Veterinary Integrative Sciences 2025; 23(3): e2025069-1-12. DOI: 10.12982/VIS.2025.069

Veterinary Integrative Sciences
ISSN: 2629-9968 (online)

Research article

# Influence of electroporation timing on CRISPR/Cas-mediated multiple gene editing in buffalo embryos

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## Abstract

Gene editing in large animals like buffalo is challenged by mosaicism, where cells contain both wild-type and mutant alleles, complicating the creation of genetically modified F0 animals in a single step. Traditionally, electroporation is performed on zygotes post *in vitro* fertilization (IVF), but mature oocytes' higher permeability suggests earlier intervention might reduce mosaicism and enhance editing efficiency. We hypothesized that the timing of electroporation before *in vitro* fertilization (IVF) can increase the rates of biallelic mutation for multiple gene knockout as the permeability of mature oocytes is greater than that of zygotes. Hence, we determined whether the timing of electroporation during *in vitro* maturation (IVM) culture enhances triple gene editing in the resulting blastocysts. Three gRNAs targeting *KDR*, *GDF9*, and *POU5F1* were simultaneously introduced into the oocytes that had been incubated for 44, 46, and 48 h from the start of the IVM culture. Electroporation with three gRNAs at 44 h and 46 h during IVM culture decreased the blastocyst formation rates and did not improve the mutation rates and target number of biallelic mutations in the resulting blastocysts. The blastocyst formation rate, mutation rates, and target numbers in the resulting blastocysts from oocytes treated by electroporation at 48 h of IVM culture were similar to those of control zygotes electroporated at 12 h after the initiation of IVF. In conclusion, multiple gene editing efficiency in the resulting blastocysts was comparable between oocytes electroporated before and after fertilization, indicating that oocytes with completed maturation time may allow better functioning of materials accepting gene editing application.

**Keywords**: Buffalo, CRISPR/Cas9, Electroporation, Gene editing, *In vitro* fertilization.

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***Article history;*** *received manuscript: 12 September 2024,*
*revised manuscript: 24 October 2024,*
*accepted manuscript: 20 November 2024,*
*published online: 25 November 2024,*
***Academic editor;*** *Kittisak Buddhachat*

# 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 and 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 challenging 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 and Lusis, 2014). We considered 3 genes in our study with nearly similar functions: *KDR* (Kinase Insert Domain Receptor), *GDF9* (Growth Differentiation Factor 9), and *POU5F1* (POU Class 5 Homeobox 1). Altering these genes can significantly enhance various traits in buffalo. KDR is crucial for angiogenesis, potentially improving growth rates, recovery, and heat stress resilience by optimizing blood flow and nutrient delivery (Napoleone et al., 2001). GDF9 plays a vital role in ovarian function, where modifications could increase fertility, enhance reproductive efficiency, and improve hormonal balance, leading to better outcomes in breeding programs (Castro et al., 2016). Meanwhile, POU5F1 is essential for embryonic development, and its alteration could result in higher embryo viability and quality, increased stem cell activity, and greater genetic diversity (Michelizzi, 2010). Another reason behind targeting these genes is that it can enhance buffalo breeding strategies while enriching our understanding of developmental biology in buffalo. This research can uncover how these genes interact during key growth and reproductive phases. Additionally, insights into genetic variations may shed light on traits associated with stress resistance and adaptability, benefiting not only buffalo breeding but also comparative genomics in other livestock species. However, 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 and 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, 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 exact mechanism behind the limitation of achieving biallelic mutations in triple gene knockout experiments despite early CRISPR/Cas9 introduction remains unclear. Inefficient delivery of CRISPR components can hinder editing outcomes, while precise timing during the gap between fertilization and genome replication is critical and difficult to achieve. The complexity of targeting multiple genes simultaneously may lead to competition for CRISPR machinery, resulting in inconsistent edits (Wang et al., 2016). Additionally, the reliance on the non-homologous end-joining repair pathway often produces indels instead of the desired mutations. Off-target effects and variations in the zygotic cellular environment further complicate the editing process (Zhang et al., 2014). These challenges highlight the need for continued refinement of approaches to enhance biallelic mutation success in gene knockout experiments. However, some researchers conducted related experiments on pigs and suggested that oocytes with completed maturation time may allow better functioning of gene-editing materials (Lin et al., 2019). 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.

# MATERIALS AND METHODS

## 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.

## 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 sulphate 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% $CO_2$ 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% $N_2$, 5% $O_2$, and 5% $CO_2$. Following coincubation, the embryos were incubated for six days at 38.5°C, 5% $CO_2$, 5% $O_2$, and 90% $N_2$ in SOF media supplemented with 5% FBS. No culture medium replacements were performed during this time.

## 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/).

**Table 1** gRNA and primer sequences used for sequencing analysis.

| Target gene (Chromosome localization*) | gRNA target sequence | PAM | Target | Strand | Forward primer | Reverse primer |
|---|---|---|---|---|---|---|
| *KDR* (Chromosome: 7, NC_059163.1) | GAGGCATCACTTG CGCGCCA | CGG | Exon 2 | Sense | GGCACAAAGT CACCCAACTT | CAGCTTGGAG GAGTGGAAAG |
| *GDF9* (Chromosome 9, NC_059165.1) | TTGCTAATTCTTCC AAGCCA | TGG | Exon 1 | Antisense | CTCCTCTTGAG CCTCTGGTG | ACAGCCCTCT CTTCTGGTCA |
| *POU5F1* (Chromosome 2, NC_059158.1) | CCCTCAGCCCGAG GGCGAGG | CGG | Exon 2 | Sense | ACAGCATGTTT TGACCCACA | CAAGACCCCA CACCAGCTAT |

*Based on NCBI: *Bubalus bubalis* isolate 160015118507 breed Murrah whole genome shotgun sequence NDDB_SH_1 (GCF_019923935).

## 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 in order to assess both the genotypes of the developing blastocysts and their capacity to develop to the blastocyst stage.

## 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 analyse 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.

## 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.

## Statistical analysis

The percentages of embryos that developed to the blastocyst stage were subjected to arcsine transformation using the formula, $Y(s)=\sin^{-1}(Z(s))$, where $(Z(s))$ is between 0 and 1. The transformed data were evaluated using analysis of variance, followed by protected Fisher's least significant difference tests. Using this combination—arcsine transformation, ANOVA, and Fisher's LSD—enhances the reliability of analyzing percentage data across multiple groups, particularly when identifying specific inter-group differences. The program used for the analysis was GraphPad Prism (California, USA). The percentages of mutated blastocysts were analyzed using chi-squared tests with Yates' correction. Differences with probability values, $P < 0.05$ were considered statistically significant.

# 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). The blastocyst formation rates of oocytes treated by electroporation before the end of the total IVM culture period were significantly lower ($P < 0.05$) than those of oocytes cultured for the total culture period. However, the blastocyst formation rate of oocytes treated by electroporation at the end of

the total culture period was similar to that of control zygotes electroporated at 12 h after the initiation of IVF.

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

| 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] |

*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 control (Figure 1A). The timing of electroporation did not influence the number of target sites with biallelic mutations in the resulting blastocysts (Figure 1B).

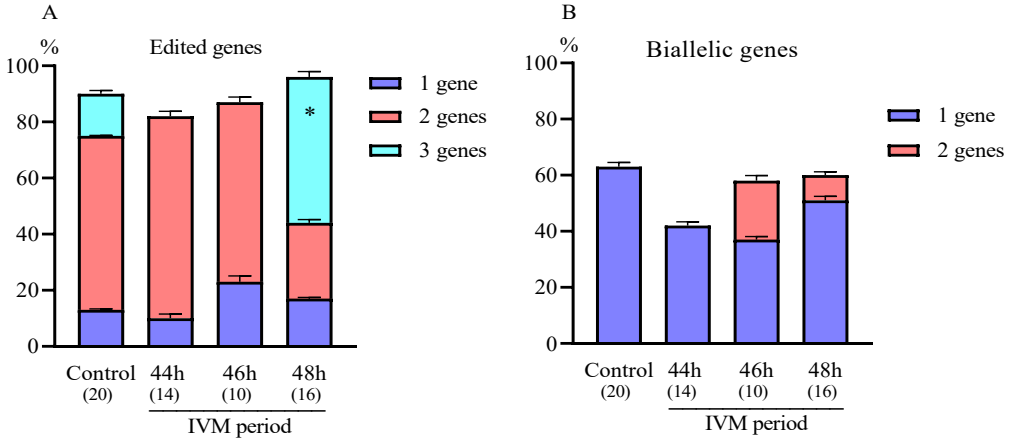


**Figure 1** Mutation (A) and biallelic mutation (B) derived from the resulting blastocysts following electroporation treatment with gRNAs targeting 3 genes during in vitro maturation (IVM). Electroporation with three gRNAs targeting KDR, GDF9, and POU5F1 was performed at the designated times from the start of the IVM culture, and then the oocytes were incubated in the same maturation medium until 48 h of the total IVM culture period. As a control, putative zygotes that were collected 12 h after the start of in vitro fertilization were electroporated with three gRNAs targeting KDR, GDF9, and POU5F1. Genotypes of blastocysts were determined using TIDE. Numbers within the parentheses indicate the total number of examined blastocysts. *P < 0.05 (chi-squared tests with Yates' correction) compared with the rate of triple mutations in the resulting blastocysts from oocytes electroporated at control.

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.

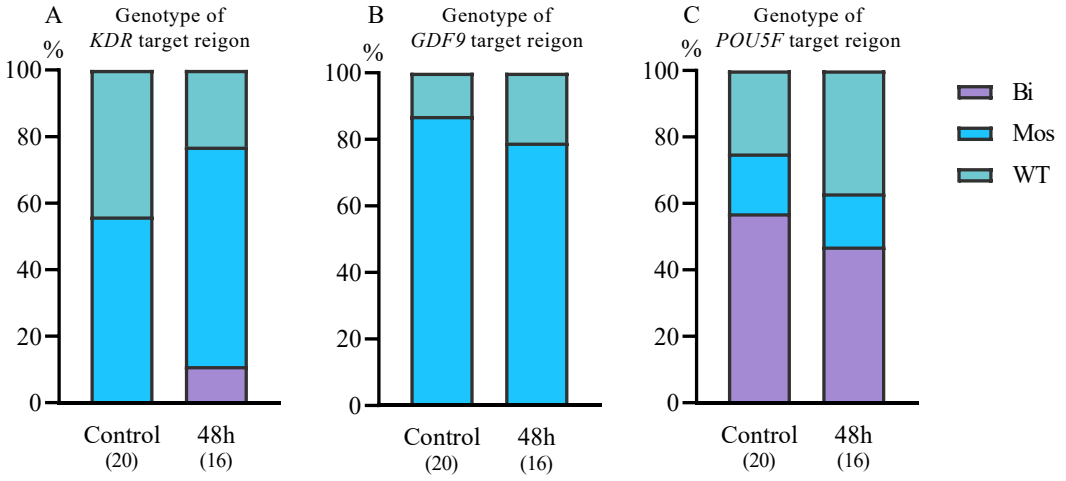


**Figure 2** Genotypes of blastocysts after individual sequencing the target sites of (A) KDR, (B) GDF9, and (C) POU5F genes. Blastocysts derived from oocytes electroporated at 48 h after the initiation of IVM culture (48 h), and that from putative zygotes electroporated at 12 h after the initiation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

# 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). Therefore, we conducted the present study to test whether the timing of electroporation during IVM culture can increase the rates of total mutations and biallelic mutations during triple gene knockout. Although no significant differences in the total mutation rates between oocytes and control zygotes electroporated simultaneously with three types of gRNAs were observed in the present study, the percentage of blastocysts carrying mutations in all three genes was higher in the group derived from oocytes that had been incubated for 48 h than those incubated for 44 h. These results indicate that oocytes that have completed their maturation time may function as gene editing material rather than oocytes in the middle of maturation culture. During IVM, immature oocytes normally undergo chromatin changes by increased condensation to prepare the genome to enter meiotic phases and accomplish the metaphase II stage (Belli et al., 2014). The evidence suggests that decondensed chromatin is a superior substrate for gene editing than condensed chromatin because its open structure makes DNA more accessible to editing tools like CRISPR/Cas9. This loose packaging is often associated with active gene regions and helpful histone modifications, allowing for

efficient and precise modifications. In contrast, condensed chromatin restricts access, making gene editing less efficient and accurate (Yoshida et al., 2007; Suzuki et al., 2014). 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). Utilizing the CRISPR/Cas9 system during oocyte maturation enables more controlled gene mutations, as editing can take place at the fertilization stage and can continue to target the paternal genome. This approach results in the creation of multiple mutated alleles in both maternal and paternal copies (Wu et al., 2018). However, contrary to our expectations, the timing of electroporation before fertilization did not improve the total biallelic mutation rates in the resulting blastocysts. Suzuki et al. (2014), suggested that the organization of oocyte chromatin during meiotic exit may resist editing machinery, resulting in only a brief window where the CRISPR/Cas9 system is effective. Mixed allele formation likely occurs when only one allele is accessible during editing, implying that maternal gene editing in this study may have happened after the initial round of DNA replication, even though the CRISPR/Cas9 system was introduced before fertilization. Additionally, the Cas9 protein may have a short half-life due to proteolysis (Yang et al., 2018), and the existing Cas9 protein activity may be insufficient to effectively initiate the gene-editing process at the time the editing machinery is active. According to Yang et al. (2014), the optimal time window for introducing CRISPR/Cas9 in oocytes is influenced by Cas9's half-life (approximately 10–24 hours in mammalian cells) and chromatin remodeling activity, which peaks during the transition from the germinal vesicle (GV) stage to metaphase II (MII). To maximize efficacy, CRISPR/Cas9 should ideally be introduced 6–24 hours before GVBD (germinal vesicle breakdown) and can remain effective for up to 12 hours post-GVBD. This timing aligns the presence of Cas9 with the period of heightened chromatin accessibility, enhancing the potential for successful genome editing (Hsu et al., 2014). However, further research is needed to clarify the mechanisms involved.

The developmental competence of oocytes treated with electroporation before the end of the IVM culture (44 h and 46 h) was lower than that of oocytes treated with electroporation at the end of the IVM culture (48 h) and control zygotes. Previous studies have shown that oocytes are more sensitive to electrical pulses than zygotes (Escriba et al., 2000). 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 and Liu, 2021). Adding antioxidants such as ascorbic acid and melatonin to culture media may be the solution to reduce oxidative stress. Parthenogenesis of matured oocytes by electroporation simulation, which will increase the blastocyst formation rate of each experimental group, is also a major concern to consider (Singh et al., 2009). Mitochondria provide the energy required for maturation and subsequent embryo development. High mitochondrial content, optimal distribution, and functional capacity are key to achieving developmental competency (Van Blerkom, 2011). Supplementation of IVM culture media with factors that support mitochondrial health like Coenzyme Q10 (CoQ10), L-carnitine, may promote better energy production for maturation and early embryo development. In this study, the blastocyst formation rate from oocytes stimulated by electroporation during maturation culture was decreased compared with the rate from control zygotes electroporated after IVF. During oocyte maturation, the negative effect of electroporation on the developmental competence of oocytes appears to be greater than the increase in blastocyst formation rate due to parthenogenesis. However, parthenogenesis caused by electroporation stimulation should be

considered in experimental design. Moreover, removing cumulus cells during IVM culture impairs oocyte nuclear maturation, fertilization, and subsequent embryo development (Wongsrikeao et al., 2005). Our findings 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 due to ROS formation. Limited exposure to light, heat, and oxygen-rich environments during culture, as these can increase ROS and stress the oocytes, can balance the ROS formation. Besides, 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 the IVF reduces sperm penetration (Kikuchi et al., 1993). Therefore, the risk of polyspermic penetration is presumably not high. However, it does complicate the interpretation of the results. The use of intracytoplasmic sperm injection may be a solution to consider.

# CONCLUSIONS

In conclusion, the results of this study suggested that oocytes with completed maturation time may allow better functioning of gene-editing materials and indicated no difference in the total mutation rates and total biallelic mutation rates in the resulting blastocysts between oocytes electroporated before and after fertilization. Although there are still some issues to be evaluated in future studies, such as the effect of electroporation before IVF on implantation, their widened time window without waiting for the completion of IVF enables flexible research design for embryonic gene modification in buffalo. Future research should aim to resolve these issues, paving the way for effective applications in buffalo breeding and biotechnology. By accessing embryos at earlier stages, researchers may introduce gene modifications at precise developmental phases, rather than being constrained by the IVF timeline. This flexibility will allow for adjusting the timing of gene editing to improve efficiency and accuracy, particularly during early zygotic divisions when chromatin is more accessible. With an extended window, gene editing tools like CRISPR/Cas9 can be applied when embryos are most responsive to modifications, enhancing the likelihood of successful integration and minimizing mosaicism. This can result in higher rates of consistent, uniform gene editing across embryonic cells.

# ACKNOWLEDGEMENTS

We thank Kabir and Son's Slaughterhouse, Khulna, Bangladesh, for supplying buffalo ovaries. We express our gratitude to Doctor's Lab and Imaging, Khulna, Bangladesh, for their assistance with genetic analysis and for providing several chemical agents for the study.

# AUTHOR CONTRIBUTIONS

**Dipankar Sardar:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Validation, Visualization, Writing – original draft, Writing – review & editing.
**Md. Taslim Hossain:** Investigation, Supervision, Validation, Writing – review & editing.
**Rudranil Baidya:** Formal analysis, Writing – review & editing.
**Adnan Habib:** Investigation, Supervision, Validation, Writing – review & editing.

## CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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How to cite this article;
Dipankar Sardar, Md. Taslim Hossain, Rudranil Baidya and Adnan Habib. Influence of electroporation timing on CRISPR/Cas-mediated multiple gene editing in buffalo embryos. Veterinary Integrative Sciences. 2025; 23(3): e2025069-1-12.