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Supplementation of rapamycin during in vitro maturation promotes oocyte quality and subsequent embryo development in bovine

Hafiza Khatun[1,2*], Md Rasadul Islam[2], Muckta Khan[1] and Fowzia Sultana[1]

Abstract

Background The developmental competence of oocyte derived from in vitro maturation (IVM) is significantly lower than those of oocyte matured in vivo. Rapamycin is an immunosuppressive substance and widely used to prolong the survival and preserve the cells culture. The objective of this research was to explore whether rapamycin supplementation during IVM enhances meiotic maturation, oocyte quality, and subsequent embryonic development.

Methods Depending on the purpose, bovine cumulus oocyte complexes were matured without (control) or with rapamycin at different concentrations (0, 1, 10, and 100 nM) for 22 h (h) followed by 6 h in vitro fertilization, and zygotes were cultured for 8 days. Following treatment, oocytes developmental competence was assessed by meiotic progression, intra-oocyte GSH synthesis, reactive oxygen species (ROS) levels, and subsequent embryonic development.

Results The results showed that addition of 1 nM rapamycin to IVM medium significantly increased the nuclear maturation (90.1 ± 1.5 vs. 81.2 ± 2.5; $P < 0.01$) and subsequent embryonic development (47.6 ± 2.2 vs. 41.5 ± 1.9; $P < 0.05$) than that observed in the control group. Consequently, compared to the control group, the relative fluorescence's intensity of ROS levels in oocyte significantly reduced by rapamycin treatment. More importantly, rapamycin supplementation during maturation significantly increased the reduced glutathione synthesis levels in oocyte compared to that observed in control, indicating the bidirectional communication between cumulus cells and oocyte becomes easy via rapamycin treatment.

Conclusions This study suggests that the presence of rapamycin during maturation can afford to enhance reduced glutathione synthesized by the oocyte, which is an internal oocyte antioxidant defense and contributes to improve subsequent high-quality embryo production.

Background

Nevertheless, the poor developmental competence of oocyte matured in vitro is a crucial issue that severely affects the outcome of assisted reproductive technologies (ART) in different species including human. The probable fact of this low efficiency is the in vitro cultural environment, that compromised cytoplasmic and nuclear maturation, which is required for the oocyte to be fertile as well as pre- and post-implantation development

*Correspondence:
Hafiza Khatun
hafizaas@bau.edu.bd
[1] Department of Animal Science, Faculty of Animal Husbandry, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh
[2] Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan

(Nagano 2019; Reader et al. 2017; Rizos et al. 2002). The production of reactive oxygen species (ROS) is an invariable phenomenon during in vitro maturation (IVM) that are generated within the cell as by-products of aerobic respiration and metabolism (Khazaei and Aghaz 2017; Lonergan et al. 2001). Upraising ROS levels beyond the physiological range triggers oxidative stress that negatively affects the developmental kinetics including morphological, ultrastructural, and transcriptional changes of the oocyte (Caetano et al. 2023; Chelenga et al. 2022; Guerin et al. 2001; Reader et al. 2017). Notably, the interaction between the oocyte and its surrounding cumulus cells is highly coordinated with oocyte developmental competence. This bidirectional communication is markedly affected by ROS, resulting decrease in the intra-oocyte GSH synthesis during IVM (Gilchrist and Thompson 2007; Hatirnaz et al. 2018). Reports of in vitro model suggested that surrounding environment of an oocyte/embryo having antioxidants either enzymatic or non-enzymatic or other regulatory molecules, provides protection against both internal and external sources of ROS production, regulates mitochondrial functions, and enhances IVF outcomes (Ren et al. 2023; Silva and Silva 2022; Yang et al. 2022a, b, c; Zhao et al. 2022). Therefore, much research has been endured to establish the use of more efficient antioxidants/molecules for oocyte/embryo production in vitro.

Rapamycin (also known by the trade names of sirolimus or rapamune) is a natural product with potent antifungal and immunosuppressive activities (Wu et al. 2023; Yoo et al. 2023). It inhibits Ser/Thr protein kinase and acts as a foremost regulator of metabolism, cellular proliferation, stress responses, growth, and cell cycle progression (Carosi and Sargeant 2023; Lenzi et al. 2023). Evidence suggested that rapamycin plays a critical role in female reproductive functions including ovarian aging (Garcia et al. 2019), folliculogenesis (Yang et al. 2018), oocytes maturation (Song et al. 2014; Long et al. 2022), and embryonic development (Lee et al. 2021). Recently, Li et al (2020) have explored that rapamycin treatment during IVM accelerated the developmental competence of embryos derived from poor quality oocytes. Therefore, it was hypothesized that rapamycin may let up oxidative and make easy cumulus cell-oocyte gap junction communications and, thereby, contribute to increase reduced glutathione (GSH) synthesized by oocyte and improve oocyte developmental competence. However, the rapamycin functions against oocyte quality during IVM as well as subsequent embryo development are still unknown.

Therefore, the aim of the present study was to explore whether the modification of IVM medium with rapamycin improves the developmental competence of bovine oocyte. First, we investigated the most suitable concentration of rapamycin for modifying IVM medium by examining the rate of oocyte maturation and subsequent embryonic development. Next, to assess the mechanism by which rapamycin promotes oocyte developmental competence, we measured the ROS and intra-oocyte GSH levels in denuded oocyte just after IVM.

Methods

Oocyte collection and IVM

Bovine ovaries were collected from a local abattoir and washed at least three times in sterile saline containing 100 IU/mL penicillin and 100 µg/mL streptomycin (Seika Pharma, Tokyo, Japan). Cumulus oocyte complexes (COCs) were aspirated from follicles (2–6 mm in diameter) using a 19-gauge needle attached to a 10-mL syringe and washed three times with TCM-199 (Thermo Fisher Scientific) containing 5% (v/v) fetal bovine serum (FBS, Thermo Fisher Scientific). A group of 50 COCs were matured in a four-well multidish (Nunc, Roskilde, Denmark) containing 500 µL TCM-199 supplemented with 5% FBS, follicle-stimulating hormone (0.02 IU/mL; Kyoritsu Seiyaku, Tokyo, Japan), and gentamicin (10 µg/mL, Nacalai Tesque, Kyoto, Japan) covered with liquid paraffin (Nacalai Tesque) at 38.5 °C for 22 h in a humidified atmosphere of 5% CO_2 in air.

Assessment of meiotic maturation

After 22 h of IVM, meiotic maturation was assessed based on nuclear stage of oocyte according to the procedures mentioned previously (Khatun et al. 2020). In brief, oocytes were denuded by pipetting in TCM-199 medium containing 0.1% hyaluronidase, washed with Dulbecco's PBS (DPBS, Sigma) containing 0.5 mg/mL polyvinylpyrrolidone (PVP; Nacalai Tesque), and mounted on microscope slides. The samples were fixed for 2–3 days with 25% (v/v) acetic acid in ethanol and stained with 1% acetic orcein (w/v) in 45% (v/v) acetic acid for 60 min. Meiotic stages of the oocytes were evaluated under a microscope (Eclipse Ti, Nikon).

Detection of oxidative stress and GSH contents in oocytes

As an oxidative stress, intracellular ROS production and GSH levels were measured in denuded oocyte just after IVM by using fluorescence probes according to the manufacturer's guidelines. The fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (DCHFDA; Molecular Probes, Eugene, OR, USA) was used to detect intracellular ROS as green fluorescence, and Cell Tracker™ Blue (Life Technologies, Carlsbad, CA, USA) was used to detect GSH level as blue fluorescence. At least 10 oocytes from each treatment group were incubated in 0.05% PVP-PBS containing 10 µM DCHFDA or Cell Tracker™ Blue for 15 min at 38.5 °C in a humidified atmosphere at

5% CO_2 in air, followed by two washes with PVP-PBS. Oocytes were then placed on a glass bottom dish, and green fluorescence emission was detected using a fluorescence microscope (EVOS® FL, Thermo Fisher Scientific). The fluorescence intensity of images was quantified using ImageJ software (version 1.55; National Institutes of Health, Bethesda, MD, USA) by counting the number of pixels after color inversion.

In vitro embryo production

In vitro fertilization (IVF) and embryo culture were carried out according to a previously described procedure (Khatun et al. 2020). Briefly, frozen semen was thawed by immersing the straw in warm water (37 °C) for 20 s. Spermatozoa were washed by centrifugation (800*g* for 10 min) in 90% (v/v) percoll solution (GE Healthcare). After removing the supernatant, the pellet was diluted with IVF100 solution (Research Institute for the Functional Peptides, Yamagata, Japan) and centrifuged at 600*g* for 5 min. The spermatozoa pellet was then diluted with IVF100 to prepare a final sperm-cell concentration of 5.0×10^6 sperm/ml. Following IVM, COCs were washed three times with IVF100, and IVF was performed at 38.5 °C in 5% CO_2 in air under humidified conditions for 6 h (25 oocytes per 100 µl sperm drop covered with liquid paraffin). After IVF, cumulus cells were removed mechanically by pipetting in CR1aa medium containing 5% FBS, and putative zygotes with polar bodies were placed into microdrops (20–25 zygotes per 50 µl drop) of CR1aa medium supplemented with 5% (v/v) FBS. The drops were then covered with liquid paraffin and cultured at 38.5 °C in a humidified atmosphere of 5% O_2 and 5% CO_2 and balanced with N_2 through Day 8 (Day 0 represented the day of insemination).

Statistical analysis

Each trial of experiment run was accompanied by parallel control and same cultural conditions. For each experiment, at least three independent replicates were performed unless specified otherwise. The results are presented as the mean ± standard error (S.E.M.). All data were analyzed using analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test. All percentage data were arcsine transformed prior to statistical analysis. A value of $P<0.05$ and $P<0.01$ was considered to be statistically significant.

Results

Rapamycin improves oocytes meiotic maturation and subsequent embryos development

At first, we examined the effect of different concentration of rapamycin (0, 1, 10, and 100 nM) on nuclear maturation of oocytes (Table 1). The result showed that only IVM with 1 nM rapamycin significantly increased the percentage of oocytes that progressed to Metaphase II (MII) stage as compared with control group (90.1 ± 1.5

Table 1 Effect of rapamycin during IVM on oocyte nuclear maturation in bovine

Rapamycin (nM)	No. of oocyte examined	Nuclear maturation stages (%)			
		GV	GVBD	MI	MII
0 (Control)	205	1.8 ± 0.6	4.5 ± 1.5	13.5 ± 2.2	81.2 ± 2.5[a]
1	205	1.1 ± 0.6	1.2 ± 0.7	7.6 ± 1.2	90.1 ± 1.5[b]
10	205	1.4 ± 0.5	4.8 ± 1.1	13.3 ± 1.9	82.5 ± 0.8[a]
100	203	1.9 ± 0.5	6.5 ± 2.2	12.5 ± 2.9	79.1 ± 3.4[a]

Slaughter house-derived bovine oocytes were matured with different concentrations of rapamycin for 22 h, and the meiotic maturation rate was examined. IVM, in vitro maturation; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, Metaphase I; MII, Metaphase II. Data are mean ± S.E.M. from five replicates. Means with different superscript letters (a,b) within column are significantly different ($P<0.01$)

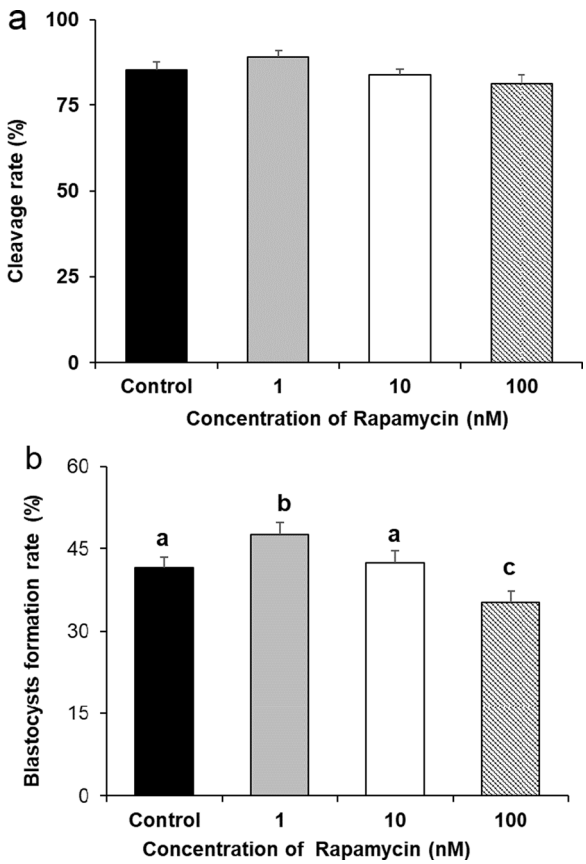

Fig.1 Effect of rapamycin during IVM on subsequent embryonic development. **a** The cleavage and **b** blastocyst developmental rates were examined on Days 2 and 8, respectively. Values are expressed as mean ± SEM from five replicates. **a**–**c** Values with different superscripts among the groups differ significantly ($P<0.05$). IVM, in vitro maturation

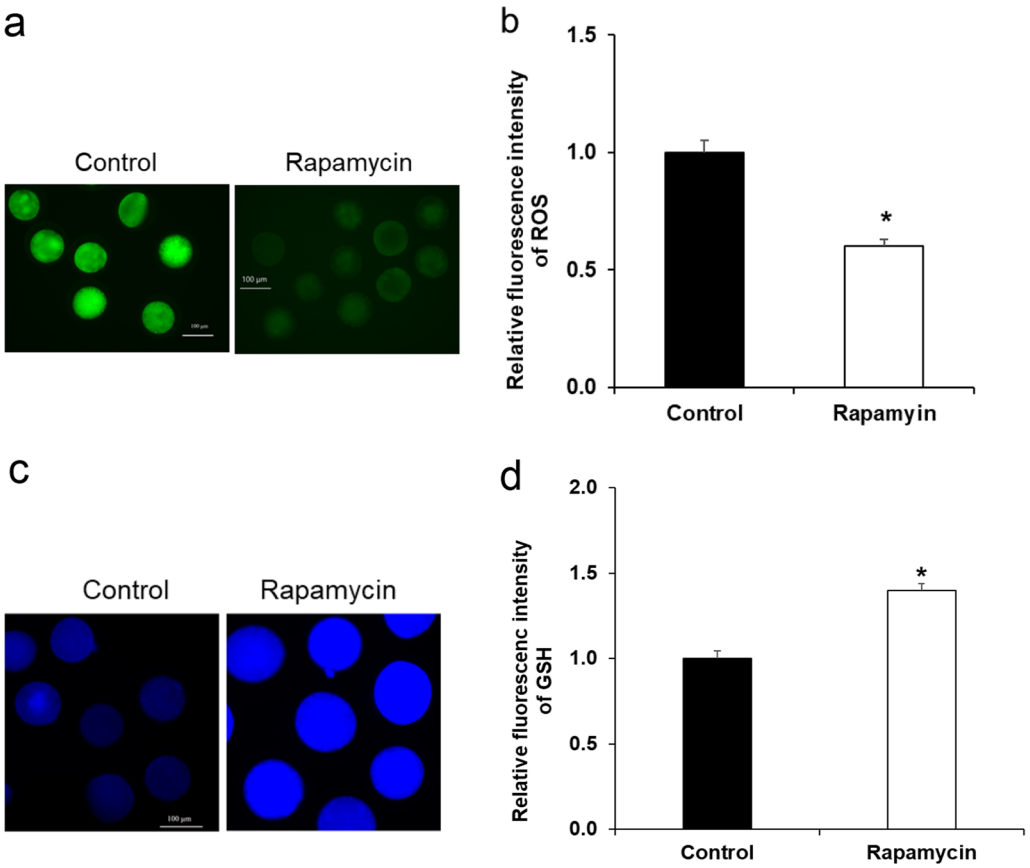

Fig. 2 Effect of rapamycin on ROS and GSH synthesis levels in bovine oocyte. **a** Representative images of ROS fluorescence (green), and **b** quantification of the relative ROS levels in denuded oocytes from the control and rapamycin groups. **c** Representative images of GSH fluorescence (blue) in denuded oocytes. **d** Quantification of the relative GSH levels in denuded oocytes from the control and rapamycin groups. Scale bar: 100 µm. Values are represented as mean ± SEM from five replicates. *$P < 0.05$; IVM, in vitro maturation; ROS, reactive oxygen species; GSH, glutathione contents

vs. 81.2 ± 2.5; $P < 0.01$). In contrast, no significant difference found in the rates of GV, GVBD and Metaphase I (MI) -stage of oocytes among the groups (Table 1). Accordingly, as shown in Fig. 1, IVM with 1 nM rapamycin significantly increased the percentage of blastocysts yield (47.6 ± 2.2 vs. 41.5 ± 1.9; $P < 0.05$) compared to control group. However, the rate of blastocysts formation was decreased by the maximum concentration (100 nM) of rapamycin during IVM ($P < 0.05$; Fig. 1b). However, no significant difference was found in the embryo cleavage rate among the groups (Fig. 1a). Based on these findings, the following experiments were performed using 1 nM rapamycin as a suitable concentration.

Rapamycin reduces oxidative stress during IVM

To explore whether IVM with rapamycin reduces oxidative stress, the intracellular ROS levels in denuded oocyte were analyzed just after IVM. The result showed that the fluorescence intensity level of ROS production is significantly higher in control group compared to the oocyte treated by rapamycin ($P < 0.05$; Fig. 2a, b).

Rapamycin enhances intra-oocyte GSH synthesis during IVM

To identify whether IVM with rapamycin supports to the acquisition of oocyte developmental competence, intra-oocyte GSH synthesis levels were measured after IVM. As shown in Fig. 2, rapamycin treatment significantly enhanced the intra-oocyte GSH synthesis levels compared to that observed in control group ($P < 0.05$; Fig. 2c, d). This finding suggested that upregulation of intra-oocyte GSH (an internal scavenger of ROS) via rapamycin treatment is associated with reducing oxidative stress in rapamycin group and improves oocytes developmental competence.

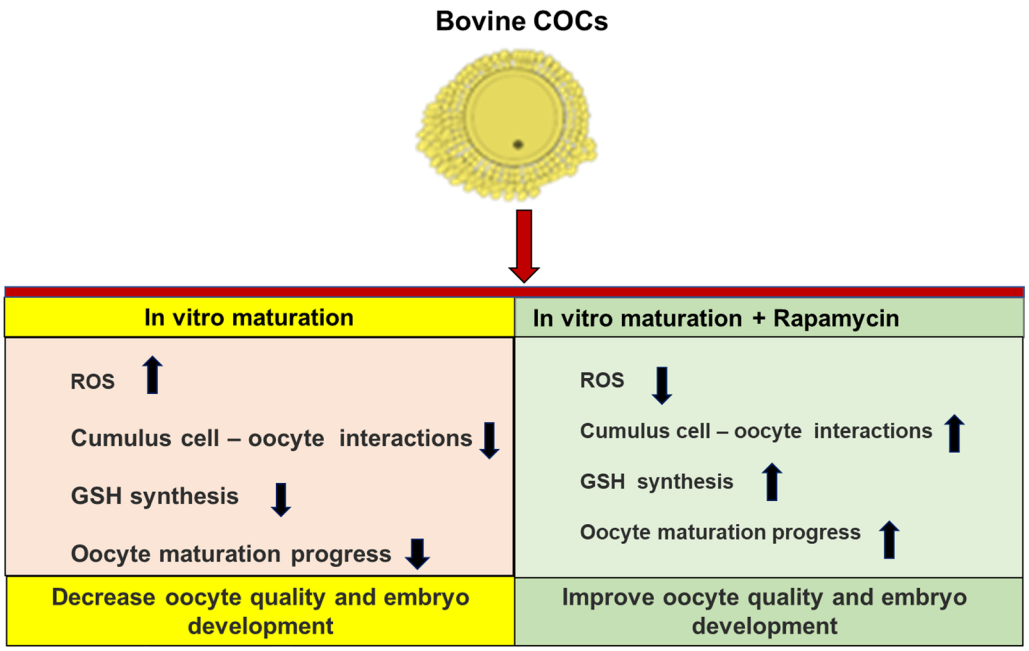

Fig. 3 The graphical representation of the mechanisms by which rapamycin improves oocyte quality and subsequent embryo development. The addition of rapamycin during maturation suppresses ROS production, which facilitates the cumulus cells-oocyte gap-junctional communication. Thereby, metabolites transfer from cumulus cell to oocyte easily, which turn on to increase intra-oocyte GSH synthesis. This high content of intra-oocyte GSH maintains intracellular homeostasis and enhances the oocyte maturation process and developmental competence. Thus, in vitro maturation with rapamycin improves the oocyte quality and subsequent embryonic development in the bovine model

Discussion

The present study explored that supplementation of rapamycin during IVM improved oocyte quality and subsequent embryonic development in bovine. To the best of our knowledge, these findings demonstrate for the first time that adequate concentration of rapamycin (1 nM) with IVM medium significantly suppressed intra-oocyte ROS production and increased intra-oocyte GSH synthesis, thus improving oocyte developmental competence.

For any chemical treatment, it is essential to optimize the timing and concentration of the chemical applied to maximize the output (Kishigami et al. 2006). Hence, we examined different working concentrations of rapamycin to evaluate their efficiency for oocyte nuclear maturation. We found that 1 nM rapamycin significantly increased the nuclear maturation compared to others group (Table 1). In bovine, Kordowitzki et al (2020) have focused that oocyte maturation and telomere length were improved by supplementing rapamycin (1 nM) with IVM medium. However, the authors of this study did not analyze the oocyte quality and subsequent embryo development. Importantly, data of developmental competence of oocytes in the present study showed that highest rate of blastocysts derived from COCs treated with 1 nM rapamycin during IVM compared to others group (Fig. 1). Similar observation also reported by in vitro studies with rapamycin in bovine (Li et al. 2020), porcine (Lee et al. 2015, 2021), mice (Long et al. 2022), and human**.** Indeed, rapamycin influenced maturation rates of oocytes in a concentration-dependent manner and in vitro culture conditions have an effect on its concentration.

Many studies reported that maintaining intracellular homeostasis via a balance between ROS and GSH levels, an internal oocyte antioxidants defense during IVM conquered the oocyte quality and subsequent embryonic development (Agarwal and Majzoub 2017; de Matos and Furnus 2000; Silva and Silva 2023; Zhang et al. 2022). The proper interaction between cumulus cells and oocyte enables the transfer of cumulus cells nutrients and metabolites into oocyte, which are typically important for synthesizing intra-oocyte GSH as well as oocyte meiosis process (Franciosi et al. 2014; Luciano et al. 2011). The synthesis of GSH in oocyte is regulated by the factors of cumulus cells (cysteine and glutamine) via gap junction communication between oocyte and cumulus cell (Mori et al. 2000). Many studies reported that the oxidative stress is a major barrier for cell to cell or oocyte communication during culture (Shaeib et al. 2016; Suzuki et al. 2000), because in vitro culture protocols associated with multiple stress factors including physicochemical (temperature, osmolality, and pH), oxidative (pro-oxidant and anti-oxidant balance), and energetic (production,

utilization and storage), are all of which can lead to ROS accumulation (Hatirnaz et al. 2018; Lonergan et al. 2001; Mauchart et al. 2023; Nagano 2019). Although normal level of ROS is beneficial for physiological processes such as tissue regeneration, hormonal signaling, intracellular redox regulation, an imbalance between the production and elimination of ROS within cells causes oxidative stress which negatively affects the developmental kinetics including morphological, ultrastructural, and transcriptional changes of the oocyte (Clerico et al. 2021; Gutierrez-Castillo et al. 2023; Martin et al. 2022; Mauchart et al. 2023). Consistent with these previous approaches, the observation in the present study suggests that modification of IVM with rapamycin markedly reduced ROS levels in oocyte compared to that observed in control group. Thereby, the upregulation of GSH levels in the present study is linked with the decreased of ROS in oocyte treated by rapamycin. Therefore, we propose that the rapamycin prevents oxidative stress and supports intra-oocyte GSH synthesis, which is essential for oocyte developmental acquisitions, because the content of intra-oocyte GSH during IVM is prerequisite for maintaining oxidative stress as well as subsequent embryo development (Guerin et al. 2001).

Conclusions

The present study represents a window of opportunity to improve bovine oocyte quality following IVM in the presence of rapamycin. Figure 3 forwards a working model for this mechanism derived from the data presented in this study. However, further studies are required to investigate whether IVM with rapamycin system improves post-implantation development and fetal viability of blastocyst.

Abbreviations

ART	Assisted reproductive technology
COCs	Cumulus oocyte complexes
GSH	Reduced glutathione
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
IVC	In vitro culture
IVF	In vitro fertilization
IVM	In vitro maturation
MI	Metaphase I
MII	Metaphase II
ROS	Reactive oxygen species

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Author contributions

HK conceived the study design, performed the experiments, analyzed data, and wrote the manuscript; Md RI analyzed data and revised the manuscript. MK conceived the study design and revised the manuscript. FS conceived the study design and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and material

The data supporting this study's findings are available on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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