EVALUATION OF PX458 LIPOFECTION AND PUROMYCIN SELECTION IN BOVINE OVIDUCT EPITHELIAL CELLS

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ABSTRACT

The epithelial cells play a significant role in the developmental and reproductive functions of the mammalian oviduct. Recent innovations in tissue culture methods have enabled long-term in vitro culturing of the bovine oviductal epithelial cells (BOECs). The sensitive phenotype and limited proliferative capacity of BOECs made efficient plasmid delivery challenging. To provide robust experimental platforms for gene-function and mechanistic studies, a reproducible, well-defined primary culture with optimised lipofection protocols has been established. In this study, we isolated primary BOECs from fresh cow oviducts, cultured and passaged them to optimise lipofection conditions and evaluate plasmid delivery and transfection efficiency. Lipofectamine™ Stem Transfection Reagent with pX458 and pMax-GFP plasmids was used to perform the transient transfection. We assessed a range of cell seeding densities (0.75-1.5×10^5 cells/ml/well) and plasmid concentrations (1-2.5 µg per well) to evaluate lipofection efficiency. The cell viability was assessed using the Trypan Blue exclusion assay and morphological evaluation of cultured cells, and GFP expression was monitored qualitatively using fluorescence microscopy. Our findings showed that 2.0 µg of pMax and 1.5 µg of pX458 with 1 × 10^5 cells per well exhibited greater GFP expression with minimal cytotoxicity. Lipofection of BOECs resulted in generally lower transfection efficiency, with pX458 exhibiting markedly lower performance than pMax. However, the successful detection of the GFP expression using pX458. Successful detection of GFP expression from pX458 supports its use as a suitable reporter model for related CRISPR/Cas9 vector systems used in genome-editing studies. A puromycin kill curve was performed to determine the effective antibiotic concentration for bovine oviductal epithelial cells, providing a basis for future CRISPR-based genetic manipulation studies.

Keywords: Bovine Oviductal Epithelial Cells (BOECs), Lipofection optimisation, pX458, pMax-GFP

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INTRODUCTION

The bovine oviductal environment plays a central role in gamete transport, fertilisation, and early embryo development. The oviduct is a multi-functional organ in the female reproductive system that basic researchers and biotechnicians in animal production focus on. Given the broad range of actions occurring in this organ, well-defined model systems must be established to elucidate the mechanisms underlying these functions. These include maturation and transport of the female and male gametes, preparation of the ideal milieu for fertilisation and embryonic development, and transport of the embryo to the uterus (Schoen et al., 2008; Kidson *et al.*, 2003)

Oviductal epithelial cells from various mammalian species have been isolated and cultured in vitro, including pig oviduct (Boullant and Greig, 1973), mouse and humans (Takeuchi et al., 1991; Bongso et al., 1989). Different studies have also reported the oviductal epithelial cell culture in the bovine species (Joshi, 1988; Hoshi et al., 1992). In bovines, BOECs co-cultures improve pregnancy and embryo development rates. A previous study has also shown their application in sperm function, including capacitation, motility, and fertilisation (Chian and Sirard, 1995). Co-culture systems using BOECs represent a valuable in vitro model for investigating the molecular and cellular mechanisms underlying key reproductive events within the oviduct, including sperm capacitation, acrosome reaction, and early embryo development, thereby contributing to the improvement of in vitro embryo production systems (Abe and Hoshi,

1997). Establishing a robust in-vitro model based on primary BOECs is therefore valuable for reproductive biology and genome-editing research. Primary BOECs present challenges in culture and genetic manipulation due to their limited lifespan, variable morphology, and relative resistance to transfection (Schoen et al., 2008). Despite the biological importance of BOECs in reproductive research, limited information is available on suitable lipofection conditions for CRISPR/Cas9 plasmids such as pX458. Therefore, this study aimed to preliminarily evaluate different lipofection conditions, including plasmid concentration and puromycin selection, in BOECs and assess their effects on transfection and cell viability.

By establishing reproducible culture and transfection conditions, this work provides a methodological foundation for the use of BOECs in functional studies relevant to reproductive biology. While the focus here is on qualitative evaluation of transfection efficiency and cell viability, the optimised protocols lay the groundwork for subsequent quantitative and genome-editing applications, including CRISPR/Cas9-mediated functional analyses. Collectively, these efforts aim to enhance the experimental utility of primary BOECs and support future mechanistic studies in bovine reproductive research.

MATERIALS AND METHODS

Experimental Animals: Bovine oviducts were collected aseptically from a healthy adult female cow from a licensed commercial abattoir near the Royal Veterinary College, University of London, UK, as a by-product of routine slaughter. The collected tissues were sequentially processed by rinsing with sterile 1× PBS to remove any impurities. These specimens were transported to the laboratory for further processing in a thermal flask, maintaining the temperature.

Isolation of bovine oviductal epithelial cells (BOECs): Bovine oviducts were thoroughly rinsed with sterile 1X phosphate-buffered saline (PBS) supplemented with penicillin (100 IU/mL) and streptomycin (100 µg/mL) antibiotic solution to remove blood and debris. Surrounding connective tissue and ligaments were carefully dissected using sterile scissors and forceps to obtain intact, straightened oviducts. The cleaned oviducts were placed in sterile Petri dishes containing pre-warmed 1× PBS to maintain tissue moisture. Luminal epithelial cells were isolated using a combined mechanical and enzymatic digestion approach. The uterine end of each oviduct was ligated using sterile suture material (Figure#1), and the other end was carefully clamped to prevent leakage.

Using a sterile syringe fitted with a needle, carefully injected 0.5-10 mL pre-warmed digested solution intraluminally until the lumen was filled and no further capacity of injection was observed. The digestive solution consisted of Hank's Balanced Salt Solution (HBSS) without magnesium and calcium supplemented with 0.05% (w/v) Trypsin III, 0.1% (w/v) bovine serum albumin (BSA), DNase I (100 µg) and 0.05% (w/v) Collagenase II (Table#1 & Table#2). The oviducts were immersed in warm HBSS containing penicillin and streptomycin and incubated at 37°C for 60-90 minutes. During incubation, tissues were inverted gently every 15 minutes to ensure uniform enzymatic exposure.

After incubation, the luminal contents comprising the dispersed cells and epithelial sheets were collected into a 50 mL tube by gently trimming off the clamp end of the oviducts. To maximise the cell recovery, the oviducts were flushed and rinsed with 1.5-2.0 mL HBSS solution, and the wash was pooled with the collected digest.

Digestion was terminated by adding 10% foetal bovine serum (FBS). The cell suspension was centrifuged at 300×g for 10 minutes. The supernatant was discarded, and the cell pellet was washed with 20 mL of culture medium supplemented with 10% FBS. Cells were then processed for subsequent culture as previously described (Way et al., 2006; Walter, 1995).

Cell culturing for cell growth: Cell pellets were resuspended in prewarmed DMEM/F12 culture medium containing 10% FBS and 1% Pen/Strep antibiotic. Cell count and viability were determined using Trypan blue.

For this, 20 µl of cell suspension was mixed with 20 µl trypan blue and then examined by loading 10 µl on a haemocytometer. Cell viability was determined by counting cells with clear cytoplasm (viable) and blue-stained cytoplasm (nonviable) (Strober, 2015). Cells were counted in five squares of the haemocytometer under a microscope. Cell viability was calculated as the percentage of unstained (viable) cells relative to the total number of cells counted. Cell concentration was adjusted to 300,000 cells/mL with DMEM/F12 culture medium, and 1 mL was plated in each well of a 24-well microplate (24 wells with lid, IWAKI, Code 3820-024). Gently mixed the cell suspension by pipetting up and down a few times to ensure even distribution and settling of cells. Plates were incubated at 37°C with 5% CO_2 and checked every 24 hours for cell growth. Culture medium was renewed every 48 hours with fresh nutrient-rich medium to ensure cell viability. Cell confluency was monitored by examining it under a microscope. Cells were further split and passaged when confluency reached up to 80-90%. Additionally, aliquots from each passage were cryopreserved at -80°C for future use.

Culture medium: Dulbecco's modified Eagle medium DMEM/F12 (Gibco #11320033) with 10% heat-inactivated foetal bovine serum (Gibco #10500064) and 1% penicillin-streptomycin (Gibco #15140122)

Culture growth conditions: Culture conditions were maintained at 37°C with 5% CO_2. The composition of the culture media and digestive solutions used for primary BOECs isolation and culture is presented in Table 2. Culture media were prepared according to day-specific requirements. The digestive solution was sterilised using a 0.22 µm filter and stored at 4°C overnight until use.

Optimisation of Liposome-mediated transfection of Bovine Oviductal Epithelial Cells (BOECs): Liposome-based transfection was optimised for bovine oviductal cells using Lipofectamine™ Stem Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA; Cat. No.STEM00001) according to the manufacturer's instructions. Lipofection parameters, including plasmid concentration and cell density, were systematically evaluated to optimise conditions for efficient lipofection into BOECs. The optimisation strategy focused on determining the effects of pMax and pX458 plasmid concentration and cell density on overall transfection rate. We used the pMax GFP plasmid as a control reporter and the pX458 gene-editing plasmid, which encodes CRISPR Cas-9 and a GFP reporter. A range of plasmid DNA quantities (1-2.5 µg per well) and cell seeding densities ($0.75\text{-}1.5\times10^5$ cells//ml/well) were examined to identify the combination that maximised uptake while preserving cellular integrity (Qurat *et al.*, 2025). All optimisation experiments evaluating plasmid concentration and cell density were performed in three independent experiments to ensure reproducibility; representative images from a single experiment are shown in Figures 3, 4(a), & 4(b). Comparative assessment of these variable conditions enabled the identification of parameters that consistently enhanced transfection efficiency, providing a standardised framework for subsequent experiments. For this purpose, different densities of bovine oviductal cells were transfected with varying concentrations of pMax and pX458 plasmids carrying a GFP reporter to assess transfection efficiency.

Lipofection Protocol: Lipofection with Lipofectamine™ Stem Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA; Cat. No#STEM00001) was performed according to the manufacturer's instructions. Cells were examined after 18-24 h of plating to observe the confluency. Cells reached approximately 70-80% confluency and formed a web-like morphology that was considered suitable for lipofection.

The stepwise protocol used for lipofection is described in Table 2. The GFP-encoding plasmids pMax and pX458 were used to assess the lipofection-mediated transfection efficiency. The transfection components were prepared in two separate tubes (Tube A and Tube B) using the Lipofectamine Stem Transfection Reagent. Both tubes were incubated at room temperature for 5 mins before the complex formation. After this, the contents of Tube B were gently poured into Tube A, gently mixed and centrifuged to collect the mixture at the bottom of the tube. This tube was then incubated for 10 mins at room temperature to allow formation of the lipid-DNA complex. The resulting lipofection mixture was then added dropwise to each well of the culture plate to evenly distribute across the well surface using a P200 micropipette. Culture plates were swirled gently for homogeneous dispersion of complexes and incubated under standard culture conditions at 37 °C. After 24-48 hours of transfection, GFP expression was analysed using fluorescence microscopy. Transfection efficiency was assessed semi-quantitatively by observing the proportion of GFP-positive cells and relative fluorescence intensity. Qualitative assessment of cell viability was also done by monitoring cell morphology, absence of membrane blebbing and adherence to the surface.

Puromycin Kill Curve and Selection Considerations: A puromycin kill curve was formed by exposing the BOECs to a range of puromycin concentrations (0-10 µg/mL) to determine the threshold required for effective selection. The minimum dose of puromycin required to kill all non-resistant cells whilst maintaining overall culture integrity was identified by monitoring cellular responses to each treatment (Kahaki et al., 2024). For this purpose, cells were treated with different concentrations of puromycin. Each treatment was performed in duplicate, and cell viability was assessed at 24 and 48 hours post-treatment to determine the percentage of viable cells at each time point. Even though the plasmid pX458 contains a puromycin resistance cassette, antibiotic-based selection was not performed in this study due to the low observed transfection efficiency (<5%). Instead, transfection efficiency was assessed using the GFP expression as a primary readout.

Statistical analysis: The present study was designed as a qualitative evaluation of pX458 lipofection and puromycin dose response in BOECs. Observations reported were based on qualitative markers, including cell morphology, GFP fluorescence and puromycin dose response. As we did not measure quantitative variables, statistical analyses were not performed.

Ethical approval: All experiments in this study were performed according to the guidelines of the Ethical Committees of the Royal Veterinary College (RVC), University of London, UK and the University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan.

RESULTS AND DISCUSSION

Isolation and Culturing of Bovine Oviductal Epithelial Cells (BOECs): Primary bovine oviductal epithelial cells (BOECs) were isolated successfully from the cow oviducts and cultured in a 37°C incubator with 5% CO2 in Dulbecco's modified Eagle medium DMEM/F12 with 10% heat-inactivated foetal bovine serum and 1% penicillin-streptomycin. The cell growth appeared as a monolayer tightly attached to the surface and highly viable. Under the microscope, BOECs exhibited adherent cells with predominantly elongated spindle-like morphology. In some areas, cells were distributed in clusters as a dense layer and formed interconnected networks. The presence of distinct cell boundaries and centrally located nuclei in several cells indicated the viable, adherent oviductal cells. Clear cell boundaries and centrally positioned nuclei, as shown in Figure#2, indicate the successful attachment and growth of the isolated oviductal cells in culture. In previous studies, similar observed growth patterns and morphological appearances have also been reported as indicative characteristics of healthy adherent epithelial cells (Way et al., 2006; Schoen et al., 2008).

Optimisation of Transfection in BOECs*:* Establishing an efficient transfection protocol with optimal efficiency is a key challenge, being a major hurdle in nonviral gene delivery methods (Young et al., 2004). Understanding the factors that determine efficient gene delivery in BOECs is crucial for the advancement of future functional studies in this reproductive tissue. Despite the significance of these parameters in obtaining high transfection efficiency, the genetic manipulation of BOECs is still not explained due to the limited studies available. Among these parameters, plasmid concentration and cell seeding density, which, to our knowledge, play key roles, have not been systematically optimised or evaluated yet in this cell type. By studying how these factors affect the transfection, this study provides significant insights into the conditions that support efficient gene delivery in BOECs, indicating their role as a model system for future functional and genome-editing studies (Qurat et al., 2025). The lipofection efficiency reported by reagent manufacturers is typically derived from commonly used cell lines, such as rodent or human cells (Shiokawa et al., 2021). However, data on the transfection efficiency in bovine-derived cells remains very limited (Hyder et al., 2020; Osorio et al., 2017). As each cell type requires specific conditions to efficiently take up the foreign DNA, optimising the key parameters is important to obtain effective transfection. In the present study, we have optimised two critical factors for lipofection, including the plasmid concentration and cell density, using Lipofectamine™ Stem Transfection Reagent. To ensure reproducibility, all optimisation experiments, including the plasmid concentration and cell density conditions, were performed as three independent experiments. To evaluate the lipofection efficiency in the BOECs, we used a transient reporter assay system utilising pMax-GFP and pX458 plasmids. Transfected cells were visualised using fluorescence microscopy by monitoring green fluorescence as a reporter of gene expression (Chong et al., 2021). Both pX458 and pMax plasmids were evaluated under identical lipofection conditions to assess GFP expression in bovine oviductal epithelial cells. Qualitative differences in GFP fluorescence were observed between the two constructs under the experimental conditions tested. However, due to differences in plasmid architecture, including vector size and promoter configuration, direct quantitative comparison of transfection efficiency between pX458 and pMax was not performed.

Optimisation of Cell Densities: To optimise transfection efficiency in bovine oviductal cells using Lipofectamine™ Stem Transfection Reagent, cells were seeded at different densities (0.75, 1.0, 1.25, and 1.5×10^5 cells/well) in 6-well culture plates. After 18-24 h of culture, when cells reached appropriate confluency, transfection was performed using 1.5 µg plasmid per well (pMax or pX458). GFP-positive cells were assessed at 24 and 48 h post-transfection by fluorescence microscopy. A greater number of GFP-positive cells were observed at a seeding density of 1×10^5 cells/well for both plasmids. However, pMax resulted in a greater number of GFP-positive cells under the experimental conditions, whereas pX458 showed low GFP expression (<5%) (Figure 3a, b).

Optimisation of Plasmid Concentration: After determining the optimal cell density for transfection, the next step was to optimise plasmid concentration. For this purpose, cells were seeded at their optimal concentration, i.e. 1×10^5 Cells/ml/well of a 6-well culture dish, and lipofection was carried out using a range of pMax and pX458 plasmid concentrations (1.0, 1.25, 1.5, 2.0, and 2.5 µg per well). Cells were imaged for GFP expression at 24 and 48 h post-lipofection. Maximum GFP-positive cells were observed with 2.0 µg for pMax (Figure 4a) and 1.5 µg for pX458 (Figure 4b).

Overall, it was observed that the liposome-mediated transfection in bovine oviductal epithelial cells was very low, consistent with the inherently restrictive nature of these primary cells, which possess robust membrane barriers and limited proliferative activity that can reduce vector uptake. Among the plasmids tested, pMax showed more frequent GFP-positive cells under the experimental conditions, whereas pX458 showed low GFP expression in comparison. Our results are consistent with those reported by Rybakovsky et al. (2019), who demonstrated that polarised, differentiated epithelial cells are inherently more difficult to transfect than less differentiated, non-polarised cells. This suggests that

similar cellular characteristics may contribute to the low lipofection efficiency observed in bovine oviductal epithelial cells.

Determination of the minimum puromycin concentration required to eliminate non-transfected BOECs: A puromycin kill curve was established to determine the antibiotic selection concentration for BOECs as part of the methodological optimisation process. This standardisation will support future studies involving CRISPR-based genetic manipulation of these cells by enabling efficient selection of successfully transfected populations. The minimum puromycin concentration required to eliminate non-transfected cells was determined to optimise selection conditions. The 48-hour post-puromycin time point enabled identification of an optimal selection concentration, which was found to be 2 µg/mL for bovine oviductal cells (Figure 5a & b). Cells were imaged under the microscope, and the % cell viability was observed for each treatment. The dead cells were indicated by suspended floccules detached from the plate surface and floating in the culture medium. The puromycin concentration was tested in the range 0.5–10 µg/mL along with a non-treated control (0 µg/mL). The percentage cell viability (%) showed that 2 µg/mL puromycin was sufficient to eliminate non-transfected cells within 48 hours. A puromycin kill curve was performed to determine the effective antibiotic concentration; however, puromycin selection was not applied due to the low transfection efficiency (<5%), and GFP fluorescence was used as the main readout. In summary, the present study demonstrates a qualitative evaluation of transfection in bovine oviductal epithelial cells based on fluorescence microscopy. We used the pMax as a positive control to assess transgene expression; the study does not include quantitative methods such as flow cytometry or any other cell lines. Cytotoxicity was assessed using Trypan Blue staining and basic morphological observations. Therefore, the findings should be interpreted as qualitative observations that may help guide future quantitative studies.

Table 1. Composition of Culture medium for Bovine oviduct cells

Culture medium		
DMEM+10% FBS+50,000IU/L Pen/Strep 1% (1ml/100ml)		
DMEM/F12	**FBS**	**Pen/Strep**
88ml	10ml	1ml
44 ml	5ml	0.5ml
22 ml	2.5ml	0.25ml

Table 2. Preparation of Lipofectamine Stem Reagent for Lipofection

Tube	**Reagent**	**Amount per well (1x 6 well)**
Tube A	OptiMEM	50 µl
	Lipofectamine stem reagent	3 µl
Tube B	OptiMEM	50 µl
	Plasmid DNA (0.5 - 2 µg/µL)	1.5 µg

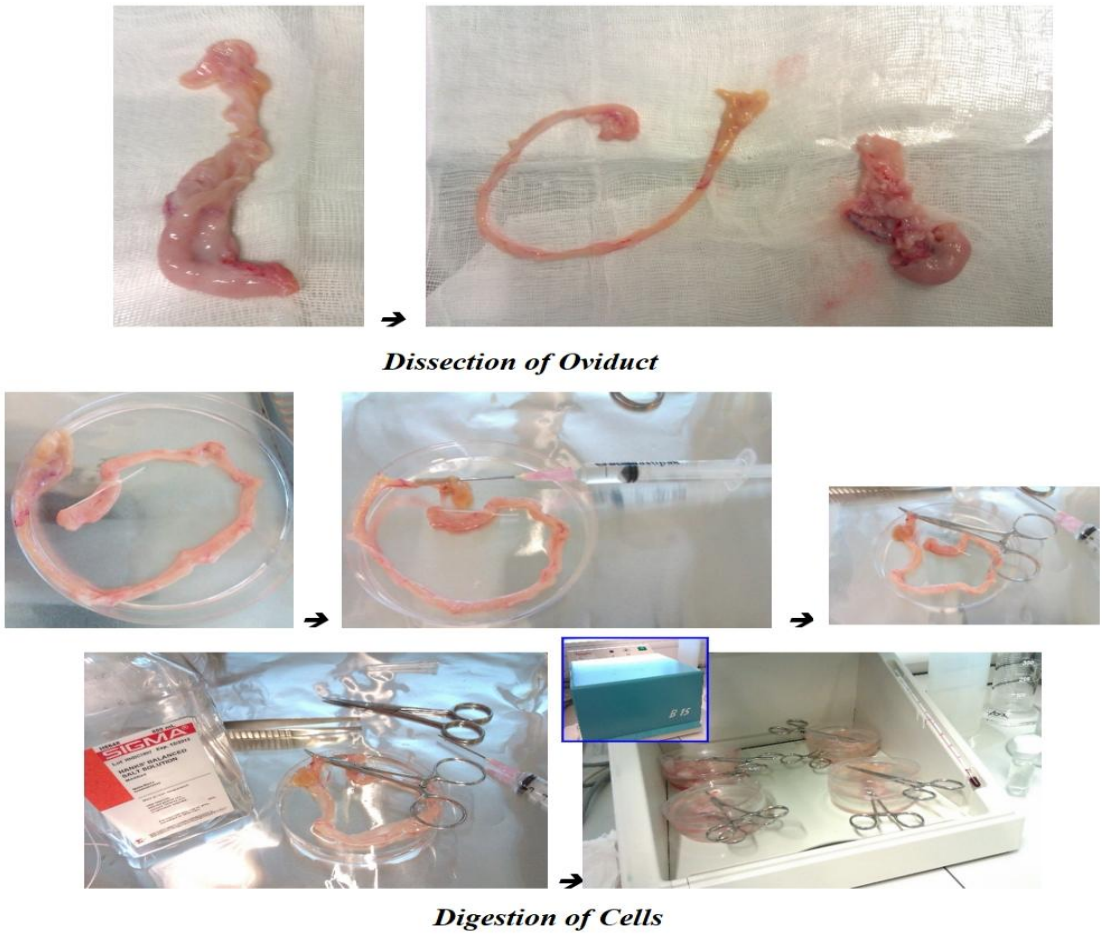

Fig. 1. Isolation and culturing of bovine oviduct cells from Cow Oviduct: *Primary cells were derived from cow oviduct tissue and cultured under standard conditions. Oviduct tissue was rinsed, enzymatically digested, and the cells were collected and plated.*

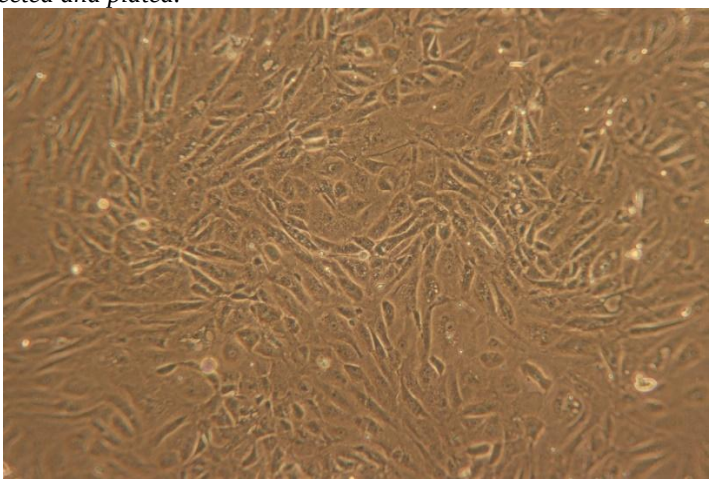

Fig. 2. Primary Bovine oviductal cells (BOECs) Cultured from cow oviducts in monolayer culture (Passage-5) (10x magnification image)

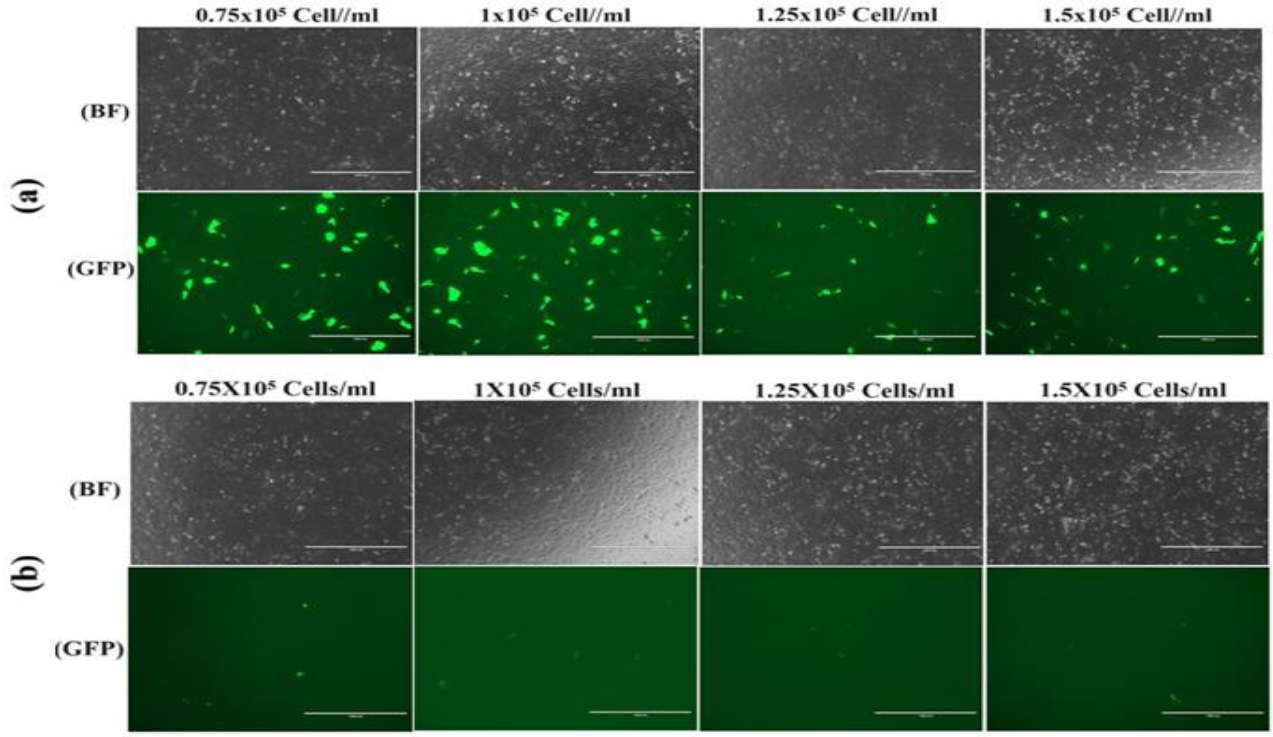

Fig. 3. Optimisation of Cell density for Transfection in Bovine Oviductal Cells: Lipofection efficiency was determined by seeding different BOECs cell densities, i.e. 0.75×10^5, 1×10^5, 1.25×10^5, and 1.5×10^5 cells per well, transfecting using a constant plasmid concentration of 1500 ng/µL of pMax GFP (a) & pX458 plasmid DNA (b). The rate of transfection was assessed 48h post lipofection by analysing GFP expression. The Brightfield (BF) and GFP fluorescence images are shown for each cell density for both plasmids.

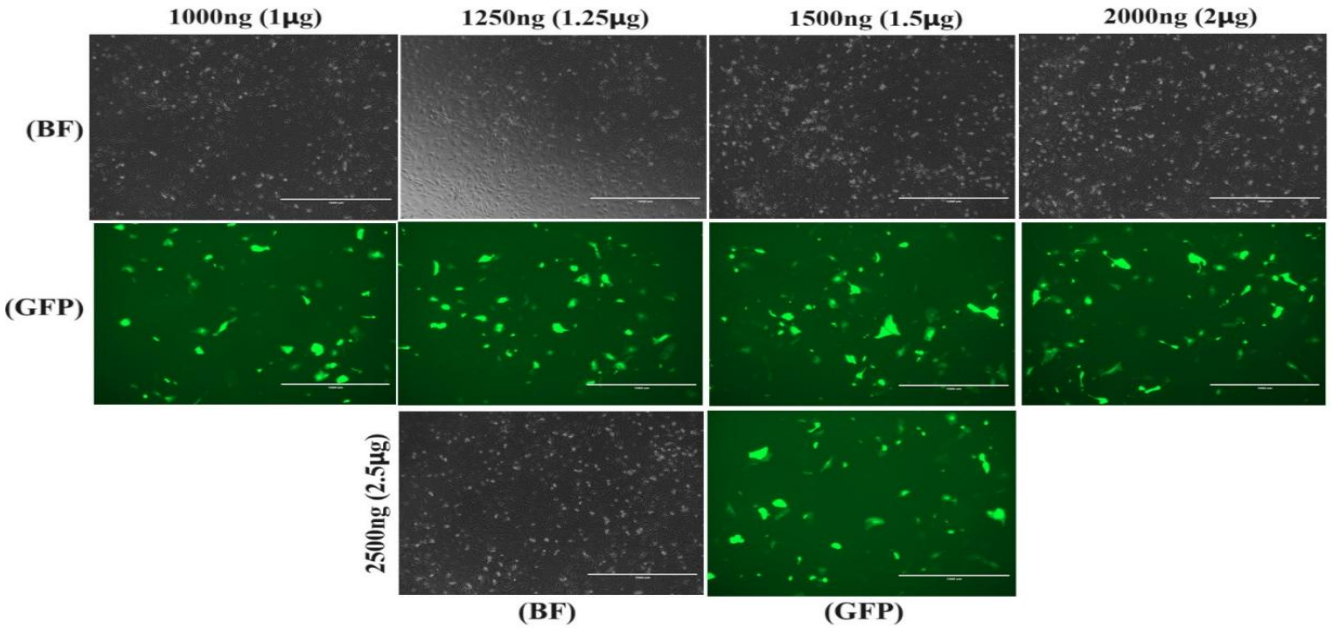

Fig. 4(a). Optimisation of pMax GFP-Plasmid concentration for Lipofection in Bovine Oviductal Cells: Optimal plasmid concentration for efficient lipofection was determined by seeding optimal cell density *$1X10^5$ Cells/ml* and transfecting with a varying range of pMax plasmid, i.e. 1000 ng/µl, 1250 ng/µl, 1500 ng/µl, 2000 ng/µl and 2500 ng/µl. The transfection efficiency was assessed at 48h post-lipofection by analysing GFP expression. The Brightfield (BF) and GFP fluorescence images are shown for each concentration of pMax-GFP plasmid.

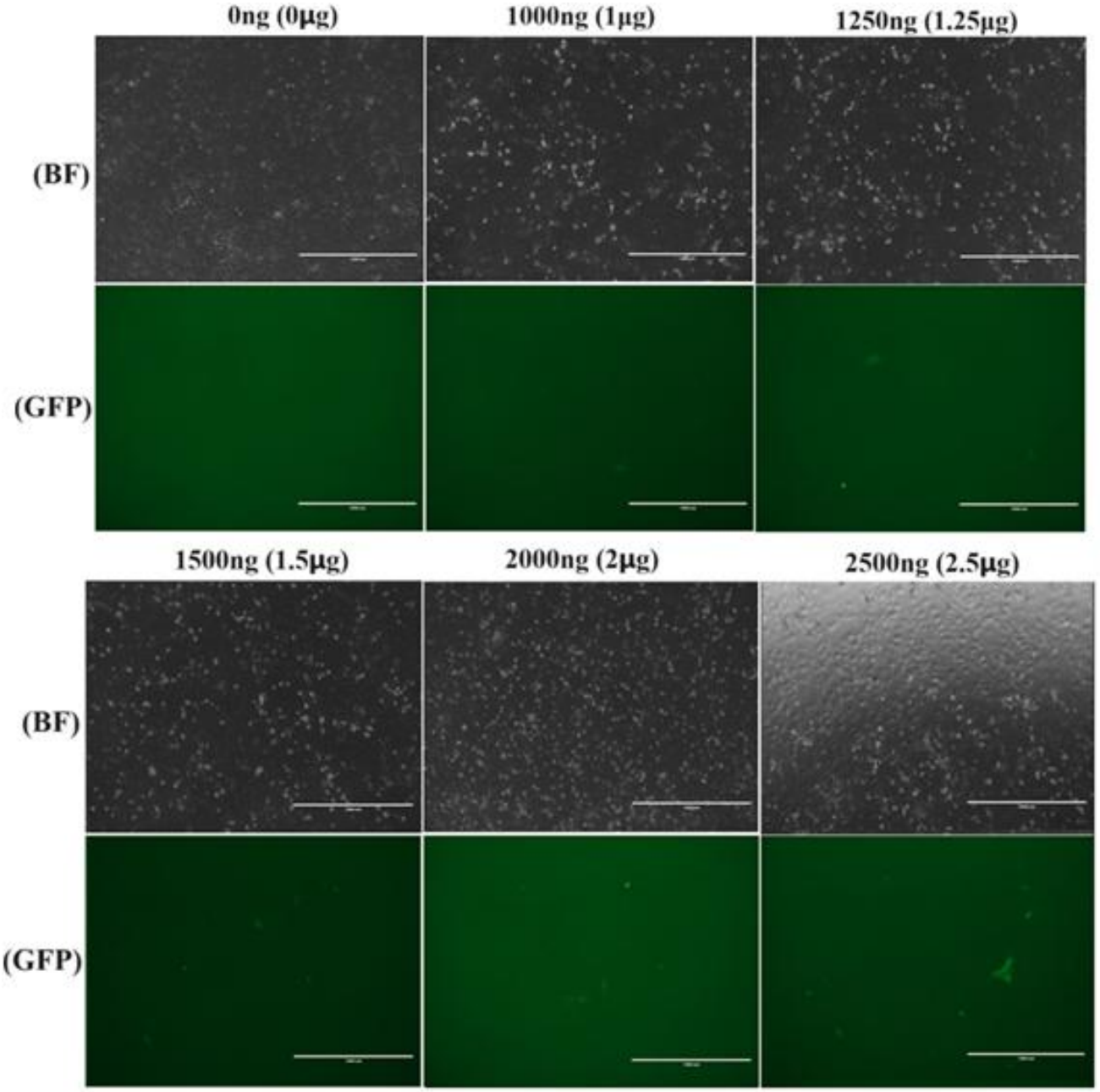

Fig. 4(b). Optimisation of pX458 Plasmid concentration for Lipofection in Bovine Oviductal Cells: Optimal plasmid concentration for efficient lipofection was determined by seeding the optimal cell density (1×10^5 cells/mL) and transfecting with a varying range of pX458 plasmid concentrations, i.e. 0 ng/µl, 1000 ng/µl, 1250 ng/µl, 1500 ng/µL, 2000 ng/µl and 2500 ng/µl. The 0 ng/µl condition consisted of cells treated with Lipofectamine in the absence of plasmid DNA and served as a lipid-only (mock-transfection) control. Transfection efficiency was assessed 48 h post-lipofection by analysing GFP expression. The Brightfield (BF) and GFP fluorescence images are shown for each pX458 plasmid concentration.

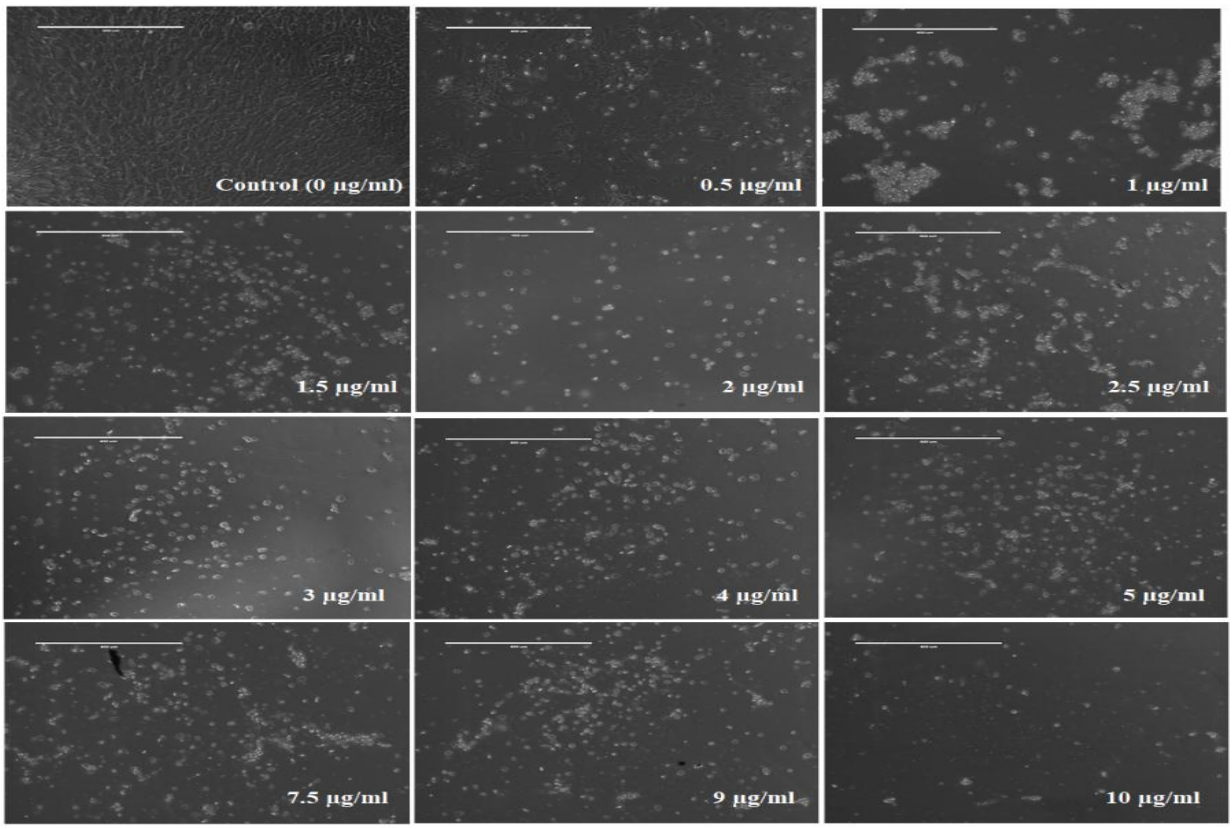

Fig. 5(a). Microscopy images of bovine oviductal cells (BOECs) after 48 hours of treatment with different doses/concentrations of puromycin (0-10 µg/mL). Minimum effective concentration of puromycin was determined by complete cell death, indicated by detached, floating cells.

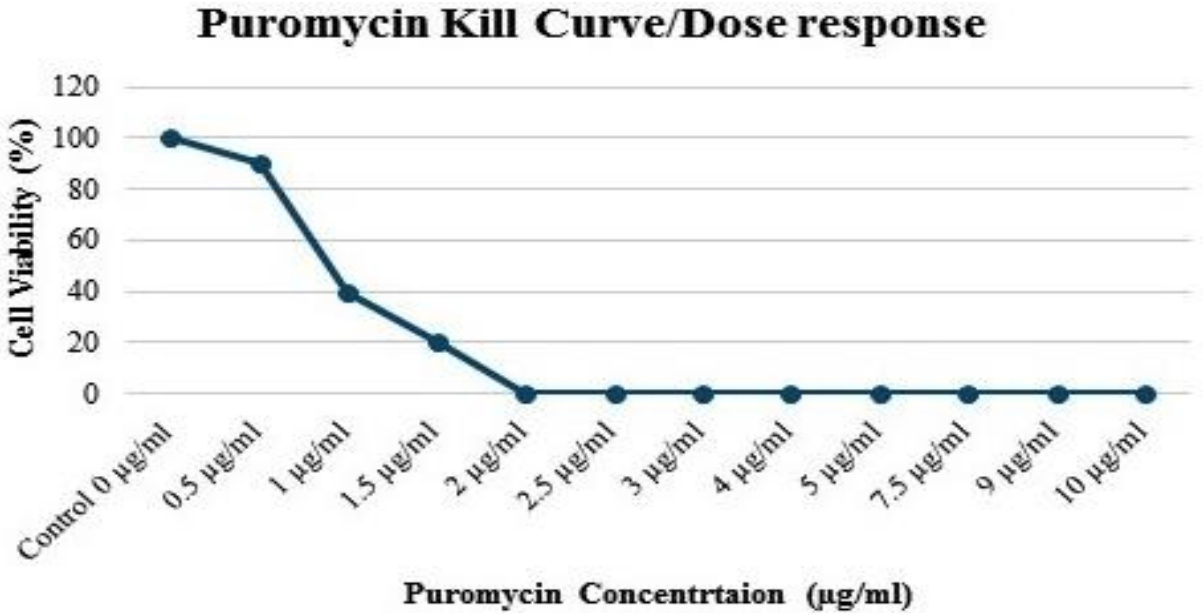

Fig. 5(b). Puromycin kill curve for BOECs showing percentage cell viability at different puromycin concentrations (0-10 µg/mL). A simple line graph was plotted with puromycin concentration (µg/mL) on the x-axis and cell viability (%) on the y-axis. The graph demonstrated that 2 µg/mL was the minimum concentration required to effectively eliminate non-transfected cells.

Conclusion: Recent advances in cell culture methods have enabled the prolonged maintenance of BOECs in vitro, providing a valuable system for reproductive and embryological research. Based on this foundation, our study outlines the isolation and establishment of primary BOEC cultures from cow oviduct tissue using simple enzymatic and mechanical approaches. Importantly, we also evaluated key determinants of liposome-mediated transfection, i.e., plasmid concentration and cell density, and identified combinations that improved transfection outcomes in these inherently challenging primary cells. This optimisation represents a meaningful advancement, as efficient gene delivery in BOECs has historically been limited by their physiological characteristics. In addition, we optimised the minimal puromycin concentration required for stringent selection in bovine oviductal epithelial cells, establishing a robust framework to efficiently enrich transfected cells for downstream applications, including future CRISPR-based studies in this system. To conclude, this study presents a significant approach for optimisation of lipofection and puromycin concentration in bovine oviductal epithelial cells (BOECs). This framework offers a reproducible and reliable platform for optimising the pX458 lipofection and puromycin selection conditions, thereby establishing a strong foundation for future CRISPR-based genetic manipulation and functional studies and advancing the use of BOECs in reproductive research.

Although this study is mainly based on qualitative analysis, it provides a primary framework to optimise lipofection parameters in BOECs and establishes the basis for future, functional, molecular and quantitative studies, thereby supporting development of a robust and validated system. Ongoing improvements in BOEC culture and transfection protocols are expected to further enhance their utility across reproductive biology research.

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