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Assessment of Temporal Effects of Electroporation on CRISPR/Cas-Driven Gene Modifications in Buffalo Embryos

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Abstract: Gene editing in buffalo faces challenges from mosaicism, where cells contain both wild-type and mutant alleles, complicating the creation of genetically modified animals in a single step. Traditionally, electroporation is done post *in vitro* fertilization (IVF) on zygotes, but the higher permeability of mature oocytes suggests earlier intervention could reduce mosaicism and improve editing efficiency. This study aimed to determine if electroporating buffalo oocytes at different stages during *in vitro* maturation (IVM) could improve biallelic mutation rates for three genes: KDR, GDF9, and POU5F1. Oocytes were electroporated at 44, 46, and 48 hours of IVM, and a control group of zygotes was electroporated 12 hours post-IVF. Each group received three gRNAs targeting the three genes. After electroporation, oocytes were fertilized and cultured to the blastocyst stage. The study assessed and compared blastocyst formation rates, mutation rates, and biallelic mutation occurrences across groups. Electroporation at 44 and 46 hours of IVM reduced blastocyst formation rates but did not significantly impact mutation or biallelic mutation rates compared to the control. Oocytes that were electroporated at 48 hours of IVM showed similar results to those that were electroporated post-IVF. This suggests that the timing of electroporation during IVM affects blastocyst formation but does not influence overall gene editing efficiency, offering a potential method for gene editing in buffalo without the need for IVF.
Keywords: Mosaicism; Biallelic mutation; Gene editing; Electroporation; Buffalo.

1. Introduction

CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated) is a revolutionary gene-editing technology that allows us to make precise changes to an organism's DNA (Xu & Li, 2020). It originated from a natural defense mechanism found in bacteria, which use CRISPR/Cas systems to defend against viral infections by cutting and destroying the DNA of invading viruses, and now has been successfully used for gene editing in several mammalian species (Liu, 2020). Water buffaloes are one of the domesticated animals in Bangladesh that have been widely used in biomedical research, including gene therapy, because of their large size, reproductive physiology, and immune system characteristics are especially useful for research purposes (Singh et al., 2020). Their large size, reproductive physiology, and immune system characteristics are especially useful for research purposes. Additionally, their genetic makeup and susceptibility to certain diseases make them relevant models for understanding human health and developing treatments (Liu et al., 2015). Research focusing on one-step multiple gene targeting in animal models has garnered significant interest in recent years due to its potential implications for understanding and treating complex human diseases. Conditions like type I diabetes, thrombosis, and liver cirrhosis often result from mutations in multiple genes, making them challenge to study and treat effectively (Singh et al., 2009). By simultaneously targeting multiple genes involved in these diseases, researchers can better mimic the genetic complexity seen in humans which would allow for a more comprehensive understanding of disease mechanisms, including interactions between genes and pathways.

By targeting multiple genes that might interact or have complementary roles, we can enhance or combine traits in ways that are more effective than single-gene modifications (Civelek & Lusis, 2014). We considered 3 genes in our study which have nearly similar functions. The genes KDR (Kinase Insert Domain Receptor), GDF9 (Growth Differentiation Factor 9), and POU5F1 (POU Class 5 Homeobox 1) play crucial roles in various biological processes in buffalo, particularly in development, reproduction, and cellular function (Michelizzi, 2010). For genome editing purposes, reducing the number of interventions means less stress and handling of the animals. This is important for maintaining animal welfare and ensuring ethical standards in livestock management (Prescott, 2020). However, one-step multiple gene targeting systems have encountered significant obstacles, with mosaic mutations being a prominent challenge (Im et al., 2016). Mosaic mutations occur when only a subset of cells within an organism undergoes successful editing, resulting in a mixture of edited and unedited cells, known as mosaicism. This phenomenon can complicate the interpretation of experimental results and hinder the generation of genetically uniform organisms (Foulkes & Real, 2013). Electroporation of Cas9 protein and guide RNA (sgRNA) into zygotes during the brief period between fertilization and the first DNA replication has been proposed as a strategy to generate non-mosaic or biallelic mutants. This approach takes advantage of the fact that editing the zygote before DNA replication ensures that any genetic modifications introduced will be present in all subsequent cells as the embryo develops (Hashimoto et al., 2016). Despite efforts to generate biallelic mutations using one-step multiple-gene targeting, challenges persist. Researchers still could not produce the desired biallelic mutation by one-step multiple gene targeting, in which the CRISPR/Cas9 system was electroporated into the putative zygotes during the gap time between the end of fertilization and onset of genome replication (Hirata et al., 2019). The mechanism behind the limitation of achieving biallelic

mutations in triple gene knockout experiments despite early CRISPR/Cas9 introduction remains unclear. Consequently, we decided to initiate a study to determine whether altering the timing of CRISPR/Cas9 introduction, either at the oocyte stage or the zygote stage, could enhance the rates of biallelic mutation. In contrast to the control group, which had the putative zygotes electroporated with pooled gRNAs targeting all three genes at 12 h after the initiation of fertilization, three gRNAs targeting the KDR, GDF9, and POU5F1 genes were simultaneously introduced into buffalo oocytes that had been incubated for 44, 46, and 48 h.

2. Materials and Methods

2.1. Ethical statement

This study protocol was reviewed, and animal experiments were permitted by the Khulna Agricultural University Animal Experimentation Ethics Committee (Approval No: AEEC/KAU/2024-01), Bangladesh.

2.2. Oocyte collection, *in vitro* maturation (IVM), and fertilization

After being slaughtered at a nearby abattoir, large black Murrah breed buffalo ovaries were taken, and they were brought to the lab at 30°C in less than an hour in phosphate-buffered saline supplemented with 50pg tetracycline/ml. Precautions were taken to minimize microbial contamination by conducting procedures in highly sterile conditions. The ovaries were treated for cumulus-oocyte complexes (COCs) recovery using 100 IU/ml penicillin G potassium and 0.1 mg/ml streptomycin sulfate after being cleaned twice in PBS and then moved to new PBS at 39 °C in a water bath. Under a stereomicroscope, follicles measuring 3-6 mm in diameter on the ovarian surface were cut with a surgical blade on a sterile dish, allowing the COCs to be seen and collected. For 24 hours, roughly 50 COCs were grown in 500 microliters of maturation medium that included tissue culture medium 199 with Earle's salts supplemented with 10% (v/v) bovine follicular fluid, 0.6 mM cysteine, 50 µM β-mercaptoethanol, 50 µM sodium pyruvate, 2 mg/ml D-sorbitol, 10 IU/ml human chorionic gonadotropin, and 50 µg/ml gentamicin. After that, the COCs were moved to a hormone-free maturation medium and cultivated for a further twenty-two hours. COCs were kept in a humidified incubator with 5% CO2 at 39°C.

The matured oocytes were subjected to *in vitro* fertilization (IVF) as described previously (Marin et al., 2019). In short, spermatozoa that had been frozen and thawed were placed into 5 millilitres of fertilization media and centrifuged at 500 × g for five minutes to remove any remaining fluid. Once again suspended in fertilization medium, the pelleted spermatozoa were adjusted to 2 × 10^6 cells/ml. Then, about 50 oocytes were placed in 500 µl of fertilization media containing10 pmol penicillamine/L plus 2 pmol sodium metabisulfite/L, 1 pmol hypotaurine/L and 20 pmol adrenaline/L, sealed with mineral oil in 4-well plates, and co-incubated for five hours at 39°C in a humidified incubator with 90% N2, 5% O2, and 5% CO2. Following coincubation, the embryos were incubated for six days at 38.5°C, 5% CO2, 5% O2, and 90% N2 in SOF media supplemented with 5% FBS. No culture medium replacements were performed during this time.

2.3. Formation of RNP complexes and electroporation of presumptive zygotes

The gRNAs were designed using the CRISPR direct web tool (https://crispr.dbcls.jp/). To minimize off-target effects, 12 nucleotides at the 3′-end of the designed gRNAs had no sequence matches in the buffalo genome other than the target regions of KDR, GDF9, and POU5F1 (Table 1), as determined using the COSMID webtool (https://crispr.bme.gatech.edu/).

Target*	gRNA target sequence	PAM	Target	Strand	Forward primer	Reverse primer
KDR-Ch7, NC_059163.1	GAGGCATCACTTGCGCG CCA	CGG	Ex. 2	Sense	GGCACAAAGTCACCCA ACTT	CAGCTTGGAGGAGTGG AAAG
GDF9-Ch9, NC_059165.1	TTGCTAATTCTTCCAAG CCA	TGG	Ex. 1	Antisen	CTCCTCTTGAGCCTCTG GTG	ACAGCCCTCTCTTCTG GTCA
POU5F1-Ch2 NC_059158.1	CCCTCAGCCCGAGGGC GAGG	CGG	Ex. 2	Sense	ACAGCATGTTTTGACCC ACA	CAAGACCCCACACCAG CTAT

Table 1 – gRNA and primer sequences used for sequencing analysis.
*Based on NCBI: *Bubalus bubalis* isolate 160015118507 breed Murrah whole genome shotgun sequence NDDB_SH_1 (GCF_019923935).

2.4. Electroporation

After removing COC and sperm 8–10 hours after insemination, the likely buffalo zygotes were twice washed with wash media and zone-thinned for 10 seconds using acid Tyrode's solution. Electroporation was performed as described previously (Punetha et al., 2024). In short, a Gene Pulser Xcell was attached to an electrode, which was placed beneath a stereoscopic microscope. After being incubated for the specified durations from the beginning of the maturation culture, the cumulus-free oocytes were cleaned with Opti-MEM I solution and positioned in a line between the electrode gap in a chamber slide that contained 10 µl of nuclease-free duplex buffer that contained 100 ng/µl of three gRNAs (IDT, Coralville, USA) and 100 ng/µl of Cas9 protein (Takara Bio, Shiga, Japan). The same maturation media was used to incubate the oocytes after electroporation for the final 48 hours of the culture period. As previously mentioned, *in vitro* fertilization was performed on the matured oocytes. The embryos were then cultivated for seven days to assess both the genotypes of the developing blastocysts and their capacity to develop to the blastocyst stage.

2.5. Analysis of the targeted genes after electroporation

To assess the effectiveness of introducing target mutations in the embryos, we examined the frequencies of base insertions or deletions (indels) in the target regions of individual blastocysts. Embryos were heated in 50 mM NaOH to extract their genomic DNA. Following neutralization, gb Ideal PCR Master Mix (Generi Biotech, Czechia) was used to perform a polymerase chain reaction (PCR) on the DNA samples following the manufacturer's instructions. The primers employed for amplification are detailed in Table 1. We used Sanger sequencing to analyze the target region sequences after purifying the PCR products using the Fast Gene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). We used an ABI 3500 genetic analyzer (Applied Biosystems, Foster City, CA, USA) and a BigDye Terminator Cycle Sequencing Kit version 3.1 (Thermo Fisher Scientific K.K., Tokyo, Japan). The TIDE (Tracking of Indels by Decomposition) bioinformatics tool (https://tide.deskgen.com/)was utilized to measure the frequency of indel mutations in blastocysts produced from oocytes that were electroporated with three different gRNAs. Blastocysts were categorized as having biallelic mutations (carrying no wild-type sequences), mosaics (containing more than one type of mutation plus wild-type sequence), or WT (carrying just the wild-type sequence) based on the target region sequences.

2.6. Experimental design

Oocytes that had been incubated for 44, 46, and 48 hours following the start of IVM culture were treated with three gRNAs targeting KDR, GDF9, and POU5F1 concurrently to test if the timing of electroporation before IVF promotes triple gene editing in the resultant blastocysts. Dulbecco's PBS mixed with 1 mg/ml hyaluronidase was used to mechanically release the oocytes that were incubated for each time point from cumulus cells. The three different gRNAs that targeted each gene were combined and added to the cumulus-free oocytes at the same time using 100 ng/µl of Cas9 protein electroporation. Following electroporation, the oocytes underwent *in vitro* fertilization (IVF) after being cultured in the same maturation media for 48 hours out of the entire culture time. Twelve hours after IVF began, zygotes were electroporated with three gRNAs simultaneously as a control. After introducing three gRNAs into oocytes, the rate at which blastocysts formed was assessed, as were the target mutations present in the subsequent blastocysts.

2.7. Statistical analysis

Arcsine transformation was applied to the percentages of embryos that reached the blastocyst stage. Analysis of variance was used to assess the converted data, and then protected Fisher's least significant difference tests were performed. The program used for the analysis was GraphPad Prism (California, USA). Utilizing Yates' correction in conjunction with chi-squared tests, the percentages of mutant blastocysts were examined. Differences with probability values, $P < 0.05$ were considered statistically significant.

3. Results

We looked at how the timing of electroporation with three different kinds of guide RNA (gRNAs) before *in vitro* fertilization (IVF) affected the development of the embryo (Table 2). When oocytes were electroporated before the conclusion of the *in vitro* maturation (IVM) culture period, their blastocyst formation rates were considerably ($P < 0.05$) lower than those of oocytes cultured for the entire duration. Nonetheless, after the entire culture period, the blastocyst formation rate of the electroporated oocytes was comparable to that of the control zygotes that were electroporated 12 hours after the IVF process began.

Electroporation time during IVM	No. of embryos examined	No. (%) of embryos*	
		Cleaved**	Developed to blastocysts
Control	290	254 (87.58±0.9)[a]	31 (10.69±3.1)[a]
44 h	236	135 (57.20±2.7)[b]	22 (16.29±1.8)[ab]
46 h	289	166 (57.43±3.9)[b]	17 (10.24±1.2)[b]
48 h	270	237 (87.78±2.9)[a]	31 (13.08±1.5)[c]

Table 2 – Effects of the timing of electroporation during *in vitro* maturation on the blastocyst formation of oocytes after *in vitro* fertilization.

*All experiments were replicated three times. Data are expressed as the mean ± SEM.

**At the appropriate intervals from the beginning of the maturation culture, three gRNAs targeting KDR, GDF9, and POU5F1 were electroporated, and the oocytes were subsequently cultured in the same maturation media for the final 48 hours of the culture period. Putative zygotes that were harvested 12 hours after IVF began were electroporated with three gRNAs as a control.

[abc]Values with different superscripts in the same column are significantly different ($P< 0.05$).

When evaluating the mutation rates by sequencing each target site in the resulting blastocysts (Figure 1), the timing of electroporation before IVF did not lead to an increase in either the overall mutation rates or the total biallelic mutation rates. Furthermore, the total mutation rates of the three types of gRNAs that were electroporated simultaneously in control zygotes and oocytes did not differ significantly. The rate of triple mutations in the resulting blastocysts from oocytes electroporated at 48 h was significantly higher ($P < 0.05$) than that of oocytes electroporated at 44 h (Figure 1A). The timing of electroporation did not influence the number of target sites with biallelic mutations in the resulting blastocysts (Figure 1B).

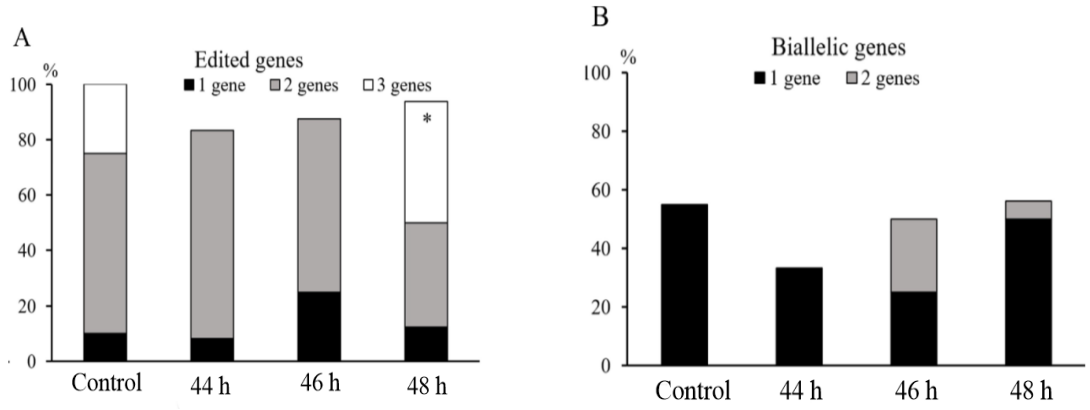

Figure 1 – The resultant blastocysts after electroporation therapy with gRNAs targeting three genes during *in vitro* maturation (IVM) had the mutation (A) and the biallelic mutation (B). At the appropriate intervals from the beginning of the IVM culture, three gRNAs targeting KDR, GDF9, and POU5F1 were electroporated, and the oocytes were subsequently cultured in the same maturation media until 48 hours into the IVM culture period. Three gRNAs targeting KDR, GDF9, and POU5F1 were electroporated into putative zygotes that were harvested 12 hours after the *in vitro* fertilization process began. This was done as a control. Using TIDE, the blastocysts' genotypes were identified. The total number of blastocysts analyzed is indicated by the numbers in parentheses. *P<0.05 compared with the rate of triple mutations in the resulting blastocysts from oocytes electroporated at 48 h.

Only the resultant blastocysts from oocytes electroporated at 48 hours and from control zygotes electroporated at 12 hours after the start of IVF showed the simultaneous insertion of triple mutations. We then contrasted the sorts of mutations made to each targeted gene in the two groups (Figure 2). The overall mutation and total biallelic mutation rates of each targeting gene in the resultant blastocysts did not differ significantly between the two groups.

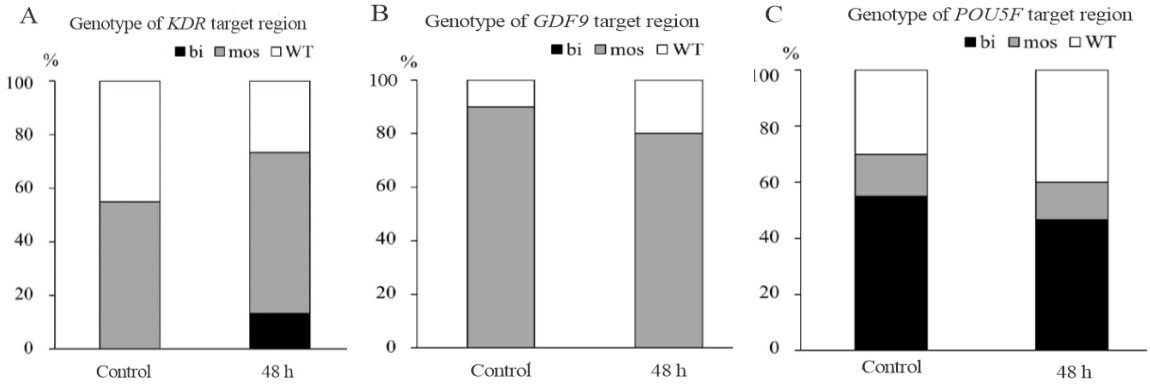

Figure 2 – Genotypes of blastocysts after individual sequencing the target sites of (A) KDR, (B) GDF9, and (C) POU5F genes. Blastocysts derived from oocytes electroporated at 48 h after the initiation of IVM culture (48 h), and from putative zygotes electroporated at 12 h after the initiation of *in vitro* fertilization (Control) were analyzed. Genotypes of blastocysts were determined using TIDE. Numbers within parentheses indicate the total number of examined blastocysts. bi: blastocysts having biallelic mutations; mos: blastocysts having mosaic mutation; WT: wild type.

4. Discussion

The timing of CRISPR/Cas9 delivery into oocytes is crucial for successful genetic modification, with mature oocytes arrested at metaphase II minimizing mosaicism and targeting specific developmental stages, while posing efficiency challenges (Hirata et al., 2019). This study explores whether electroporating buffalo oocytes during the IVM process can enhance biallelic and total mutation frequencies for triple gene knockouts. Findings suggest that oocytes at the end of maturation may be more effective for gene editing than those at intermediate stages. During IVM, oocytes undergo chromatin remodeling, transitioning through meiotic phases to reach metaphase II, driven by various molecular factors like histone modifiers, chromatin remodelers, and transcription factors (Belli et al., 2014). The evidence suggests that decondensed chromatin is a superior substrate for gene editing than condensed chromatin because decondensed chromatin is more likely to target editing machinery due to protein depletion during decondensation (Suzuki et al., 2014; Yoshida et al., 2007). This work could contribute to the understanding of why a more effective CRISPR/Cas9 system is supported by mature oocytes that make it through the maturation stage and on to the fertilization stage.

Gene editing activities can continue after genome replication in multi-cell stage embryos when CRISPR/Cas9 is introduced into fertilized oocytes, leading to mosaicism (Chang et al., 2013). The CRISPR/Cas9 system can edit the paternal genome continuously during fertilization, potentially enhancing control over gene mutations during oocyte maturation and resulting in multiple mutated alleles in both paternal and maternal genomes (Wu et al., 2018). However, electroporating before fertilization did not increase biallelic mutation rates in blastocysts. Suzuki et al. (2014), suggested that the brief tolerance window for CRISPR/Cas9 is due to oocyte chromatin organization during meiotic departure, which may resist the editing apparatus. Mixed-allele cases likely arise when only one allele is available during editing. This implies that, although CRISPR/Cas9 was introduced before fertilization, maternal gene editing might have occurred after the initial DNA replication. Additionally, the Cas9 protein may have a short half-life due to proteolysis (Yang et al., 2018), possibly limiting its activity during gene editing. Additional research is required to clarify these mechanisms.

This study found that oocytes electroporated at 48 hours of IVM culture and control zygotes had higher developmental competency compared to oocytes electroporated at 44 and 46 hours. Earlier research indicates that oocytes are more sensitive to electrical pulses than zygotes (Hirata et al., 2019). During electroporation, the creation of membrane pores allows reactive oxygen species (ROS) to enter cells, impacting membrane structure and inducing oxidative stress, which can alter cellular behavior and facilitate the entry of exogenous materials (Yadav et al., 2021; Yang & Liu, 2021). Another important factor to take into account is the parthenogenesis of developed oocytes via electroporation simulation, which will raise each experimental group's blastocyst formation rate (Singh et al., 2009). In contrast to control zygotes electroporated after *in vitro* fertilization, the blastocyst formation rate from oocytes driven by electroporation during maturation culture was lower in this investigation. The detrimental impact of electroporation on an oocyte's developmental capacity throughout maturation seems to outweigh the parthenogenesis-induced increase in the rate of blastocyst formation. However, the experimental design should take electroporation stimulation-induced parthenogenesis into account. Additionally, removing cumulus cells during IVM culture hinders the nuclear maturation, fertilization, and subsequent development of the embryo in oocytes (Wongsrikeao et al., 2005). Our findings therefore suggest that, in addition to causing a stress response due to membrane damage, electroporation of cumulus-free oocytes during IVM may also result in future embryonic developmental arrest, presumably as a result of ROS formation. Buffalo IVF methods continue to face a continuous challenge from polyspermic penetration (Funahashi, 2003). Using our IVF system, the normal and polyspermic fertilization rates were approximately 40 to 50%, and < 15%, respectively (Kadoom et al., 2014). Removal of cumulus cells before IVF reduces sperm penetration (Kikuchi et al., 1993). Consequently, there is probably not a great chance of polyspermic penetration. It does, however, make it more difficult to evaluate the findings. One option to think about is intracytoplasmic sperm injection.

5. Conclusion

This study demonstrates that using fully matured buffalo oocytes for electroporation, whether before or after fertilization, does not significantly alter mutation rates or biallelic mutations in resultant blastocysts. This expands the timeframe for gene editing without waiting for IVF completion, offering greater flexibility in research design. While promising, further investigation is needed to understand the impact of pre-IVF electroporation on implantation success and to delve into the underlying mechanisms of gene editing efficiency in matured oocytes. Future research should aim to resolve these issues, paving the way for effective applications in buffalo breeding and biotechnology.

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