptoms, and also permits a better understanding of the underlying disease mechanisms. Limb-girdle muscular dystrophy recessive 1 (LGMDR1), previously known as LGMD2A, is an autosomal recessive myopathy described in humans that is caused by a deficiency of the muscle-specific calcium-activated neutral protease 3 (calpain 3, CAPN3) [1,2]. This disease is characterized by progressive weakening of the shoulder, pelvic and proximal limb muscles and usually appears in children and young adults [2]. Currently, the precise pathophysiological mechanism of this disease is unknown. There is no effective treatment for LGMDR1, resulting in a loss of ambulation within 20 years after disease onset in most patients [2]. In 2000, the first animal knock-out (KO) for *CAPN3* was generated in mice [3] and in addition, knock-in (KI) mice with specific mutations of *CAPN3* have been generated [4]. However, while murine *CAPN3* mutants exhibit some characteristics of LGMDR1, including dystrophic symptoms, disease severity is markedly greater in human patients [2,5]. Thus, the lack of suitable animal models for this disease the necessitates development of complementary animal models using species that are physiologically and anatomically closer to humans, such as the pig [6,7].

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The development of CRISPR-Cas9 editing technology has become an important tool for production of KO/KI animal models [8–10]. In 2014, the first KO pigs were generated using the CRISPR-Cas9 system [8,9] via intracytoplasmic microinjection of Cas9 mRNA and sgRNAs into zygotes. Since then, a number of genetically modified pigs have been produced with different intended uses in biomedicine, such as for xenotransplantation [11,12], human disease models [13–17], and agriculture [18].

Currently, there are two popular approaches to producing genetically modified pig embryos by CRISPR-Cas9: somatic cell nuclear transfer (SCNT) [8] and the delivery of CRISPR-Cas9 into oocytes or embryos [9]. The first consists of producing somatic cells with the desired mutation, via transfection of somatic cells with CRISPR-Cas9 constructs and their subsequent fusion with enucleated *in vitro* matured oocytes [19]. The second involves intracytoplasmic microinjection of CRISPR-Cas9 using plasmid DNA [20], RNA [8,9] or ribonucleoprotein complex (RNP) [21] into oocytes or zygotes. In recent years, electroporation of oocytes and zygotes has also been used to perform CRISPR-Cas9 gene editing and was first reported in mouse embryos [22]. This process consists in subjecting the cells to a pulsatile electric current to induce reversible plasma membrane breaks that allow entry of external macromolecules into the cytoplasm. The first reported use of this technique in pig embryos was in 2016 [23], leading to production of KO piglets by electroporation [16,23–28].

Most gene editing studies in pigs have targeted zygotes for electroporation [16,23–32], but generation of edited embryos has also been reported after porcine oocyte electroporation [32]. Nevertheless, embryos derived by oocyte electroporation have been shown to have poorer developmental outcomes compared with those produced from zygote electroporation [32]. In previous studies, we demonstrated that injection of CRISPR-Cas9 into *in vitro* matured porcine oocytes before fertilization is a suitable strategy to minimize mosaicism without reducing the mutation rate [17] and we have recently explored the use of Aphidicolin, a reversible inhibitor of eukaryotic nuclear DNA replication for reducing the mosaicism [33]. Since oocyte microinjection leads to reduced mosaicism, on of our objective was improving the conditions for generating KO embryos by oocyte electroporation to achieve equivalent rates of embryo quality and mutation relative to oocyte microinjection.

These methodological optimization experiments were performed in the context of studies designed to generate pig models of LGMDR1 by mutating the gene encoding CAPN3. In the literature, hundreds of allelic variants of *CAPN3* have been registered as variants producing muscular dystrophies included in the pathophysiology of LGMDR1 [1,2,34]. For the generation of porcine embryos to model LGMDR1, we have designed two separate gene editing strategies: a) generating *CAPN3* KO embryos that have a deletion of the start codon (Fig. 1); and b) generating embryos with a dysfunctional *CAPN3* with a frameshift mutation in exon 22 of this gene (Fig. 1), which causes a severe form of the disease predominant in the Basque cohort of LGMDR1 patients [34]. In this study, we tackle both the gene editing strategies to generate pig models for LGMDR1 and methods to maximize oocyte electroporation conditions for gene editing.

Therefore, the objective of the current study was to optimize porcine oocyte electroporation to maximize embryo quality and mutation rate in order to efficiently generate LGMDR1 porcine models.

2. Material and methods

2.1. Ethical issues

The study was developed according to the Spanish Policy for Animal Protection (RD 53/2013), which conforms to the European Union Directive 2010/63/EU regarding the protection of animals used in scientific experiments. This project was approved by the Ethics Committee at the University of Murcia (Reference CBE 195/2019, CCEA 525/2019) and Murcia Regional Government for the use of Genetically Modified Organisms (Reference 01/2016, activities A/ES/16/79, Facilities A/ES/16/I-22 and I-23).

2.2. Culture media reagents

All chemicals were purchased from Sigma-Aldrich Quimica, S.A. (Madrid, Spain) unless otherwise indicated.

2.3. Design of sgRNAs

CAPN3 targeting guide RNA sequences were designed using software available from the National Center for Biotechnology of the Spanish National Research Council (CNB-CSIC; https://bioinfogp.cnb.csic.es/tools/breakingcas). Three different guides were designed (Fig. 1) flanking the start codon in exon 1, sgRNA #3 57bp upstream of the start codon and sgRNA #6 and sgRNA #7 53bp and 117 bp downstream of the start codon, respectively. Separately, sgRNA #22 was designed to target codon R788 in exon 22 of *CAPN3*. The sgRNAs and Cas9 protein were purchased from IDT (Integrated DNA Technologies, Leuven, Belgium) and RNP complexes were prepared according to manufacturer recommendations.

2.4. Oocyte in vitro maturation (IVM)

Cumulus-oocyte complex (COCs) were obtained from gilt ovaries from the slaughterhouse and processed as previously described [35]. Briefly, ovaries were transported to the laboratory in saline solution at 38 °C, washed once in 0.04% cetrimide and twice in saline, both at 38 °C. COCs were collected by aspiration from follicles between 3 and 6 mm diameter, washed in Dulbecco's PBS with 1 mg/mL polyvinyl alcohol (DPBS-PVA), and then washed again in maturation medium (NCSU-37) [36]. Groups of 50–55 COCs were subsequently cultured in 500 μL of NCSU-37 supplemented with 10% (v/v) porcine follicular fluid, 1 mM dibutyryl cAMP, 10 UI/mL eCG and 10 UI/mL hCG for 20–22 h at 38.5 °C under 5% CO_2 and 7% O_2 conditions, followed by an additional 20–22h in NCSU-37 lacking dibutyryl cAMP, eCG and hCG.

2.5. Evaluation of macromolecule delivery to oocytes by electroporation

To evaluate the capacity to introduce macromolecules, *in vitro* matured and decumulated oocytes were cultured in DPBS-PVA with 2 mg/mL tetramethylrhodamine-labelled dextran (TMRD) (3000 MW; Molecular Probes, Inc. Eugene, OR, USA) as previously reported for rat oocytes [22]. Oocytes were electroporated with different numbers of pulses and a negative control without electroporation was performed. After electroporation, the localization of TMRD was determined by epifluorescence microscopy (Nikon Eclipse 90i) and images were collected to measure fluorescence intensity using ImageJ (version 1.52a, National Institutes of Health, USA). The relative intensity was calculated using the maximal fluorescence signal as 100% of the signal and different groups were compared.

2.6. CRISPR-Cas9 delivery

After IVM, 50 μL of 0.5% hyaluronidase was added to each well containing 500 μL NCSU37 and COCs, and incubated for 5 min at

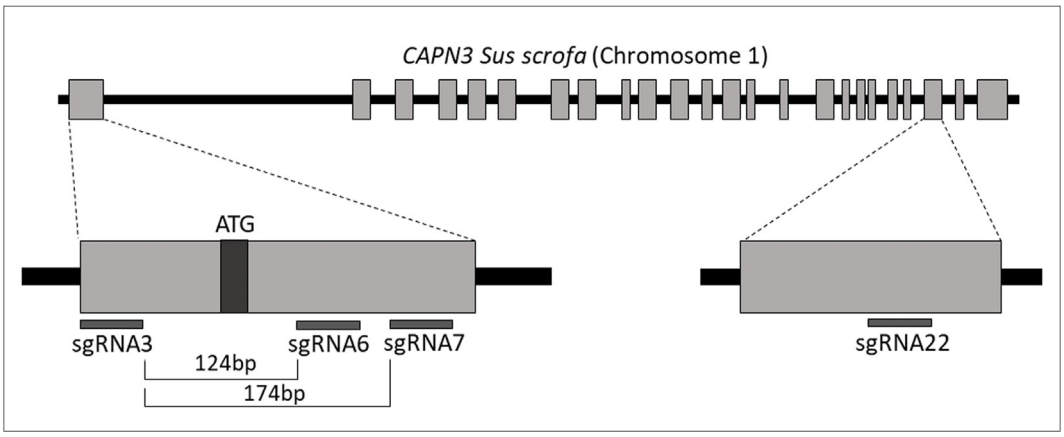

Fig. 1. Scheme of *CAPN3* gene in chromosome 1 of the domestic pig (*Sus scrofa*) with location of single guides RNAs (sgRNA) designed to generate a KO embryo for *CAPN3*. Two different strategies were designed to produce porcine embryos with LGMDR1. The deletion of start codon in exon 1 (sgRNA3, sgRNA6 and sgRNA7) and the generation of a mutation with a frameshift in exon 22, which causes a severe form of LGMDR1 predominant in the Basque country population. bp: base pair.

38.5 °C under 5% CO_2 and 7% O_2 conditions. The matured COCs were manually decumulated by pippeting until most of the cumulus cells were removed [17].

2.6.1. Electroporation

Before electroporation, decumulated oocytes were washed in Opti-MEM I Reduced Serum Media (Thermofisher, Waltham, MA USA). Subsequently, groups of 50–100 oocytes were transferred to a droplet containing CRISPR-Cas9 RNPs, placed on a slide between 1 mm gap electrodes (45–0104, BTX, Harvard Apparatus, USA) connected to ECM 830 Electroporation System (BTX, Harvard Apparatus, USA), and electroporated using 2, 4 or 6 pulses, at 30 V, 1 ms pulse duration and 100 ms pulse interval [37].

2.6.2. Microinjection

Before microinjection, decumulated oocytes were washed in DPBS-PVA, transferred in pairs into 6 μL DPBS-PVA drops covered by mineral oil, and microinjected with CRISPR-Cas9 RNPs, essentially as described [17].

2.7. In vitro fertilization (IVF)

IVF was performed essentially as described previously [35]. Depend on the treatment, the oocytes were pre-treated (electroporation or microinjection) before IVF. Briefly, *in vitro* matured oocytes were washed in TALP medium [38] supplemented with 1 mM sodium pyruvate, 0.3% BSA, and 50 μg/mL gentamycin (IVF-TALP), and transferred in groups of 50–55 oocytes to wells containing 250 μL IVF-TALP medium. Oocytes were inseminated with frozen-thawed ejaculated spermatozoa from a fertile boar after being selected for motility by a swim-up procedure [17]. Briefly, one 0.25 mL semen straw was thawed in a water bath (30 s, 38 °C) and semen was diluted in 2 mL NaturARTsPIG sperm swim-up media (Embryocloud) at 38 °C. Sperm selection was performed by adding 1 mL sperm swim-up media in a conical tube and layering 1 mL thawed-diluted sperm beneath the media, incubating (38 °C, 20 min, 45° angle), and removing 500 μL of the top medium by gentle aspiration. The concentration of selected motile was adjusted to 1×10^4 sperm/mL in IVF-TALP and oocytes were inseminated with 250 μL sperm suspension (final IVF well volume 500 μL). Gametes were cocultured at 38.5 °C, 5% CO_2, and 7% O_2 for 20–22 h.

2.8. In vitro embryo culture and embryo quality evaluation

After gamete co-incubation, putative zygotes were cultured in NCSU-23 medium supplemented with 5 mM sodium lactate, 0.5 mM sodium pyruvate, and both essential (1% v/v) and nonessential (1% v/v) amino acids [35] and cultured for 24 h at 38.5 °C, 5% CO_2, and 7% O_2. Cleavage rates were then evaluated and 2–4 cell embryos transferred to NCSU-23 medium supplemented with 5.5 mM glucose and essential (1% v/v) and nonessential (1% v/v) amino acids [35] until 156 h after insemination. After *in vitro* culture, blastocysts rate was determined, and blastocyst were collected and some were fixed and stained with Hoechst 33342 to evaluate the number of cells [39]. Mutation rates were evaluated in the remaining individual unfixed embryos as described below.

2.9. Mutation analysis in blastocysts

The zona pellucida was digested with 0.5% pronase (Protease from Streptomyces griseus, P8811, Sigma-Aldrich, Madrid, Spain) and subsequently blastocysts were washed in nuclease free water and stored individually in a minimal volume at −80 °C until analysis. DNA extraction and PCR were performed using a Phire Animal Tissue Direct PCR Kit (Thermofisher, Waltham, MA USA). Genomic DNA was extracted following the dilution protocol of this kit. One microliter per sample was used for 12.5 μL of PCR reaction containing 0.5 μM primers. The PCR cycling times included an initial denaturation for 5 min at 98 °C followed by 40 cycles (denaturation 5 s at 98 °C, annealing 5 s at 64.9 °C for exon 1 primers and 64.8 °C for exon 22 primers, extension 20 s at 72 °C) and a final extension for 1 min at 72 °C.

Mutation of exon 1 (Fig. 2A) was by evaluation of the desired deletion (deletion by cut of both sgRNA), so it was detected by observation of a shorter amplicon using agarose gel electrophoresis (1.5% agarose in TAE). Mutation of exon 22 (Fig. 2B) was determined using a fluorescent PCR-capillary gel electrophoresis technique [17,40]. PCR was carried out using 6-FAM-labelled FW primers. After PCR, samples were diluted 1:100 in TE buffer and 1 μL of the mixed samples was added to a clean 1.5 mL tube with 11.5 μL Hi-Di™ formamide (Thermofisher) and 0.1 μL GeneScan™ 500 LIZ Size Standard (Applied Biosystem, Thermofisher). The sample was incubated (3 min at 95 °C), immediately chilled on ice for 2 min, and analysed by capillary gel electrophoresis on a 3500 Genetic Analyzer (Applied Biosystems, Thermofisher). The details of the instrumental protocol were similar to that previously described

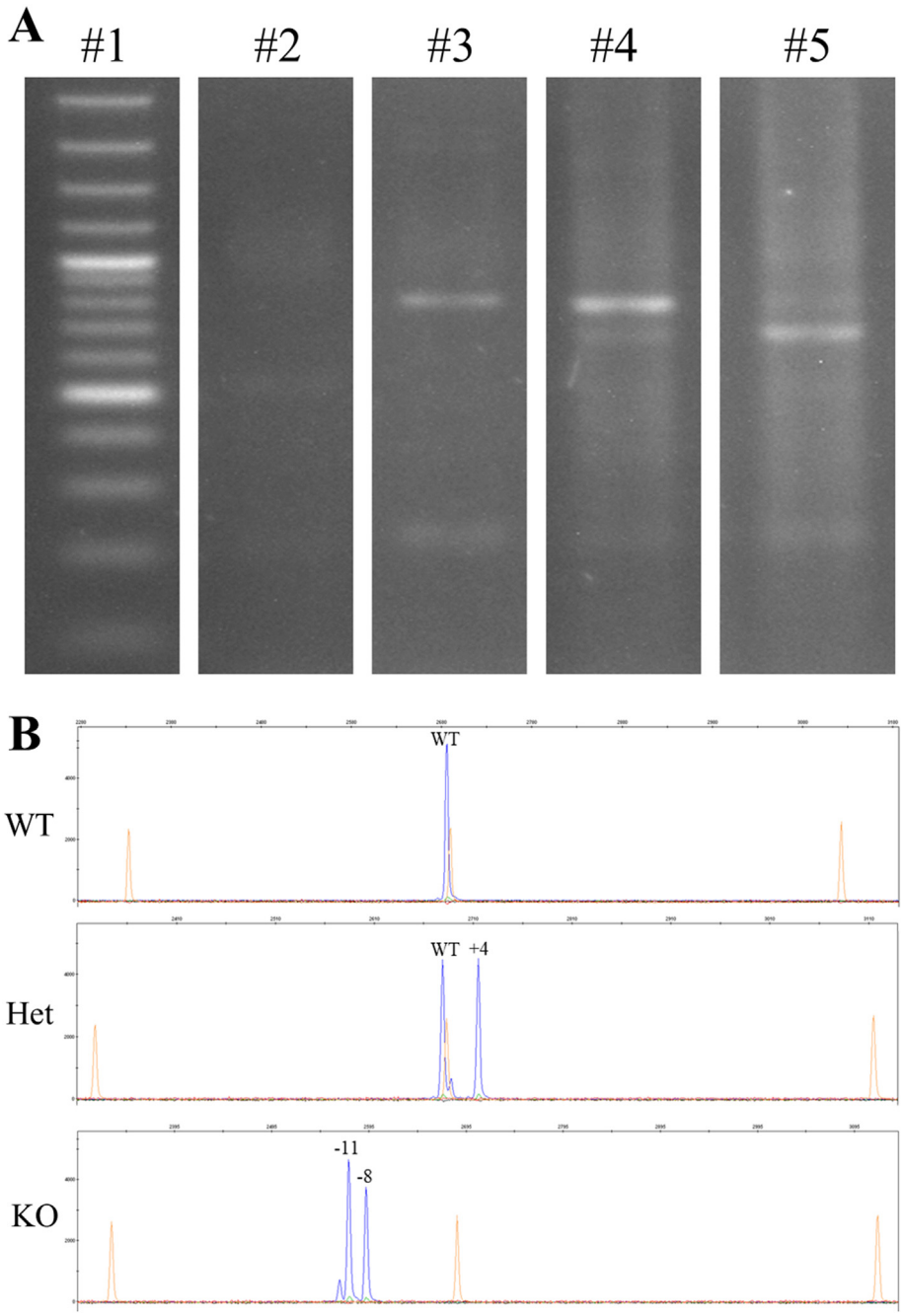

Fig. 2. Mutation analysis. A agarose electrophoresis to evaluate deletion in exon 1. Note: the size marker on lane #1, negative control on lane #2, wildtype amplicon on lane #3, heterozygous sample (WT amplicon + amplicon with deletion) on lane #4 and doble KO sample (amplicon with deletion) on lane #5. B capillar electrophoresis to evaluate mutation in exon 22. WT: sample with wildtype allele. Het: sample with wildtype allele and KO allele (insertion of 4 base pairs). KO: sample with KO alleles (deletion of −11 base pairs and −8 base pairs).

[40]: capillary length: 50 cm; polymer: POP7; dye set: G5; run voltage: 19.5 kV; pre-run voltage: 15 kV; injection voltage: 1.6 kV; run time: 1330 s; pre-run time: 180 s; injection time: 15 s; data delay: 1 s; size standard: GS500 (−250) LIZ; size-caller: SizeCaller v1.10. Results were analysed using Gene Mapper 5 (Life Technologies). Embryos were considered to be wild type (WT) when the peak obtained by capillary electrophoresis was the same size as the control peak.

Other peaks of different sizes with respect to the control peak were considered to be knock-out (KO) and when more than two peaks were detected in a sample, embryo was considered as mosaic.

2.10. Statistical analysis

All data analysis was performed using SYSTAT 13. The variables in all experiments were tested for their normality by a Shapiro-Wilk test. Data that were not normally distributed were analysed by a Kruskal-Wallis test. When data had significant differences ($p < 0.05$), values were compared by a Conover-Inman test for pairwise comparisons. Data with normal distribution were analysed by one-way analysis of variance (ANOVA). When data showed significant differences ($p < 0.05$), values were compared by a pairwise multiple comparison post hoc test (Tukey).

2.11. Experimental design

In order to optimize the electroporation delivery of sgRNAs, we first evaluated the effect of the number of pulses on the capacity to deliver macromolecules and on the embryo development (blastocyst rate). Later once the electroporation system was optimized, the objective was to maximize the efficiency of generating CALP3 KO blastocytes with minimal mosaicism using alterations to the number of electrical pulses, the sgRNA concentration and different combinations of sgRNAs (Fig. 3).

2.11.1. Experiment 1: Evaluation of the effect of number of electrical pulses on macromolecule delivery into in vitro matured oocytes by electroporation

To evaluate macromolecule delivery into the oolemma, oocytes were exposed to TMRD and were electroporated using 0 (control group), 2, 4 or 6 pulses (Fig. 3). After electroporation, the localization of TMRD was observed using an epifluorescence microscope. The relative intensity was calculated using the maximal signal as 100% of the signal and different groups were compared. Two replicates were performed.

2.11.2. Experiment 2: Effect of number of electrical pulses on embryo development

To evaluate any possible detrimental effect of electroporation on embryo development, groups of 50 *in vitro* matured oocytes were electroporated in DPBS using 0 (control group), 2, 4 or 6 pulses without CRISPR-Cas9 (Fig. 3). After electroporation, oocytes were *in vitro* fertilized and cultured until 156 h after IVF. Cleavage rate, blastocyst rate and number of cells per blastocyst were evaluated to determine any potential damage induced by different numbers of electroporation pulses. Three replicates were performed.

2.11.3. Experiment 3: Effect of number of pulses on CAPN3 KO embryo generation by electroporation

Once we confirmed that the electroporation conditions allowed macromolecule cross through the zona pellucida (ZP) and oolemma, the next step was to evaluate the effect of these conditions on generation of KO embryos using a strategy with two different sgRNAs (sgRNA3 + sgRNA6, Fig. 3). Cas9 protein and sgRNAs were mixed at 100 ng/μL Cas9 and 12.5 ng/μL each guide (1:1 Cas9:sgRNA molar ratio). Five experimental groups of oocytes were *in vitro* fertilized and *in vitro* cultured until blastocyst stage as follows: a) control (without electroporation or microinjection), b) RNP electroporation with 4 pulses, c) RNP electroporation with 6 pulses, d) RNP microinjection, and e) DPBS microinjection (microinjection control). Cleavage and blastocyst rates were compared between groups and 156 h after insemination blastocysts were stored to be genetically evaluated. The percentage of embryos with deletions and the percentage of embryos with homozygous deletions were calculated to compare the efficiency of different conditions. Four replicates were performed.

2.11.4. Experiment 4: Effect of sgRNA concentration on the generation of CAPN3 KO embryos

With the aim of improving mutation rates, the sgRNA concentration was doubled (Fig. 3) to deliver 25 ng/μL of each guide (sgRNA 3 + sgRNA 6, 1:2 Cas9:sgRNA molar ratio). For this experiment, two experimental groups were used: a) RNP electroporation with 4 pulses, and b) RNP microinjection. Embryos were *in vitro* cultured until day 6 and percentage of embryos with deletions and the percentage of embryos with homozygous deletions were calculated to compare the efficiency of different conditions. Four replicates were performed.

2.11.5. Experiment 5: Efficiency of different strategies to generate LGMDR1 models

With respect to embryo quality and mutation rate, the most efficient conditions of previous experiments, electroporation (1:2 Cas9:sgRNA molar proportion) and microinjection (1:1 Cas9:sgRNA molar proportion), were directly compared. For this experiment, two different combinations of sgRNAs against the start codon of *CAPN3* were used: sgRNA 3 + sgRNA 6 and sgRNA 3 + sgRNA 7 (Fig. 1). In addition, these guides were used together with a guide against exon 22 of *CAPN3* (Fig. 1). Four groups were designed for this experiment (Fig. 3): a) 3 + 6+22 electroporation, b) 3 + 6+22 microinjection, c) 3 + 7+22 electroporation, and d) 3 + 7+22 microinjection. Embryos were evaluated as explained for experiment 3. The percentage of embryos with deletions and the percentage of embryos with homozygous deletions were calculated to compare the efficiency of the 3 + 6 or 3 + 7 combinations, and the percentage of embryos with a mutation in exon 22 were evaluated by capillary electrophoresis. Five replicates were performed.

3. Results

3.1. Effect of number of pulses on macromolecules delivery into in vitro matured oocytes by electroporation

In the control group (non-electroporated oocytes) no fluorescence was observed (Fig. 4A) and when electroporation was performed, we observed a pulses-dependent increase in the relative intensity of fluorescence in oocyte cytoplasm (Fig. 4B–D) as a consequence of the TMRD intake. Specifically, increased number of pulses led to a proportional increase in fluorescence intensity (2 pulses 31.6%; 4 pulses 58.5% and 6 pulses 70.9%; $p < 0.05$, Fig. 4E).

3.2. Effect of number of pulses on embryo development

We found that the cleavage rate was significantly increased in embryos exposed to electroporation (ranged 69.9–78.8%) compared with the control group (49.3%) (Table 1, $p < 0.01$). The application of 4 pulses was associated with the highest cleavage rate. However, blastocyst rate (percentage of blastocysts per initial oocytes) showed differences only between 2- and 4-pulse groups ($p = 0.03$), being higher in the 4-pulse group. Notably, the mean number of cells per blastocyst was significantly lower in electroporated groups compared with the control (Table 1, $p = 0.03$).

3.3. Effect of number of pulses and sgRNA concentration on CAPN3 KO embryo generation by electroporation

After the delivery of RNP 3 + 6 against *CAPN3* (Table 2), no significant differences were found in the cleavage rate in microinjection groups and 4-pulse group respect to the control group. Nevertheless, cleavage rate was significantly higher in 4-pulse group relative to the microinjection groups ($p < 0.01$) and significantly lower in the 6-pulse group relative to the control and microinjection groups ($p < 0.01$).

Blastocyst rates were lower in all manipulated groups with respect to the control group (Table 2, $p < 0.01$), and the lowest value was found in the 6-pulse electroporation group with significant difference relative to all other groups (control microinjection, microinjection, and 4 pulses).

Regarding mutation parameters, the percentage of embryos with at least one deletion was significantly higher in the microinjection group than in the electroporation groups (40 *vs.* 19% Table 2, $p = 0.04$) and no differences were found between 4 or 6 pulses. The percentage of embryos with a biallelic mutation, that is both alleles having a deletion, showed a similar pattern in all groups ranged

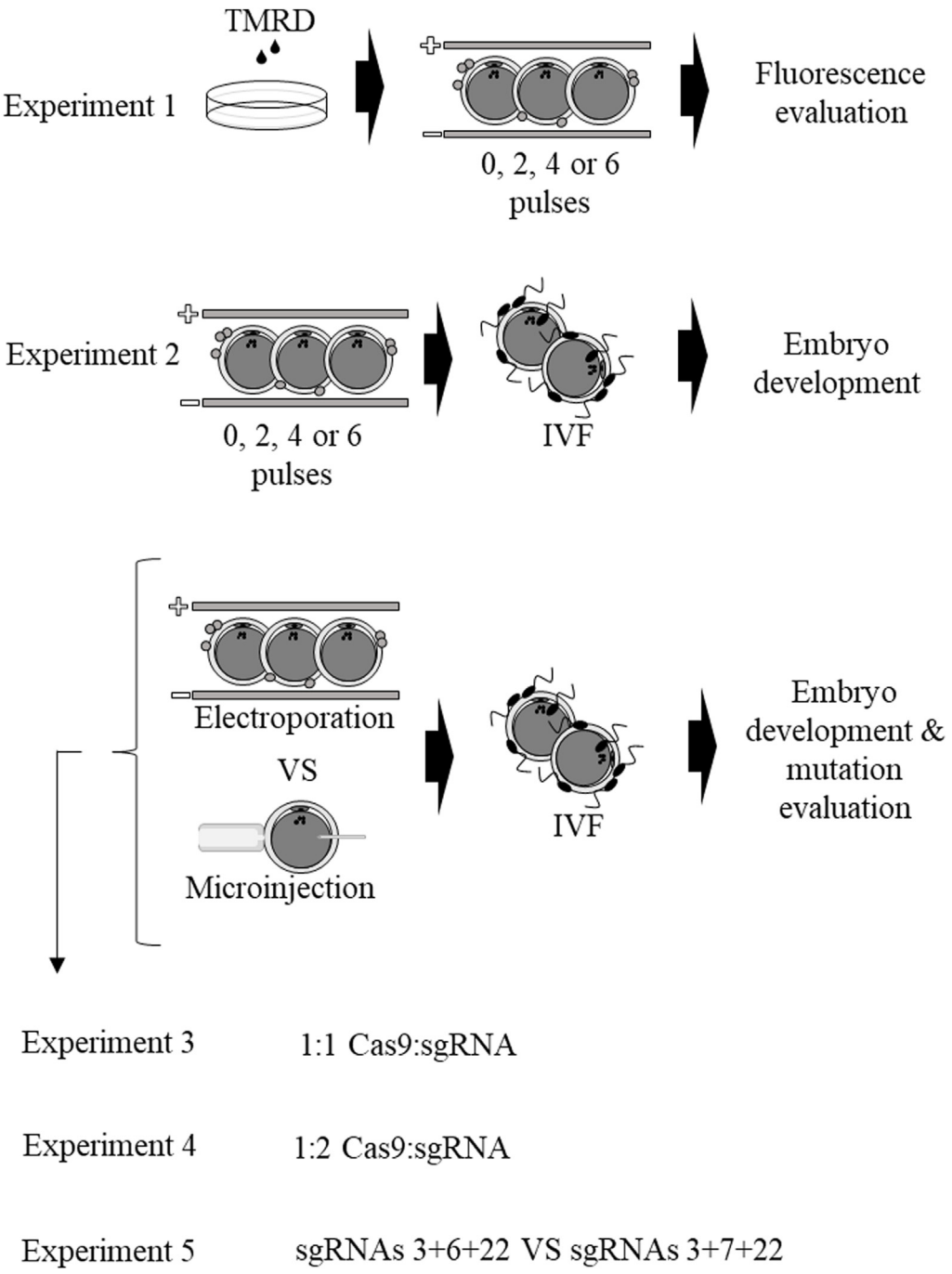

Fig. 3. Experimental design. Experiment 1: Evaluation of the effect of number of pulses in macromolecule delivery into *in vitro* matured oocytes by electroporation. Tetramethyl rhodamine-labelled dextran (TMRD). Experiment 2: Evaluation of the effect of number of pulses on embryo development. Experiment 3: Effect of number of electroporation pulses on *CAPN3* KO embryo generation, where oocytes were microinjected or electroporated with an equimolar concentration of RNP (100 ng/μL Cas9 and 12.5 ng/μL each guide). Experiment 4: Effect of sgRNA concentration on the generation of *CAPN3* KO embryos, where oocytes were microinjected or electroporated with a double molar concentration of sgRNA relative to Cas9 (100 ng/μL Cas9 and 25 ng/μL each guide). Experiment 5: Efficiency of different strategies to generate LGMDR1 models, where the efficiency of guides sgRNAs 3 + 6+22 and sgRNA 3 + 7+22 were compared by microinjection and electroporation.

6.5–15.6% (Table 2, $p > 0.05$).

When the concentration of sgRNA was doubled (from 12.5 to 25 ng/μL) for electroporation, no differences were found in the deletion or biallelic deletion rates between electroporation and microinjection groups (Fig. 5).

3.4. Efficiency of different strategies to generate LGMDR1 models

After the optimization of electroporation conditions in the previous experiments (4 pulses of electroporation) and changes to the ratio of sgRNA:Cas9, the efficiency of electroporation was tested

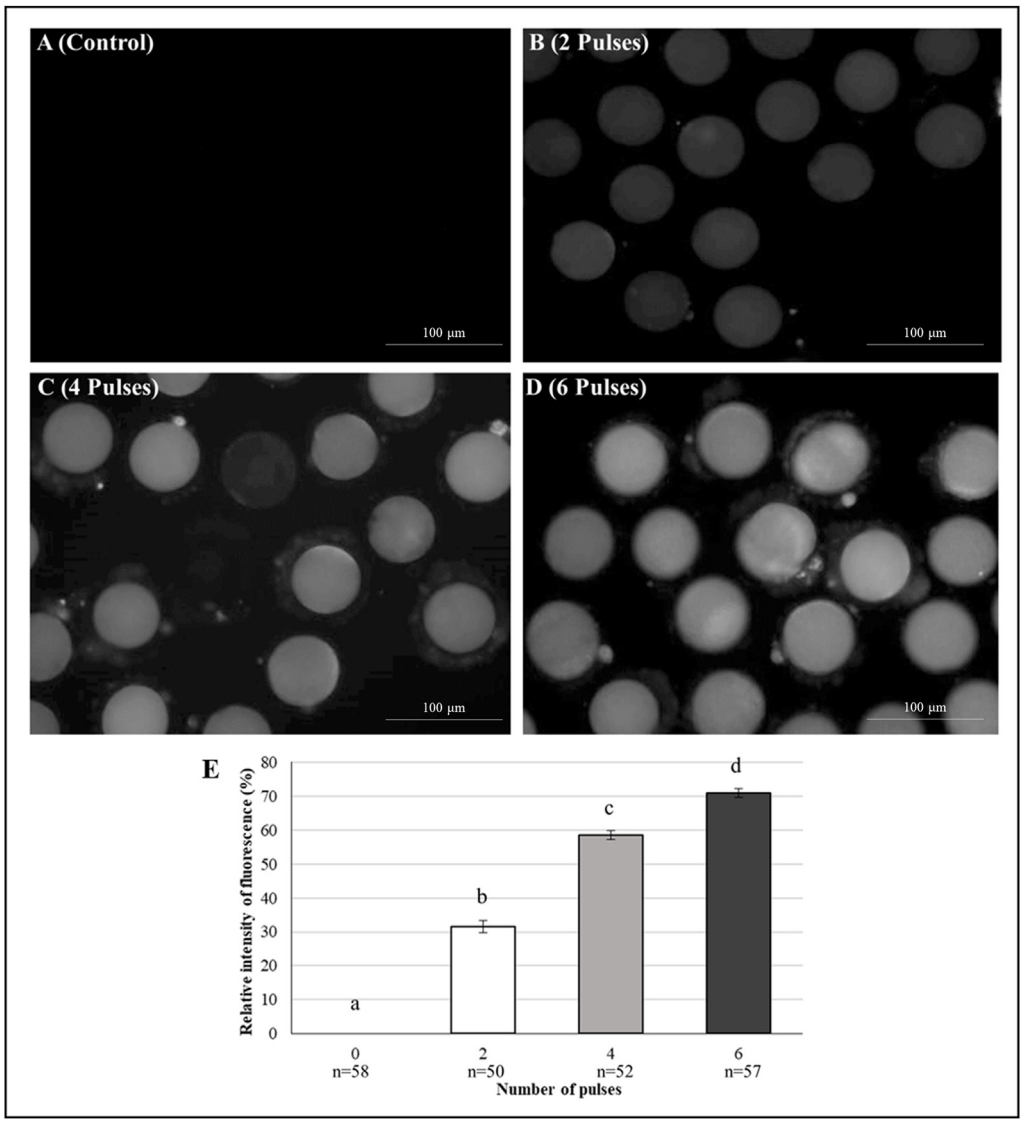

Fig. 4. Oocytes electroporated with tetramethyl rhodamine-labelled dextran (TMRD) (A non electroporated, B-D electroporated with the noted number of pulses). E results of relative intensity of fluorescence (RIF) with DTMR electroporation. Data expressed as mean ± SEM. [a-d] Values in the same column with different superscripts are significantly different ($p < 0.05$).

in comparison to microinjection to analyse different strategies to generate embryo models of LGMDR1 (Fig. 1). As expected, cleavage rate was higher in the electroporation groups compared with microinjection groups ($p < 0.01$) without effect of guide combination (3 + 6+22 *vs.* 3 + 7+22). No differences were found in blastocyst rate (Table 3).

Regarding mutation parameters (Table 3), no differences were found between either the methods (electroporation *vs.* microinjection) or the combination of guides (3 + 6+22 *vs.* 3 + 7+22) in the percentage of embryos with a deletion in exon 1 and mutations in exon 22 (Table 3). Mutant rate ranged between 46.2 and 51.6% of embryos with a deletion in exon 1, 16.7–20.5% with a biallelic deletion in exon 1, 66.2–78.6% with a mutation in exon 22, and 0–3.2% for biallelic mutations.

As shown in Fig. 5, the strategy followed to produce pig models of LGMDR1 made it possible to produce a high percentage of

Table 1
Effect of number of electroporation pulses without CRISPR/Cas9 in embryo development. Data expressed as mean ± SEM.

	Control No electroporated	2 pulses electroporation	4 pulses electroporation	6 pulses electroporation	p value
n	140	146	146	132	
Cleavage rate[a] (%)	49.3 ± 4.2[a]	69.9 ± 3.8[b]	78.8 ± 3.4[c]	66.7 ± 4.1[b]	<0.01
Blastocyst rate[b] (%)	30.7 ± 3.9[ab]	22.6 ± 3.5[a]	38.4 ± 4.0[b]	27.3 ± 3.9[ab]	0.03
Cells/blastocyst[c]	56.5 ± 4.3[a]	44.2 ± 3.6[b]	43.3 ± 2.2[b]	41.5 ± 2.2[b]	0.03

[a-b] Values in the same column with different superscripts are significantly different ($p < 0.05$). n, number of analysed samples.
[a] Two cell embryos per total number of inseminated oocytes.
[b] Blastocyst obtained per total number of inseminated oocytes.
[c] Mean number of cells per blastocyst.

Table 2
Effect of number of pulses of CRISPR-Cas9 electroporation and microinjection against *CAPN3* on embryo development. Data expressed as mean ± SEM. *In vitro* matured oocytes were electroporated or microinjected at 100 ng/μL Cas9 and 12.5 ng/μL each guide.

	Control No microinjected/electroporated	Microinjection Control	Microinjection	4 pulses electroporation	6 pulses electroporation	p value
n	210	213	209	207	198	
Cleavage rate[a] (%)	69.5 ± 3.2[ab]	63.4 ± 3.3[a]	63.2 ± 3.3[a]	72.9 ± 3.1[b]	42.9 ± 3.5[c]	<0.01
Blastocyst rate[b] (%)	32.9 ± 3.2[a]	21.1 ± 2.8[b]	22.5 ± 2.9[b]	21.7 ± 2.9[b]	11.1 ± 2.2[c]	<0.01
Deletion rate[c] (%)	-	-	40.0[a] (18/45)	19.1[b] (9/47)	19.4[b] (6/31)	0.04
Biallelic deletion[d] (%)	-	-	15.6 (7/45)	10.6 (5/47)	6.5 (2/31)	0.46

[a-c] Values in the same column with different superscripts are significantly different ($p < 0.05$). Microinjection control group: oocytes microinjected without RNP; n, number of inseminated oocytes.
[a] Two cell embryos per total number of inseminated oocytes.
[b] Blastocyst obtained per total number of inseminated oocytes.
[c] Percentage of embryo with one allele with desired deletion relative to total embryos.
[d] Percentage of mutant embryos with both alleles with desired deletion with respect to total embryos.

embryos with at least one type of mutational candidate to generate a pig with mutations in *CAPN3* protein. These results demonstrated a range between 77 and 87.3% of embryos with mutations.

4. Discussion

The use of electroporation techniques to induce reversible membrane breaks to allow the passage of biomacromolecules like CRISPR-Cas9 RNPs had been described in different types of cells [41]. However, the delivery of macromolecules into oocytes and zygotes is more difficult than for other cells due to the presence of the ZP, which blocks molecules larger than 170 kDa in matured oocytes and 110 kDa in zygotes [42].

Differences in porcine ZP permeability have also been demonstrated by Hirata et al., 2019 [32] where the mutation rates of *in vitro* matured oocytes electroporated with RNPs was higher than in zygotes with low numbers of electroporation pulses. Since the size of Cas9 protein is nearly 160 kDa, inaccessibility of zygotes to this protein is an important limiting factor, which is not a concern for oocytes. Despite this issue, previous studies produced mutant embryos and piglets using zygote electroporation with mRNACas9 [23] and RNP [16,23,30,32,43], obtaining a higher mutation rate when Cas9 protein was used because Cas9 protein is smaller than Cas9 mRNA.

Optimization of electroporation conditions is important to maximize rates of mutation with low embryo damage. In previous studies, adjustment of electroporation parameters like pulse duration [23], number of pulses [23,32], voltage strength [43], and use of unipolar or bipolar currents [43], were evaluated. Our use of fluorescent macromolecules [22] as a penetrance reporter helped us to optimize these in a faster and cheaper way than performing CRISPR-Cas9 gene editing because it was not necessary to carry out embryo culture and genetic assays.

Our results confirmed that the degree of TMRD uptake through electroporation-initiated membrane pores was entirely due to the pulse number - the greater the number of pulses, the greater the uptake. Indeed, we did not identify a maximum number of pulses required to saturate TMRD, so it is possible that more pulses with the same conditions could increase the TMRD concentration into the cytoplasm. However, since 6 electroporation pulses with CRISPR-Cas9 ribonucleoprotein reduced the percentage of blastocysts formed, exceeding this pulse number is unlikely to be fruitful. These data agree with reduced blastocyst rates shown in previous experiments in porcine zygotes and matured oocytes when the number of pulses was higher than five [23,32].

In the electroporated groups the mean number of cells per blastocyst were significantly lower than control. One possible cause of this fact could be that electroporation led to fusion of oocyte with polar body, which could lead to smaller cell numbers. However, this is an unlikely scenario because the electric potential difference (voltage) that we used in this study was low (30V). Previous studies used at least 150V to induce parthenogenetic activation of oocytes [44]or approximately 200V to produce cell fusion between somatic cells and enucleated oocytes for SCNT [44,45].

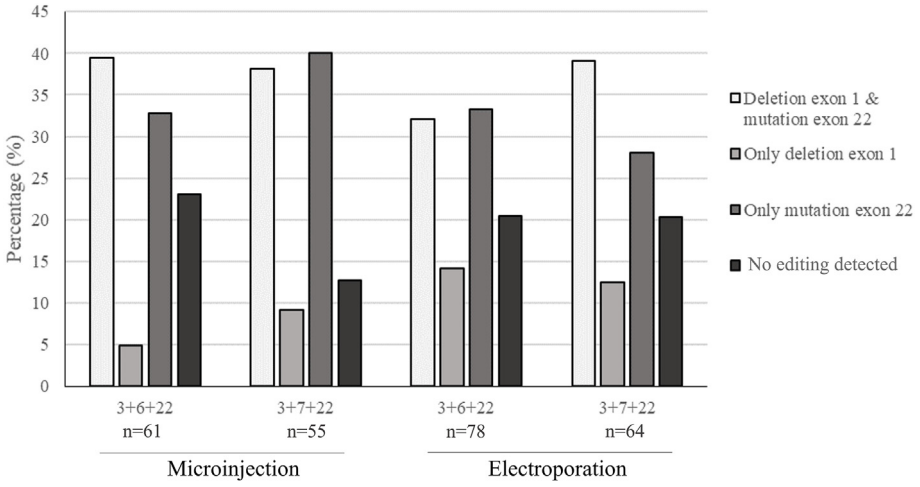

Fig. 5. Distribution of embryos produced with different strategies to generate LGMDR1 models. Guides 3, 6 and 7 were designed against exon 1 and guide 22 against exon 22. *In vitro* matured oocytes were electroporated with 150 ng/μL Cas9 and 25 ng/μL each guide and microinjected with 150 ng/μL Cas9 and 12.5 ng/μL each guide. Resulting embryos were classified into groups based on presence of deletion in exon 1 and mutation in exon 22, only deletion in exon 1, only mutation in exon 22 and without any mutation detected.

Table 3
Efficiency of different strategies to generate LGMDR1 models. Guides 3, 6 and 7 were designed against exon 1 and guide 22 against exon 22. *In vitro* matured oocytes were electroporated at 150 ng/μL of Cas9 and 25 ng/μL of each guide and microinjected at 150 ng/μL of Cas9 and 12.5 ng/μL of each guide. Data of embryo development were expressed as mean ± SEM and gene edition parameters were expressed as percentage.

Procedure		Microinjection		Electroporation		p value
sgRNA		3 + 6+22	3 + 7+22	3 + 6+22	3 + 7+22	
n		247	256	452	461	
Cleavage rate[a] (%)		46.2 ± 3.2[a]	41.4 ± 3.1[a]	61.1 ± 2.3[b]	62.5 ± 2.3[b]	<0.01
Blastocyst rate[b] (%)		21.5 ± 2.6	17.2 ± 2.4	19.9 ± 1.9	18.9 ± 1.8	0.65
Exon 1	Deletion rate[c] (%)	47.4 (37/78)	50.6 (40/79)	46.2 (36/78)	51.6 (33/64)	0.90
	Biallelic deletion[d] (%)	20.5 (16/78)	20.3 (16/79)	16.7 (13/78)	17.2 (11/64)	0.90
Exon 22	Mutation rate[e] (%)	73.0 (46/63)	78.6 (44/56)	66.2 (53/80)	68.8 (55/80)	0.43
	Biallelic mutation[f] (%)	3.2 (2/63)	1.8 (1/56)	1.3 (1/80)	0 (0/80)	0.46

[a-b] Values in the same column with different superscripts are significantly different ($p < 0.05$). n, number of inseminated oocytes.
[7]Percentage of mutant embryos in exon 22 without mutations in exon 1.
[a] Two cell embryos per total number of inseminated oocytes.
[b] Blastocyst obtained per total number of inseminated oocytes.
[c] Percentage of embryos with one allele with desired deletion in exon 1 with respect to total embryos.
[d] Percentage of mutant embryos with both alleles with desired deletion in exon 1 with respect to total embryos.
[e] Percentage of embryos with at least one mutated allele.
[f] Percentage of mutant embryos with both mutant alleles with respect to total embryos.

In our study, embryos produced by electroporation had similar mutation rates with 4 or 6 electroporation pulses, but the blastocyst rate was worse in 6 pulse embryos than in 4 pulse embryos. A lower mutation rate was found with electroporation compared with microinjection at the same conditions of RNP delivery with a 1:1 equimolar RNP proportion. These results agree with previous studies where CRISPR-Cas9 microinjection and electroporation were compared in porcine 1 and 2 cell embryos [46].

In previous studies, porcine zygotes were electroporated with a concentration of 50 ng/μL Cas9 and 200 ng/μL sgRNA [16,23,30,32,43] (1:16 Cas9:sgRNA molar ratio), or 100 ng/μL Cas9 and sgRNA [46] (1:4 Cas9:sgRNA molar ratio). In our first experiment electroporating RNP, we used an equimolar proportion of Cas9 and sgRNA, with a concentration of 100 ng/μL Cas9 and 12.5 ng/μL each guide. The lower concentration of sgRNA we used compared with previous experiments could explain the relatively lower mutation efficiency that we observed. This hypothesis was confirmed in our following experiment in which mutation rates were higher with double gRNAs concentrations. On the other hand, we also found that the target deletion in exon 1 of *CAPN3* plateaued at roughly 50% of embryos containing at least one deleted allele. This limitation may be due to requirement for both sgRNAs effectively generate the desired mutation and because our strategy detected only the target deletion, and not the specific INDELs produced by each sgRNA.

Between the two different strategies we used to generate models for LGMDR1 we found no differences in embryo development or mutation rates. We designed this strategy to use 3 sgRNAs that simultaneously remove the start codon and generate INDELs in exon 22, facilitating higher rates of mutations. We found that nearly 80% of embryos having at least one type of mutation and three types of possible embryos: KO of *CAPN3* in which the start codon was deleted, embryos with the *CAPN3* protein having an early stop codon in exon 22 and without a mutation in exon 1, and few wild-type embryos. It should be noted that the efficiency of the system may be higher because mutations caused by sgRNA 6 or 7 without deletion of start codon were not evaluated and these could produce a mutation via the generation of INDELs with a frameshift in part of the 20% of embryos in which no mutation was detected.

5. Conclusion

Production of porcine KO embryos by oocyte electroporation is easier and faster than by microinjection and it does not require highly qualified personnel and expensive equipment. The optimization of this process in term of maintaining embryo quality and maximizing mutation efficiency could allow us to produce more embryos at any given time, which will increase KO throughput at the farm. We defined the optimal electroporation conditions to reach similar rates of KO embryos compared with the tedious microinjection approach, which led to generation of the best strategy for producing porcine LGMDR1 models.

CRediT authorship contribution statement

Sergio Navarro-Serna: Experimental design, Embryo production and collection, Embryo genotyping, Formal analysis, and, document writing. **Martxel Dehesa-Etxebeste:** Experimental design, Investigation, sgRNA design. **Celia Piñeiro-Silva:** Embryo production and collection, Embryo genotyping. **Raquel Romar:** Experimental design, Formal analysis, and document writing. **Jordana S. Lopes:** Embryo production and collection. **Adolfo López de Munaín:** Experimental design, Funding acquisition. **Joaquín Gadea:** Experimental design, Formal analysis, and, document writing, Funding acquisition, Supervision, and, Project administration, All authors have approved the definitive version of this article.

Declaration of competing interest

None of the authors have any conflict of interest to declare.

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