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Impact of TGF-β and ERK1/2 inhibition on preimplantation embryo development and quality

Faezeh Mohammadi Ghazani [1, 2#], Fatemeh Shirvanizadeh [2#], Seyedeh-Nafiseh Hassani [3], Azam Dalman [2], Nahid Nasiri [2, 4], Maryam Zarei [2], Saman Maroufizadeh [5], Poopak Eftekhari-Yazdi [2*]

[1] Faculty of Development of Biology, University of Science and Culture, Tehran, Iran
[2] Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
[3] Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
[4] Department of Photo Healing and Regeneration, Medical Laser Research Center, Yara Institute Academic Center for Education, Culture and Research (ACECR), Tehran, Iran
[5] Department of Biostatistics and Epidemiology, School of Health, Guilan University of Medical Sciences, Rasht, Iran

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ABSTRACT

Objective(s): Preimplantation embryo culture is a critical phase in assisted reproductive technologies. The use of small molecules to modulate signaling pathways has emerged as a strategy to improve embryo viability. SB431542 inhibits the TGF-β pathway, while PD0325901 inhibits the MEK/ERK pathway. This study investigates whether their combined application affects embryonic development and cellular composition during early culture, independent of stem cell derivation.

Materials and Methods: To assess the effects of SB431542 and PD0325901 on early embryo development, mouse embryos at 2PN, two-cell, and eight-cell stages were collected *in vivo* and cultured in LIF-supplemented medium containing both molecules until the blastocyst stage. Embryo quality, apoptosis, and developmental progression were assessed using differential staining, TUNEL assay, and continuous monitoring throughout the culture period.

Results: Embryos treated with SB431542 and PD0325901 showed a slightly higher rate of blastocyst formation than controls, though the difference was not statistically significant. Degeneration and apoptosis rates were also comparable between groups. However, treated embryos exhibited a significant reduction in ICM cells at early stages, suggesting compromised inner cell mass development. These findings indicate that dual inhibition may not enhance embryo quality under the tested conditions.

Conclusion: SB431542 and PD0325901, when applied during preimplantation embryo culture, do not significantly improve developmental progression or reduce degeneration and apoptosis. The observed decrease in ICM cells suggests limited benefit for embryo culture protocols designed to support early development. Further studies are needed to evaluate their relevance in stem cell derivation contexts.

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Introduction

Mouse embryogenesis is divided into two distinct phases: preimplantation and post-implantation. The preimplantation phase is a meticulously regulated and critical stage of fetal development, initiated with a single cell (the oocyte) and progressing through the morula stage to the blastocyst stage. This initial phase is essential for establishing a successful mammal pregnancy (1, 2).

Following its formation, the zygote travels through the fallopian tube toward the uterus, undergoing sequential divisions into the two-cell, four-cell, eight-cell, and sixteen-cell (morula) stages before reaching the blastocyst phase. The blastomeres undergo compaction at the 16-cell morula stage, forming two distinct cell layers: the trophectoderm (TE) and the inner cell mass (ICM).

The protein Octamer-binding Transcription factor 4 (OCT4) becomes detectable at the eight-cell stage and is pivotal for ICM development. Concurrently, the primary trophectoderm marker, Caudal Type Homeobox 2 (CDX2), is expressed at the morula stage, marking the initiation of the cavitation process (3, 4). The ICM gives rise to the three embryonic germ layers through subsequent cell divisions, ultimately forming the entire organism. For this reason, these cells are referred to as pluripotent.

Meanwhile, trophectoderm cells expressing CDX2 play a pivotal role in placenta formation (5). The proper progression of the cell cycle during embryogenesis is governed by a complex network of regulatory pathways,

*Corresponding author: Poopak Eftekhari-Yazdi. Department of Embryology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran. Tel: +98-2122339901, Fax: +98-2122339916, Email: eftekhari@royaninstitute.org
# These authors contributed equally to this work

among which the Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinases 1 and 2 (MAPK/ERK1/2) signaling pathway is particularly significant. This pathway is primarily activated by fibroblast growth factor (FGF) proteins (6, 7).

FGF ligands are expressed in preimplantation embryos, with FGF4 being the predominant ligand, highlighting its critical role in preimplantation embryogenesis (8). Although FGF is not essential for murine stem cell proliferation, it is indispensable for their differentiation into neural and non-neural lineages during stem cell development and embryogenesis. Evidence from studies reveals that mouse embryos lacking FGF4 fail to survive shortly after implantation. Consistently, embryos with FGF4 mutations implant approximately 4.5 days after fertilization but do not survive beyond 1–2 days post-implantation (9).

Exogenous FGF4 supplementation in embryo cultures promotes the progression of the ICM toward the formation of primitive endoderm and yolk sacs, which are derivatives of the hypoblast (10, 11). Although the precise mechanisms underlying FGF's role in ICM differentiation remain unclear, it is hypothesized that individual ICM cells respond variably to differing levels of FGF4 signaling. Epiblast cells secrete FGF4, while hypoblast cells express its receptor, Fibroblast Growth Factor Receptor 2 (FGFR2), at high levels. This interaction induces the expression of genes such as Sox17, Gata6, and Gata4 in the hypoblast, all associated with development of the primary endoderm (11, 12).

The MAPK/ERK signaling pathway is also activated by other growth factors, such as Leukemia Inhibitory Factor (LIF)(13). LIF exerts its function by activating Signal Transducer and Activator of Transcription 3 (STAT3) downstream of ERK phosphorylation, disrupting FGF's autocrine activity and enhancing the maintenance of pluripotency. Without LIF, the FGF/ERK pathway prepares cells for a transient state in which they are predisposed to adopt a neural fate. During this state, cells are highly responsive to sustained Notch and FGF signaling but remain susceptible to fate redirection by other inducing agents, such as members of the Transforming Growth Factor-β family (TGF-β)(6, 14).

The TGF-β signaling pathway, a key regulator in embryogenesis, plays multifaceted roles during preimplantation development (15). This pathway is essential for the proliferation and maintenance of pluripotency in murine embryonic stem cells. However, several studies have demonstrated that inhibiting TGF-β signaling does not impair the self-renewal capacity of these cells (16, 17). TGF-β signaling is intricately modulated through its interactions with other pathways, notably the Hippo signaling pathway, which is particularly significant during preimplantation development (18, 19).

Recent advancements in stem cell culture techniques have significantly improved the efficiency of generating and maintaining murine embryonic stem cells. By employing chemical inhibitors that target specific signaling pathways to inhibit differentiation, it is now possible to effectively produce and propagate murine stem cells. These embryonic stem cells, derived from the ICM of blastocysts, were initially isolated by culturing blastocyst-stage embryos or their ICMs on mitotically inactive murine embryonic fibroblasts (MEFs) in a medium supplemented with bovine fetal serum. Although these culture conditions, which include serum and a feeder cell layer, are widely used due to their effectiveness in supporting genetic manipulation, they are not considered optimal for embryonic stem cell production (20).

In 2008, researchers identified a breakthrough method to maintain the pluripotency of murine embryonic stem cells using a combination of three small molecule inhibitors targeting FGF receptors, MAP kinase (MEK) 1/2, and Glycogen Synthase Kinase 3 (GSK3), collectively referred to as "3i." This inhibitor combination enabled the maintenance of pluripotency without requiring serum or LIF. Moreover, the 3i approach facilitated the derivation of embryonic stem cells from diverse murine strains, overcoming previous challenges in their generation (21). In 2010, a more potent MEK inhibitor, PD0325901, was introduced, eliminating the need for the FGF receptor inhibitor. This led to a simplified combination of two small molecules, PD0325901 and CHIR99021, termed "2i." This approach proved superior to the earlier "3i" formulation when used with basic stem cell culture media. These compounds target and inhibit the proteins ERK1/2 and GSK3, enhancing the stability and growth of murine stem cells (22).

Using these chemical inhibitors in a simplified culture medium eliminated the need for exogenous growth factors, effectively maintaining stem cells in a robust pluripotent state. Shortly after that, the addition of LIF to the 2i medium was shown to yield even better outcomes (23).

By 2014, researchers demonstrated that combining the TGF-β signaling pathway and ERK1/2 inhibitors could further strengthen pluripotency maintenance (24, 25). Specifically, the small molecule SB431542, which inhibits the type I TGF-β receptor (ALK4/5/7) and PD0325901, formed the "R2i" combination. This approach was found to facilitate the derivation of competent and proliferative mouse stem cells from blastocysts of strains previously unable to generate cell lines (23, 24).

Mouse stem cells cultured under R2i conditions exhibited enhanced colony growth and consistent expression of key transcription factors, including Nanog and Stella. Additionally, R2i-treated cells showed a reduced propensity for differentiation, as evidenced by lower expression levels of genes such as Left-Right Determination Factor 1 (lefty1), lefty2, and Brachyury compared to cells cultured with the 2i combination. Furthermore, R2i conditions demonstrated superior effectiveness in preserving karyotypic stability during extended cell passages (24, 25). Hassani *et al.* (2014) proposed that R2i conditions could significantly influence blastocyst formation (26).

This study aims to evaluate the impact of the R2i small molecule combination on embryonic division and preimplantation development. To achieve this, we will compare an experimental group cultured in a basal embryonic medium (T6 supplemented with 4 mg/ml BSA) enriched with LIF and the R2i combination to a control group maintained in the basal embryonic medium alone. The quality of blastocysts will be assessed by analyzing their morphology, total cell count, and apoptosis incidence in both the experimental and control groups.

Materials and Methods

Experimental group

This prospective case-control study utilized adult male and female NMRI mice aged six to eight weeks. To induce

superovulation, female mice received a single intraperitoneal injection of 7.5 IU/ml pregnant mare's serum gonadotropin (PMSG; Intervet Folligon 5000 IU, Holland). Forty-eight hours later, a second intraperitoneal injection of 7.5 IU/ml human chorionic gonadotropin (hCG; Organon 500 IU, Holland) was administered. Immediately following the hCG injection, females were placed with males for mating. Successful copulation was confirmed by the presence of a vaginal plug, checked between 13 and 16 hours post-hCG injection. Mice without a vaginal plug were excluded from the study.

Two groups of embryo culture without treatment were considered the control group, and embryo culture under treatment with R2i was considered the test group.

Mated females were euthanized by cervical dislocation. In each group, embryos at the zygote, two-cell, and eight-cell stages were isolated from their fallopian tubes. These embryos were cultured until the blastocyst stage, which occurred within 4.5 to 5 days (Figure 1).

The study received approval from the Medical Ethics Committee of the Royan Institute in Tehran, Iran, Ethical code: NO.IR.ACECR. ROYAN. EC.93.1141).

To assess outcomes, blastocysts were examined for morphology and the rate of development to the blastocyst stage. Differential staining was used to quantify cell numbers, while apoptotic cells were detected through TUNEL staining (27).

Counting blastocyst cells

Embryos were stained immediately upon reaching the expanded blastocyst stage to quantify the total number of cells in each embryo and differentiate between ICM and TE cells. This differentiation was achieved using two fluorescent dyes: propidium iodide (PI)(Sigma, USA) and bisbenzimide (Hoechst 33258; Calbiochem, USA)(28).

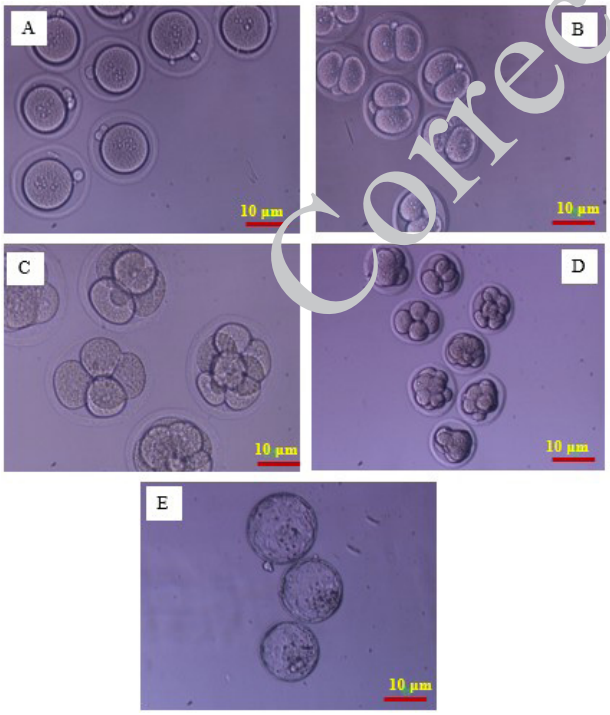

Figure 1. Different stages of mouse embryo development *in vitro*
A: 2PN embryo. B: 2 Cell embryo. C, D: 4-8 Cell and Morula embryos. E: Expand Blastocyst
The experimental group was cultured in T6 medium supplemented with 4 mg/mL BSA, 10 µM SB431542, 1 µM PD0325901, and 1000 IU of recombinant LIF. The control group, by contrast, was cultured in a T6 medium containing only 4 mg/mL BSA
2PN :2 pronuclear; BSA: Bovine Serum Albumin; IU: international units; LIF: Leukemia Inhibitory Factor

Differential staining

Expanded blastocyst-stage embryos were carefully removed from the culture medium in a dark environment and transferred to a four-well plate containing 500 µl of PI solution for 10 sec. Subsequently, the embryos were transferred to a well containing 500 µl of bisbenzimide solution, where they were incubated overnight (12-18 hrs) at 4 ˚C in the dark (28)(Figure 2).

Examining cell death in blastocysts

To evaluate programmed cell death in blastomeres within the blastocyst, the TUNEL staining method was employed using 4′,6-diamidino-2-phenylindole (DAPI) dye to quantify apoptotic cells (29).

TUNEL staining

Nuclear DNA fragmentation, a hallmark of programmed cell death in blastomeres, was assessed using the TUNEL kit (Roche, Germany). Initially, blastocysts were removed from the culture medium and washed in a 0.5% PBS Tween (Sigma, USA) solution. They were then fixed in PBS containing 4% paraformaldehyde and stored overnight at 4 ˚C. After washing, the blastocysts were permeabilized in 0.1% Triton X-100 for 1 hour, then washed again before incubation with 50 µl of TUNEL mixture for 1 hr at 37 ˚C, protected from light.

After incubation, the embryos were washed and transferred to a 50-µl drop of 1% DAPI for 10 min at room temperature in the dark. The embryos were rewashed and

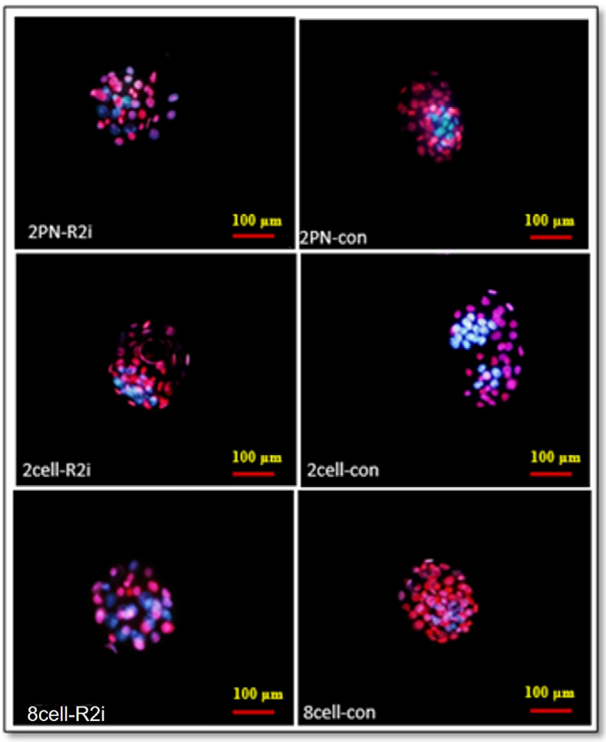

Figure 2. Differential staining of blastocysts derived from 2PN, two-cell, and eight-cell mouse embryos in R2i-treated and control groups
2PN :2 pronuclear; R2i: SB431542 and PD0325901

Table 1. Developmental progression of mouse embryos in the R2i (Test) and control

Embryonic stage	2 Pro Nucleus (2PN) (n)		2 Cell (n)		8 Cell (n)	
	Control	Test	Control	Test	Control	Test
Early embryo	75	84	73	81	77	84
Expanded blastocyst	48 (64%)	66 (78.5)	51 (69.86)	62 (76.54)	51 (66.23)	59 (70.23)

mounted on slides using a mounting medium (DAKO, England). A small slide was gently placed over the embryos to mark their positions. The slides were examined under a fluorescent microscope at 550 nm. DNA fragmentation was identified by bright-green-staining nuclei in the TUNEL reaction, while nuclei with a fragmented appearance and chromatin aggregation were classified as apoptotic (29).

Statistical analysis

Data are presented as mean±standard deviation (SD). Graphs were generated using GraphPad Prism software. Statistical analyses were conducted using t-tests, one-way analysis of variance (ANOVA), and Tukey's post hoc test. A p-value<0.05 was considered statistically significant.

Results

A total of 474 early-stage embryos, specifically at the 2PN, two-cell, and eight-cell stages, were retrieved from the fallopian tubes of 51 pregnant mice. These embryos were individually cultured in the control and test (R2i) groups until they reached the expanded blastocyst stage. Across eight independent trials, 337 blastocysts were successfully obtained from the cultured embryos (Table 1).

Comparative analysis of embryo development to the expanded blastocyst stage

The comparative analysis evaluated the percentage of embryos progressing from the initial culturing phase to the expanded blastocyst stage in both R2i-treated and control groups.

In the R2i-treated group, the average developmental potential of embryos cultured to the blastocyst stage was as follows: 2PN embryos (0.79±0.11), two-cell embryos (0.77±0.06), and eight-cell embryos (0.70±0.11). By contrast, the control group exhibited developmental potential for 2PN embryos (0.64±0.11), two-cell embryos (0.70±0.05), and eight-cell embryos (0.66±0.10) under the same conditions.

While the R2i group consistently showed higher developmental percentages across all stages, the difference in developmental success rates between the R2i-treated and control groups was not statistically significant. Figure 3 illustrates the developmental percentages of embryos reaching the expanded blastocyst stage.

Analyzing the percentage of embryonic degeneration during development to the expanded blastocyst stage

The analysis of embryonic degeneration from the start of culture to the extended blastocyst stage indicated the following degeneration percentages in the R2i-treated group: 2PN embryos (0.17±0.13), two-cell embryos (0.07±0.02), and eight-cell embryos (0.01±0.02). In comparison, the control group exhibited degeneration rates of 2PN embryos (0.20±0.05), two-cell embryos (0.06±0.03), and eight-cell embryos (0.19±0.11). Although the differences between the treated and control groups were not statistically significant, R2i-treated embryos cultured from the zygote (2PN) and eight-cell stages demonstrated lower degeneration percentages than their control counterparts. While not statistically significant, these findings suggest a potential trend of reduced degeneration in the R2i-treated embryos during development (Figure 4).

Analyzing the average number of ICM cells in blastocyst-stage embryos

The analysis of inner cell mass (ICM) cell counts in blastocysts derived from cultured embryos revealed the following averages in the R2i-treated group: 2PN embryos (12.1±2.13), two-cell embryos (11.7±2.26), and eight-cell embryos (14.7±2.00). In comparison, the control group

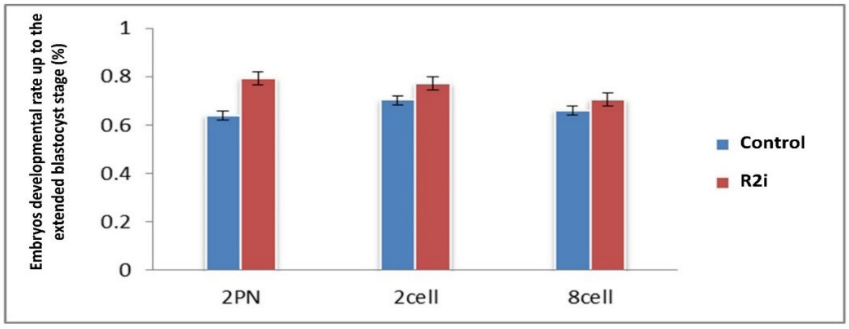

Figure 3. Developmental rates of 2PN, two-cell, and eight-cell mouse embryos to the extended blastocyst stage in R2i-treated and control groups
The differences in developmental success rates between the R2i-treated and control groups were not statistically significant. Data are expressed as mean±SE, n=3. *P-value*<0.05 was considered statistically significant
R2i: SB431542 and PD0325901, 2PN:2 pronuclear

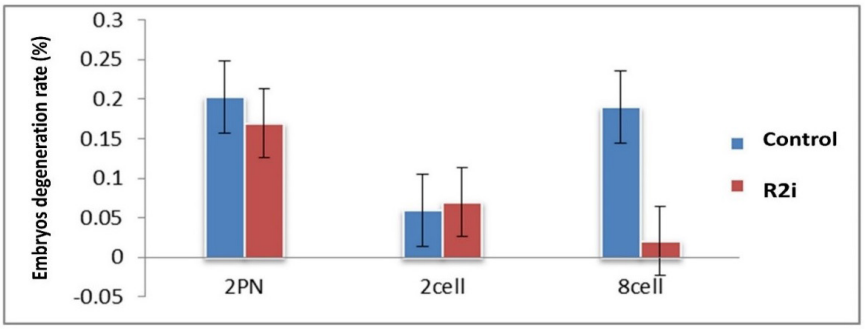

Figure 4. The degeneration rates of 2PN, two-cell, and eight-cell mouse embryos during development to the extended blastocyst stage were not statistically significant between the R2i-treated and control groups
Data are expressed as mean±SE, n=3. *P-value*< 0.05 was considered statistically significant
R2i: SB431542 and PD0325901, 2PN: 2 pronuclear

exhibited higher averages: 2PN embryos (14.6±1.89), two-cell embryos (15.4±2.67), and eight-cell embryos (14.1±1.59).

Statistical analysis indicated that the control group had a significantly higher average number of ICM cells than the R2i-treated group, particularly in the 2PN and two-cell embryo categories. This difference highlights a notable reduction in ICM cell counts in embryos treated with R2i. The findings suggest that while R2i treatment supports blastocyst formation, it may impact the proliferation or maintenance of ICM cells, especially in earlier embryonic stages (Figure 5).

Analyzing the average number of TE cells in blastocyst-stage embryos

The analysis of trophectoderm cell counts in blastocyst-stage embryos derived from cultured embryos revealed the following averages for the R2i-treated group: 2PN embryos (49±1.98), two-cell embryos (40.4±11.28), and eight-cell embryos (34.5±3.40). In the control group, the corresponding averages were 2PN embryos (41.9±6.29), two-cell embryos (44.4±6.89), and eight-cell embryos (34.8±7.89).

In the two-cell and eight-cell groups, R2i-treated embryos had a lower average number of TE cells than the control group, although the difference was not statistically significant. Conversely, the 2PN group treated with R2i showed a higher average number of TE cells than the control group, with this difference approaching statistical significance (P=0.06). These findings suggest a potential influence of R2i treatment on TE cell proliferation, particularly in early-stage embryos, warranting further investigation (Figure 6).

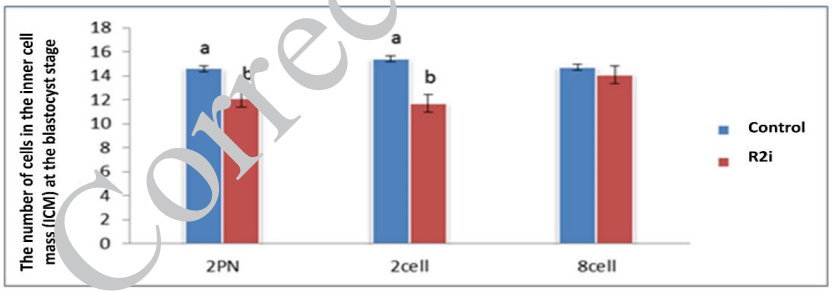

Figure 5. The average number of ICM cells in blastocyst-stage embryos derived from 2PN, two-cell, and eight-cell mouse embryos between the R2i-treated and control groups
The average number of ICM cells was significantly higher in the control group compared to the R2i group. Different letters indicate significant differences (P<0.05). Data are expressed as mean±SE, n=3
R2i: SB431542 and PD0325901, 2PN: 2 pronuclear, ICM: Inner Cell Mass

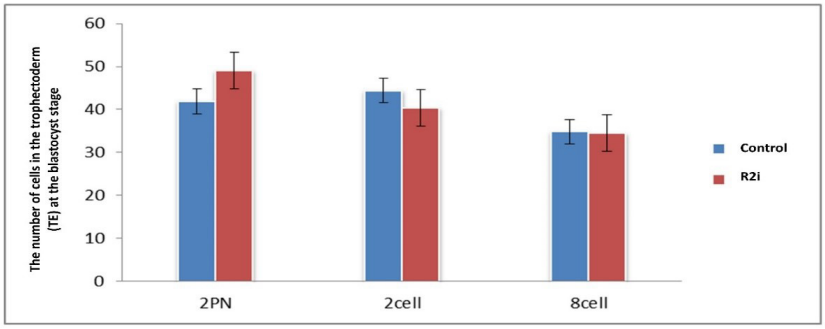

Figure 6. The average number of trophectoderm cells in blastocyst-stage embryos derived from 2PN, two-cell, and eight-cell mouse embryos was not statistically significant between the R2i-treated and control groups
Data are expressed as mean±SE, n=3. *P-value*<0.05 was considered statistically significant
R2i: SB431542 and PD0325901, 2PN: 2 pronuclear, ICM: Inner Cell Mass

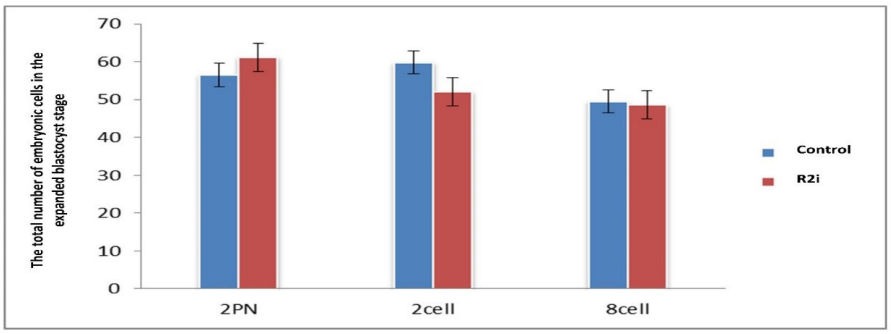

Figure 7. The mean total cell number in expanded blastocyst-stage embryos derived from 2PN, two-cell, and eight-cell mouse embryos did not differ significantly between the R2i-treated and control groups
Data are expressed as mean±SE, n=3. *P-value*<0.05 was considered statistically significant
R2i: SB431542 and PD0325901, 2PN: 2 pronuclear

Analyzing the average total cell count in blastocyst-stage embryos

Analysis of total cell counts in embryos at the expanded blastocyst stage revealed no statistically significant difference between the R2i-treated and control groups. In the R2i-treated group, the average total cell counts were 61±2.11 for 2PN embryos, 52±1.12 for two-cell embryos, and 48.6±8.93 for eight-cell embryos. In the control group, the corresponding averages were 56.5±6.11 for 2PN embryos, 59.8±7.03 for two-cell embryos, and 49.5±8.51 for eight-cell embryos.

While the R2i-treated two-cell and eight-cell groups exhibited slightly lower average cell counts than the controls, and the 2PN group showed a modest reduction, none of these differences were statistically significant. These findings suggest that R2i treatment does not significantly affect the total cell count in embryos at the expanded blastocyst stage (Figure 7).

Comparative analysis of average cell counts in the inner cell mass, trophectoderm, and total blastomeres of blastocysts treated with R2i

This study compared the average cell counts of the ICM, TE, and total blastomeres in blastocysts derived from embryos treated with R2i and their respective controls. The analysis included embryos originating from the 2PN stage and progressing to two-cell and eight-cell stages, then to the blastocyst stage.

The findings demonstrated significant differences in ICM cell counts among embryos treated from the two-cell and eight-cell stages. Trophectoderm cell counts also differed significantly between embryos treated from the 2PN stage and those treated from the eight-cell stage. Furthermore, a significant difference in total blastocyst cell counts was observed between embryos treated from the 2PN and eight-cell stages. These results indicate that the stage of treatment initiation influences the cellular composition of blastocysts, with notable differences observed in both the ICM and TE populations, as well as in total blastomere counts (Figure 8).

Comparison of the ICM index (Ratio of ICM to total cell count)

The analysis of the average ICM to total cell ratios (ICM/Total) in blastocysts revealed the following results for R2i-treated embryos: 0.20±0.02 for 2PN, 0.23±0.05 for two-cell, and 0.29±0.29 for eight-cell stages. In the control group, the corresponding ratios were 0.26±0.04 for 2PN, 0.26±0.04 for two-cell, and 0.30±0.06 for eight-cell stages.

Notably, the average ICM/Total ratio for the R2i-treated 2PN embryos was significantly lower than that of the control group (P=0.002). This result highlights a potential effect of R2i treatment on the proportional development of ICM cells at this early stage (Figure 9).

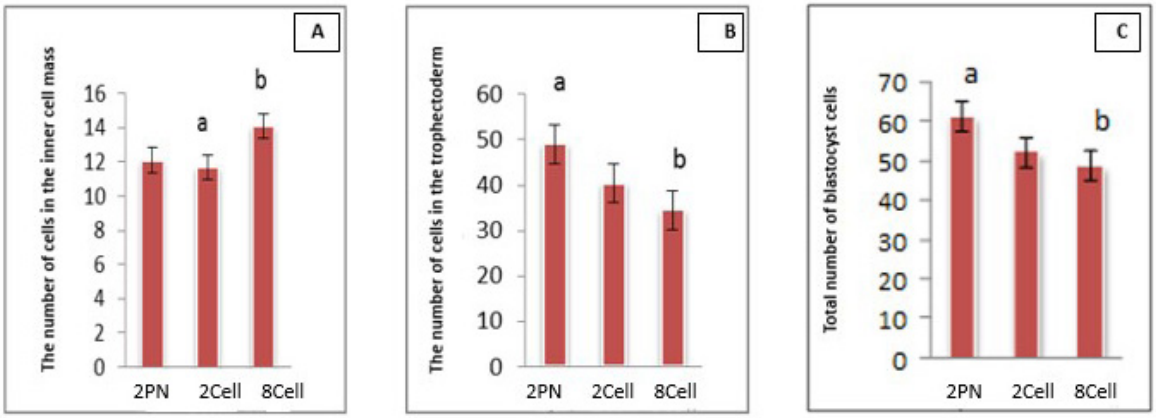

Figure 8. The average counts of ICM cells, TE cells, and total blastomeres in blastocysts derived from 2PN, two-cell, and eight-cell mouse embryos in the R2i-treated groups showed significant differences
Data are expressed as mean±SE, n=3
R2i: SB431542 and PD0325901, 2PN: 2 pronuclear, ICM: Inner Cell Mass

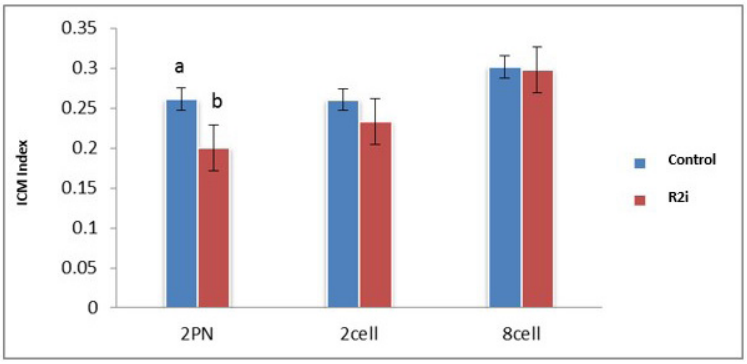

Figure 9. The inner cell mass index (ICM/total cell ratio) of expanded blastocyst-stage embryos derived from 2PN, two-cell, and eight-cell mouse embryos between the R2i-treated and control groups
Data are expressed as mean±SE, n=3
2PN :2 pronuclear; R2i: SB431542 and PD0325901. ICM: Inner Cell Mass

Analyzing programmed cell death (Apoptosis) in blastomere cells of embryos at the blastocyst stage

The analysis of apoptotic blastomeres in blastocysts revealed the following averages for the R2i-treated groups: 1.2±1.13 for 2PN embryos, 0.9±0.73 for two-cell embryos, and 0.5±0.73 for eight-cell embryos. In the control group, the corresponding averages were 1.5±1.43 for 2PN embryos, 1.0±1.05 for two-cell embryos, and 0.9±1.28 for eight-cell embryos.

While the R2i-treated groups exhibited lower average numbers of apoptotic cells across all stages (2PN, two-cell, and eight-cell) compared to the control groups, these differences were not statistically significant. The results indicate that R2i treatment does not significantly affect programmed cell death in blastomeres at the blastocyst stage (Figure 10).

Staining of blastocysts by TUNEL assay

The results of staining blastocysts with Kit Tunnel across different groups are consistent with those shown in Figure 11.

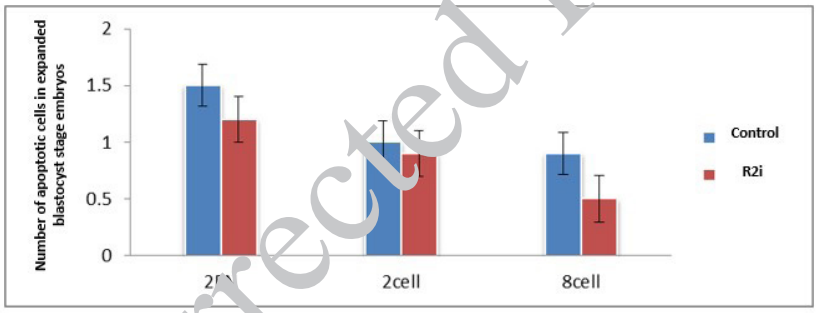

Figure 10. The number of apoptotic cells in expanded blastocyst-stage embryos derived from 2PN, two-cell, and eight-cell mouse embryos did not differ significantly between the R2i-treated and control groups
Data are expressed as mean±SE, n=3. *P*-value<0.05 was considered statistically significant
R2i: SB431542 and PD0325901, 2PN: 2 pronuclear

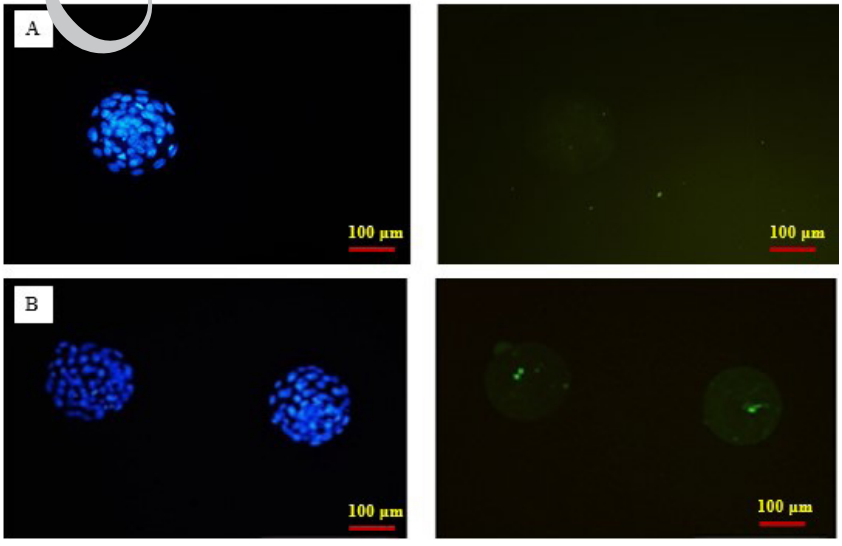

Figure 11. TUNEL staining of mouse blastocysts for detection of apoptotic cells
A: Blastocyst without apoptotic cells, B: Blastocysts with apoptotic cells

Discussion

An essential aspect of assisted reproductive technologies and *in vitro* embryo culture is the identification of non-invasive, cost-effective criteria for selecting embryos with the highest developmental potential. Two commonly used approaches are morphological assessment during early cleavage stages and extended culture to the blastocyst stage. Reaching the blastocyst stage reflects successful progression through key embryonic milestones, including the formation of the inner cell mass and trophectoderm—structures critical for fetal development and placental function. (30). Conversely, generating a large number of embryos at minimal cost remains a key objective in developmental biology. Reaching this objective depends on optimizing *in vitro* culture conditions to support high-quality embryonic development, particularly during the critical transition to the blastocyst stage, when lineage specification and structural organization begin to emerge.

In 2012, Hassani *et al.* developed a combination of small molecules that inhibit the FGF and TGF-β pathways, enabling the successful generation of stem cell lines from mouse strains that had previously been unable to produce them. This innovative approach, termed R2i, achieved a remarkable 100% success rate (31).

In 2014, Hassani *et al.* demonstrated that supplementing embryo culture media with R2i significantly enhanced blastocyst formation. Through systematic testing of various formulations aimed at deriving stem cell lines from single blastomeres of *in vivo* embryos, they found that culturing NMRI mouse embryos at the two-cell stage in media enriched with LIF and R2i—up to the point of zona pellucida hatching—substantially improved both blastocyst development and overall embryonic progression compared to alternative conditions. These findings highlight the potent role of R2i in supporting early-stage embryogenesis and optimizing culture environments for stem cell derivation (26).

Building on its established use for deriving robust stem cell lines, this study shifts focus to *in vitro* embryo culture by examining R2i's impact on preimplantation development. We evaluated whether supplementing preimplantation media with R2i enhances embryo progression and overall quality. These findings could inform optimized culture and transfer protocols, addressing the key question: can R2i improve preimplantation embryo development and thereby advance reproductive biology?

Members of the TGF-β superfamily exhibit dynamic changes in the expression levels of their receptors, ligands, and pathway components during preimplantation development (32). These fluctuations suggest that the TGF-β signaling pathway remains active during this critical developmental stage, although its precise role remains unclear. TGF-β has been reported to accelerate embryonic development to the blastocyst stage and, in some instances, to increase the number of ICM cells in cultured embryos (16, 33, 34).

Similarly, studies on the influence of FGF in mammalian preimplantation embryos have shown that FGF significantly accelerates blastocyst formation and progression (35, 36). While some research suggests that LIF does not directly affect division rates or progression to the morula and blastocyst stages, it has been shown to improve embryo quality (37, 38). Conversely, other studies have reported that LIF may inhibit blastocyst development in both mice (39, 40) and cows (41, 42). However, the most well-established role of LIF appears to be its significant impact on blastocyst hatching and its ability to enhance uterine receptivity for implantation (37).

Although R2i supplementation did not produce a statistically significant increase in blastocyst formation compared to controls, treated embryos showed a non-significant upward trend in blastocyst development rates. This pattern hints at a potential promotive effect of R2i on preimplantation progression. To clarify these preliminary findings, future studies should dissect the contribution of each small-molecule inhibitor, explore their combinatorial synergy, and examine interactions with endogenous paracrine factors. Incorporating larger sample sizes and molecular endpoints will be essential for validating and optimizing R2i-enhanced culture protocols to bolster embryonic competence.

Apoptosis, or programmed cell death, is commonly observed in embryos, including those that appear morphologically normal at the blastocyst stage. This phenomenon primarily occurs in the ICM of blastocysts. Studies on human embryos have reported significant apoptotic indices in morphologically healthy blastocysts, suggesting that apoptosis plays a vital role in regulating cell numbers during natural development (43). Research in other mammals has shown that *in vitro*-cultured blastocysts exhibit more apoptotic cells than those developed *in vivo* (44-47).

Stewart *et al.* found that inhibiting the FGF signaling pathway did not affect the blastomeres' apoptosis (48). No data are available on the effects of TGF-β and its inhibition on apoptosis in preimplantation blastomeres. However, it has been established that LIF exerts an anti-apoptotic effect through one of its three signaling pathways (49).

In this study, we assessed apoptosis in cultured blastocysts and found no statistically significant difference in mean apoptosis rates between R2i-treated and control groups. Nevertheless, R2i-exposed embryos displayed a consistent, non-significant trend toward lower apoptosis. This apparent reduction may stem from the anti-apoptotic actions of LIF included in the R2i.

A direct correlation was observed between prolonged *in vitro* culture and increased apoptosis, consistent with earlier reports. Blastocysts exposed to R2i showed a non-significant trend toward fewer apoptotic cells, implying a more supportive culture environment. However, this apparent benefit may stem primarily from LIF's anti-apoptotic properties rather than the small-molecule inhibitors. Dissecting the individual contributions of each R2i component will require targeted follow-up studies.

This study assessed embryo development to the blastocyst stage and monitored degeneration rates. Although the lower degeneration observed in R2i-treated embryos did not reach statistical significance, treated groups consistently exhibited fewer degenerated embryos than controls. These results suggest that R2i-supplemented culture conditions may support healthier embryo development.

Building on the observed trends in degeneration rates, we examined how culture duration specifically affected R2i-treated embryos. We found that embryos exposed to R2i for shorter intervals were significantly less prone to degeneration than those maintained in culture for longer

periods. This pattern suggests that the R2i medium may more faithfully replicate the dynamic conditions of the *in vivo* environment, thereby bolstering embryo viability when culture times are minimized. Future work should define the optimal exposure window for R2i's protective effects and assess how varying culture durations interact with each component of the medium.

Complementing our analyses of culture timing and degeneration, we next quantified lineage allocation within blastocysts to define R2i's impact on cell composition. R2i-treated embryos showed a significant reduction in ICM cell numbers compared to controls, with the most pronounced decrease in embryos cultured from the 2PN and two-cell stages under extended exposure. Within the treated cohorts, those exposed from the two-cell stage showed greater ICM decline than those exposed from the eight-cell stage, suggesting that shorter R2i exposure windows may mitigate adverse effects on ICM differentiation. Additionally, the observed reduction in TE cells in the eight-cell group relative to other groups further supports the conclusion that a shorter R2i treatment duration exerts a milder effect on cell differentiation and overall cell composition. These findings underscore the critical need to optimize both the timing and length of R2i supplementation to preserve balanced ICM and TE populations during preimplantation development.

A 2014 study on human embryos treated with the TGF-β pathway inhibitor SB431542 reported a significant increase in NANOG-positive blastomeres within the ICM, while no effect was observed on GATA6-positive cells. This study demonstrated that embryos treated with SB431542 showed a substantial increase in fetal ICM cells compared to the control group (50). Similarly, research by Chow *et al.* found that TGF-β accelerates embryonic development to the blastocyst stage and, in some cases, increases the number of ICM cells (16). These findings suggest that inhibiting the TGF-β pathway may inversely reduce ICM cell numbers.

In parallel, multiple studies have demonstrated that fibroblast growth factor (FGF) enhances the number of pluripotent cells within the ICM. Conversely, inhibition of the FGF signaling pathway via MAPK has been shown to exert anti-proliferative effects (35, 36). These findings align with the results obtained in this article.

Reducing the number of ICM cells is often regarded as a negative outcome, as embryos with higher cell division rates and more significant cell numbers are traditionally considered of superior quality in morphological assessments (52). However, a 2014 study demonstrated a direct correlation between increased fetal cell numbers at specific stages and a higher incidence of aneuploidy, challenging the assumption that higher cell counts always indicate better embryo quality (53).

Conversely, research on human embryos has identified numerous apoptotic markers in morphologically normal blastocysts, suggesting that programmed cell death is critical in regulating cell numbers during natural development (43, 54). This finding highlights the importance of maintaining a balanced, controlled number of cells rather than striving for excessive proliferation, as such equilibrium may provide a more favorable environment for preimplantation embryos.

Based on these insights, it is proposed that combining R2i treatment with the deliberate modulation and reduction of ICM cell numbers could have a restorative effect on preimplantation embryos. While this approach may initially appear counterintuitive, it could enhance embryo quality by optimizing the number of essential ICM cells required to form the embryo's body. To validate this hypothesis, further research is required to examine the expression of epiblastic and hypoblastic markers and to evaluate the potential of embryos treated under these conditions to achieve viable, healthy births.

Previous studies have shown that FGF4 is secreted to stimulate the proliferation of TE cells, a critical population during early embryonic development (55, 56). Additionally, high levels of FGF2 have been observed to enhance both the percentage of blastocyst formation and the number of TE cells (38, 57).

Inhibiting FGF signaling via MAPK inhibition will reduce the number of TE cells. This anticipated effect was observed in the two-cell and eight-cell R2i-treated groups, which showed lower TE cell numbers than their respective controls. Notably, these groups were exposed to R2i for shorter durations than the 2PN group. Interestingly, TE cells in the R2i-treated 2PN group exhibited a nearly significant increase (P=0.06) in cell numbers compared to the control group, indicating a distinct response to prolonged R2i exposure.

When comparing R2i-treated groups, a significant difference in trophectoderm (TE) cell counts was observed between embryos originating from the 2PN and eight-cell stages. Embryos cultured from the 2PN stage exhibited a higher number of TE cells, underscoring the influence of treatment duration on TE proliferation. Combined with prior studies, our results indicate that R2i reduces TE cell numbers in embryos developing beyond the eight-cell stage, implicating R2i-sensitive pathways in later-stage modulation of the TE population. Trophectoderm cells are essential for placenta and extraembryonic organ formation and, therefore, for successful implantation and embryonic support. The decrease in TE cells associated with R2i treatment, however, does not provide clear evidence of improved overall embryo quality. These observations are consistent with the expected consequences of inhibiting FGF signaling. Further comprehensive studies are required to determine whether R2i's modulation of TE cells has net positive or negative effects on implantation success and long-term developmental viability. Analysis of total cell counts in developed blastocysts revealed no statistically significant differences between R2i-treated groups and their respective controls. Within the R2i cohort, however, embryos cultured from the 2PN stage contained significantly more total blastocyst cells than those cultured from the eight-cell stage. This increase appears driven by a higher number of trophectoderm (TE) blastomeres, plausibly resulting from prolonged exposure to leukemia inhibitory factor (LIF). LIF is known to promote proliferation and, under extended treatment, may preferentially support TE expansion, accounting for the elevated total cell counts observed in 2PN-derived blastocysts.

The data indicate that LIF increases overall embryonic cell numbers, driving proliferation primarily within the trophectoderm (TE) compartment. Concurrent inhibition of key signaling pathways by R2i shifts lineage allocation, reducing the proportion of cells specified to the inner cell mass (ICM). As culture duration lengthens, this diversion becomes more pronounced, producing progressively fewer

ICM cells while TE cell numbers expand.

This pattern reveals a trade-off between TE proliferation and ICM allocation in extended *in vitro* culture: prolonged exposure to proliferation-promoting factors favors extraembryonic lineage growth at the expense of pluripotent lineage establishment. Optimizing culture regimens will therefore require balancing signals that support necessary TE expansion with those that preserve or restore adequate ICM cell numbers for proper embryonic development.

The ratio of ICM cells to total blastocyst cells is a key indicator of preimplantation embryo quality, typically around 25% for normally developed embryos and up to 40% for *in vivo*-produced embryos (58-60). In this study, the ICM ratio declined among the R2i-treated groups. Embryos cultured from the 2PN stage exhibited ICM ratios of 20% and 23%, falling below the 25% benchmark for optimal quality. In contrast, embryos cultured from the eight-cell stage presented a higher ICM index of 29%.

Despite the apparent enhancement in the ICM ratio observed in the eight-cell group, this metric may not accurately reflect improved embryo quality. The elevated ratio is likely a consequence of reduced trophectoderm cell numbers and overall blastocyst cell count, which artificially inflate the proportion of inner cell mass. These findings underscore the importance of cautious interpretation of the ICM index, particularly under experimental conditions such as R2i treatment that can significantly alter total cell numbers and cellular composition.

Comprehensive functional and long-term studies are required to determine whether R2i-based culture modifications can be translated into clinically meaningful improvements in embryo transfer success and offspring health.

Conclusion

This study revealed that treatment with SB431542 and PD0325901 (R2i) during preimplantation embryo culture led to a significant reduction in the number of inner cell mass (ICM) cells, indicating a potential compromise in pluripotent cell formation. However, no statistically significant differences were observed between the treated and control groups in blastocyst formation, degeneration, or apoptosis rates. These findings suggest that while R2i treatment does not adversely affect overall embryonic progression, it may not enhance embryo quality under the tested conditions. Therefore, the utility of R2i appears limited for promoting healthy embryo development and may be more applicable to specific research settings—such as stem cell derivation—than to broader reproductive applications aimed at improving fetal viability or live birth outcomes.

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Ethical Approval

All experimental procedures involving animals were conducted in accordance with institutional guidelines and approved by the relevant ethics committee.

Availability of Data and Materials

All data analyzed during this study are included in this published article, as well as supporting information.

Consent for Publication

All authors favored the manuscript.

Animal Ethics Approval

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with national and institutional guidelines for the care and use of laboratory animals.

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Authors' Contributions

P EY, S NH, and N N conceptualized the study and designed the experiments. F MGH carried out the experimental procedures. F MGH, F SH, P EY, A D, and M Z wrote the manuscript. S M performed statistical analysis. All authors reviewed and approved the final version of the manuscript before submission.

Conflicts of Interest

The authors declare that they have no competing interests.

Declaration

To prepare this study, the authors used Microsoft Copilot to reduce textual overlap and improve language and grammar. All content was subsequently re-reviewed and thoroughly edited by the authors to ensure accuracy and integrity.

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