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Administration of the mTOR Activator MHY1485 Does Not Impede the Developmental Processes After Fertilization

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Abstract

Mammalian target of rapamycin (mTOR) regulates numerous cellular processes such as survival, apoptosis, and autophagy. The mTOR signaling has been studied in different cells, but its role during the development of embryos is unclear. Here, we inspected the impact of MHY1485, a specific mTOR activator, treatment post-fertilization, on the developmental capacity of bovine pre-implantation embryos. The initial results of microscopic investigations showed high cleavage and day-8 blastocyst development rates following the incubation of presumptive zygotes with MHY1485. Additionally, the administration of mTOR activator did not increase, but rather decreased, the reactive oxygen species (ROS; $p < 0.05$) and apoptotic DNA fragmentation ($p > 0.05$) in the developing embryos. The RT-qPCR did not show any up-regulation of the apoptosis-related gene caspase 3 upon addition of MHY1485. Using immunofluorescence, high levels of PI3K ($p < 0.05$) and low levels of caspase 3 ($p > 0.05$) were observed in MHY1485-treated embryos. To conclude, mTOR activation post-fertilization does not hinder, but could boost, the development of pre-implantation embryos. This paves the way for more in-depth studies to elucidate the role of mTOR signaling throughout the different embryonic stages.

Keywords: mTOR; in vitro culture; cleavage; blastocyst

1. Introduction

Mammalian target of the rapamycin (mTOR) is a negative regulator of autophagy, the process by which the unnecessary proteins are removed. The two complexes of mTOR are complex 1; mTORC1 and complex 2; mTORC2, with different sensitivity to rapamycin, a natural immunosuppressive product that induces autophagy, have been identified (1). The mTORC1 is the rapamycin-sensitive TORC1 complex that commonly termed mTOR (Raptor) regulatory proteins, while mTORC2 is the insensitive rapamycin-companion of mTOR (Rictor). In mammals, the mTORC1 pathway is involved in cell growth, metabolism, proliferation, transcription, and protein synthesis. mTOR is negatively regulated by both tuberous sclerosis complexes 1 (TSC1 and TSC2) while it activated by the protein kinase B; the AKT, through inhibition of TSC2. Once activated, mTORC1 exerts its regulatory function via the direct phosphorylation of p70S6K ribosomal kinase (S6K) and the eukaryotic translation initiation factor 4E-binding protein 1. The dysfunction of mTOR signaling has been observed under different conditions and diseases such as type 2 diabetes, obesity, and tumor progression (1-3).

Although the function of mTOR signaling has been widely studied in various cells and tissues, its actual role in embryonic development is controversial. It has been reported that the mTOR is critical for growth of early embryos and stem cells (4, 5). The inhibition of mTOR by rapamycin, and hence inducing autophagy, negatively affected the fertilization and developmental rates of vitrified

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mouse oocytes (6). In addition, it delayed the development of primordial follicles that was overturned upon the use of PI3K/mTOR stimulators (7-10). It also arrested bovine oocyte meiotic maturation at metaphase I (MI) stage (11), and leads to oocyte loss and destruction within the follicles (12). Disruption of the kinase domain of mTOR in mice decreased the proliferation of homozygous embryonic cells of and consequently led to the death of embryos after implantation (4). However looked normal, their trophoblast and inner cell mass (ICM) of homozygous blastocysts was unable to proliferate when cultured in vitro (4). Previously, treatment of blastocyst with the mTOR inhibitor INK128 prolonged the survival compared to untreated and rapamycin-treated embryos (13). This could be related to the induction of autophagy leading to increase cell survival rate (14). However, mTOR silencing in mice did not affect the follicle development and fertility, but the PI3K/AKT signaling was activated even in the absence of mTOR to compensate the shortage of mTOR and supporting the normal development of ovarian follicles (15). In addition, we previously reported that the PI3K/AKT/mTOR signaling-related markers were highly expressed in embryos developed after oocyte treatment with different antioxidants such as melatonin and vanillic acid (16, 17). This reflected the importance of mTOR for enhancing the quality of oocytes and embryo developmental competence in addition to its critical role in fertility and follicle activation (6-10, 18-21).

On the other hand, the above mentioned findings were contradicted by others that showed that the inhibition of mTOR (mTORC1) signaling rescued the developmental capacity of both oocytes and embryos (22-25). In one study, rapamycin, applied after sperm injection, the period that called the activation period, enhanced the percentage of developed blastocysts (22). Using parthenogenetic activation (PA) and somatic cell nuclear transfer (SCNT), rapamycin treatment to poor quality oocyte at the timing of in vitro maturation (IVM) significantly improved the embryo development (24). In another study, rapamycin treatment during in vitro culture (IVC) after parthenogenetic activation (PA) enhanced the developmental rates (25). This might indicate that the effect of rapamycin on induction of autophagy might favor the embryo development only under stress condition such as using low-quality oocytes (24). Previously, it has been shown that cells under nutrient rich conditions tended to activation of mTORC1 for inhibition of autophagy, while the opposite action was induced under stress or starvation environment (26). Also, it was reported that mTORC1 activity, in MII- stage oocyte, was increased and was observed to decline after fertilization (5, 27).

Since the actual role of mTOR signaling in embryonic development is still controversial, the current study aimed to clarify the effect of mTOR activation, using MHY1485, post-fertilization on the developmental competence of bovine embryos. MHY1485 is a cell-permeable triazine derivative that inhibits autophagy through the activation of mTOR (28) and it was previously reported that ovary treatment with MHY1485 increased ovarian weight and follicle development in mice (29). Day-4 cleavage and day-8 blastocyst development rates, the apoptosis and oxidative stress, the survival- related markers were also inspected following MHY1485 treatment.

2. Materials and Method

2.1. In Vitro Maturation of Oocytes

The experimental work was executed based on GNU guidelines (GNU-230425-A0088). Ovaries from bovine were collected from abattoir and arrived to our lab in thermal bottles, and within 2 h after slaughter. COCs were collected from follicles using needles and vacuum pump. Oocytes with intact cumulus cells were used. Samples were washed in TL-HEPES medium and IVM medium (30) and cultured in 4-well plates in IVM medium at approximate density 50 oocytes per well for 24 h, and at 38.5°C and humidified conditions.

2.2. In Vitro Fertilization (IVF)

After IVM , frozen semen was thawed at 37°C then washed in warm DPBS, centrifuged, and the pellet was incubated with 500 µL heparin (20 µg/mL) prepared in IVF medium as previously mentioned (30). Sperm was diluted in IVF medium then the mixtures were added to oocytes, and incubated for 20 h in humidified conditions.

2.3. In Vitro Culture (IVC) and Blastocyst generation

The cumulus cells were gently removed from oocytes post-fertilization by pipetting while presumptive zygotes were collected and washed three times with SOF-BE1 medium (30), then cultured in the SOF medium in the presence or absence of the mTOR activator MHY1485 (0, 0.1, 1.0, 10µM) and kept at 38.5°C, under 5% CO2. The cleavage of embryos was monitored after4 days following fertilization, while the 8-16 cells-stage embryos were re-suspended in fresh medium and re-incubated at the same condition for another 4 days. Day-8 blastocyst were either kept at −80°C for future use in total RNA extraction or stored at 4°C in 4% paraformaldehyde for use in staining experiments. For experiments that required live cells, blastocysts were immediately used without fixation.

2.4. Detection of Apoptosis and Intracellular Reactive Oxygen Species (ROS)

To investigate the blastomeric apoptotic levels, TUNEL assay was performed as shown before (31). Briefly, blastocysts (n = 10) were permeabilized with triton X-100 for 30 min, then washed in PBS and incubated for 1 h with the TUNEL reagent. Next, samples were incubated with DAPI staining for 10 min, then spotted on glass slides and covered with coverslips. The bright red TUNEL-positive cells, which reflect apoptosis levels in the nuclei, and DAPI-stained nuclei appeared in blue color were analyzed under epifluorescence microscope (Olympus, IX71). The experiment was repeated three times.

The intracellular content of ROS was investigated in day-8 blastocysts (n = 10) using H2DCFDA (2,7-dichlorodihydrofluorescein diacetate). Briefly, blastocysts were incubated with 5 μM H2DCFDA in dark for 15 min, then washed in PVA-PBS and examined under fluorescence microscope. The fluorescence intensities were analyzed using ImageJ software.

2.5. Quantitative Reverse Transcription PCR (RT-qPCR)

Blastocysts (n = 5) were used for RNA extraction. The concentrations of RNA were checked using NanoDrop 2000c spectrophotometer. For RT-qPCR, the cDNA was synthesized using Bio-Rad iScript cDNA synthesis kit.

The qPCR was performed using iQ-SYBR GREEN Supermix (Bio-Rad Laboratories) as following, diluted cDNA were added to primers mixture, and iQ-SYBR GREEN Supermix. Each cDNA sample was applied in duplicate Caspase 3 (Forward: CCC AAG TGT GAC CAC TGA AC; Reverse: CCA TTA GGC CAC ACT CAC TG), expression level was calculated using the ΔΔCt method in relation to GAPDH housekeeping gene (Forward: CCC AGA ATA TCA TCC CTG CT; Reverse: CTG CTT CAC CAC CTT CTT GA).

2.6. Immunofluorescence analysis

Paraformaldehyde-fixed day-8 blastocysts; around 15 per group, were permeabilized for 20 minutes using Triton X-100. Blastocysts were incubated with blocking buffer, then subjected to overnight incubation, at 4°C, with antibodies (anti-caspase 3, anti-PI3K, and anti-phosphorylated AKT (pAKT). Following washing, Alexa Fluor 488- and 568-labelled secondary antibodies (Santa Cruz Biotechnology) were added to embryos followed by incubation for 90 min with shaking. The nuclei were stained for 10 min using 1μg/mL DAPI, then the blastocysts were washed in PVA-PBS, mounted on slides, visualized using confocal microscope and analyzed using ImageJ software.

2.7. Statistical Analyses

Statistical analyses were performed using GraphPad Prism software. The differences between the treated and the control were analyzed using unpaired student t-test and Wilcoxon's matched-pairs signed-ranks test whereas the multiple comparison was used for experiments encompassing more than two groups. Data are shown as SEM, and the statistical significance is considered when p-value is less than 0.05.

3. Results

3.1. Effect of MHY1485 on embryonic development

Initial experiments were performed to test the effect of mTOR activator MHY1485 on embryo development and quality of produced embryos when applied after in vitro fertilization (IVF). For doing this, presumptive zygotes were maintained in 10 fold serial dilutions of MHY1485 (0, 0.1, 1.0, 10 μM) during the first phase of in vitro culture (IVC1; the first 72 hours post-fertilization), then left for blastocyst development in untreated culture medium (IVC2). As seen in figure 1A, the day-4 cleavage rate was increased in the groups treated with 1.0 μM of MHY1485 compared to the untreated control (72.80 ± 0.97%, and 83.20 ± 1.91% for the control and the 1 μM treatment respectively; $p < 0.05$). Similarly, day-8 blastocyst rate was significantly enhanced in 1.0 μM-treated group compared to the untreated control (31.17 ± 2.40%, and 39.00 ± 2.22% for the control and the 1.0 μM treatment respectively; Figure 1B). Oppositely, presumptive zygotes exposed to high concentration of MHY1485 (10.0 μM) exhibited lower rates of day-4 cleavage and blastocyst development (Figure 1A and IB) but only the day-4 cleavage rate decline reached the significant difference ($p < 0.05$).

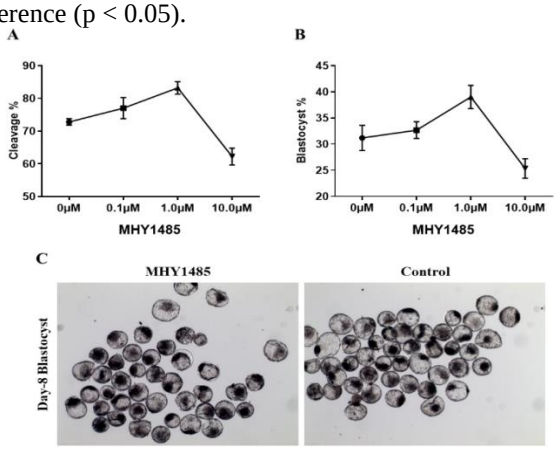


Figure 1. The effect of MHY1485 supplementation on the cleavage, blastocyst development and quality. The percentage of total cleavage (A) and Blastocyst (B) recorded on day 4 and day 8 post-fertilization, respectively **(C)** Morphology of day-8 developed embryos at day-8 post fertilization in the mTOR activator and control groups.

3.2. MHY1485 decreases the DNA fragmentation in developed embryos

Moreover, the effect of MHY1485 on the quality of blastocysts was also investigated using the TUNEL assay, the DNA fragmentation detector. As seen in figure 2, moderate decrease in TUNEL-positive cells was observed in embryos developed under MHY1485 treatment compared to the untreated control (4.5 ± 0.64 for treatment versus 6.00 ± 0.89 for control), but this decrease did not reach the statistical difference ($p > 0.05$).

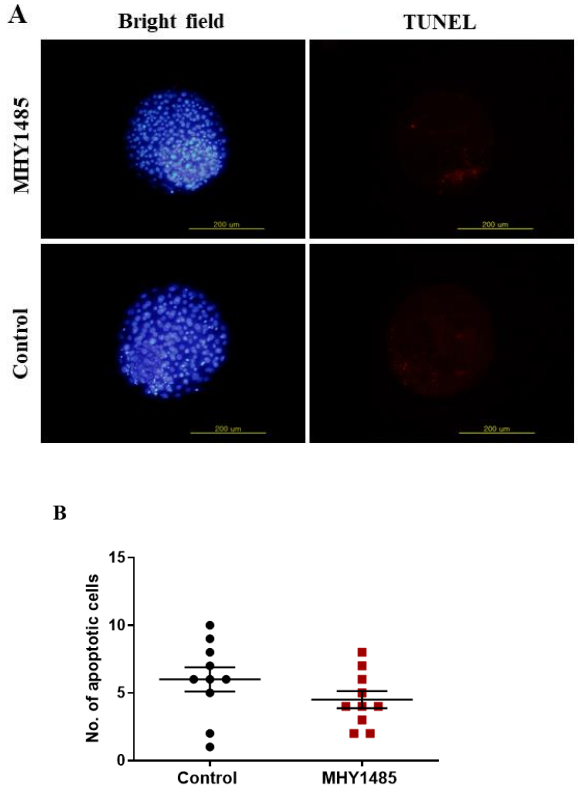


Figure 2. The impact of MHY1485 supplementation on the DNA fragmentation in day-8 blastocyst. (A) Microscopic determination of TUNEL staining in embryos (B) Total number of apoptotic cells tested using TUNEL assay. Scale bar, 200 µm.

3.3. MHY1485 partially alleviates embryonic apoptosis and survival rates in developed embryos

We moved forward to explore the functional role of the mTOR activator MHY1485 on apoptosis in the in vitro developed embryos. For doing this, the intracellular levels of reactive oxygen species (ROS) were investigated using H2DCFDA staining. As shown in figure 3A and B, the fluorescence intensity corresponding to the ROS level was lower in MHY1485 group ($p < 0.05$).

Besides, we used the RNA of blastocysts and day-8 fixed blastocysts for investigating the level of the apoptotic marker caspase-3 following MHY1485 treatment at both transcriptional and translational levels using RT-qPCR and immunofluorescence respectively. As shown in Figure 4, the mRNA and protein expression levels of caspase-3 displayed a slight decrease under MHY1485 treatment but this effect was not statistically significant.

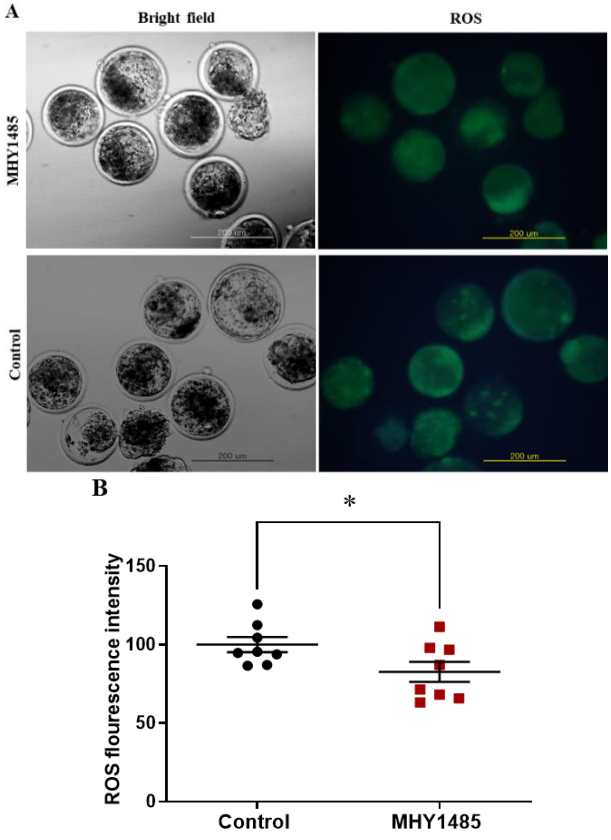


Figure 3. ROS levels in day 8 blastocysts. (A) ROS microscopic determination using H2DCFDA staining of bovine day 8 blastocysts derived from MHY1485 and control culture treatment. Scale bar, 200 µm. (B) Mean values of the integrated optical density of ROS staining as analyzed by ImageJ software.

Next, the levels of the survival proteins PI3K and pAKT were checked, these markers that are involved in the protection of cells from apoptosis and promoting cellular survival under oxidative stress condition, demonstrated the significant increase in PI3K ($p < 0.05$), while a slight increase was detected in pAKT, this increase did not reach to the significant difference (Figure 5A-D).

4. Discussion

Developmental defects can occur during the in vitro production (IVP) of embryos of domestic animals, and improving the culturing conditions, is crucial for production of high-quality embryos in assisted reproductive technology. Different studies have elucidated the developmental events and the

underlying mechanisms for the improvement of embryo development. Mammalian target of rapamycin (mTOR) is a nutrient sensor and growth promoter in mammalian cells. The mTOR signaling has been shown to regulate different processes in different cells.

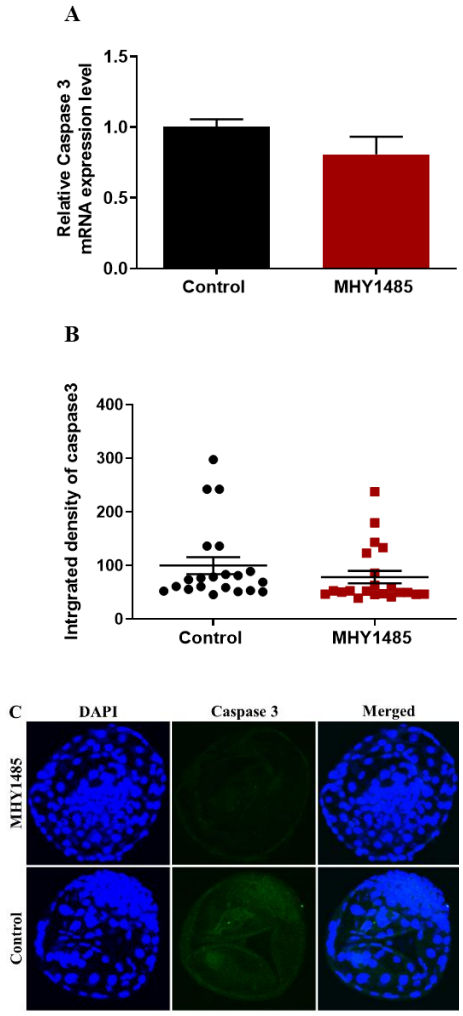
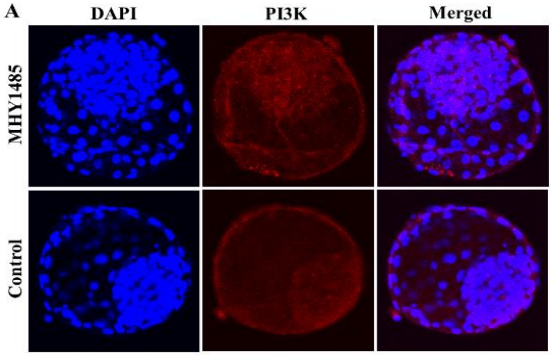


Figure 4. The impact of MHY1485 supplementation on apoptosis. **(A)** Relative expression of mRNA of caspase-3 apoptosis gene. **(B)** Fluorescence intensity of Caspase 3 in embryos. **(C)** Caspase 3 staining of day 8 blastocysts in the mTOR activator and control groups.



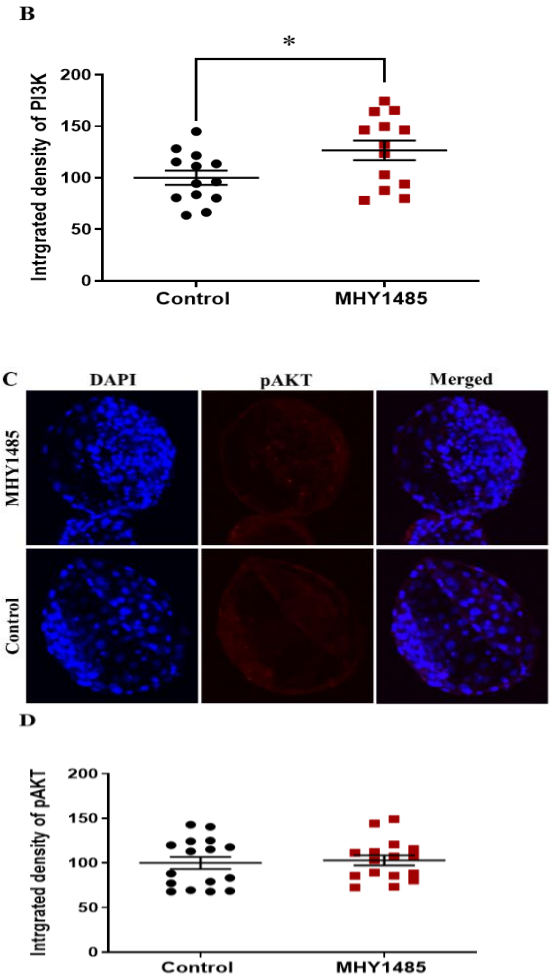


Figure 5. Effect of MHY1485 supplementation on the survival rate in blastocyst. **(A)** PI3K staining in embryos in MHY1485 and control groups. **(B)** Integrated optical density of the PI3K antibody staining in embryos. **(C)** pAKT staining of day 8 blastocysts in the mTOR activator and control groups. **(D)**Integrated optical density of the pAKT antibody staining in blastocyst.

In the context of early embryonic development, mice ovaries incubated with MHY1485 showed increase in weight and inducing the development of follicles when cultured in vitro as well as transplantation in adult ovariectomized animals (29). Although this might reflect the ability of the mTOR activation in promoting follicle growth, the exact impact of mTOR activation following the process of fertilization is unclear.

Here, we sought to investigate the impact of mTOR activation post-fertilization on the development of embryos. For doing this, the specific activator MHY1485 was administered at different micromolar concentrations whereas the cleavage and blastocyst development rates were initially monitored. Interestingly, embryos exposed to 1.0 µM of MHY1485 for 72 h post-fertilization boosted both

cleavage and blastocyst development levels compared to untreated embryos. To investigate the quality of the in vitro produced embryos, the DNA fragmentation in MHY1485-treated embryos was checked, using the TUNEL apoptotic assay. A lower incident of DNA fragmentation in the MHY1485-generated blastocyst was detected as compared to control. We and others previously showed that embryos with low TUNEL staining were characterized by an increase in the total number of cells in blastocyst; the indicator for embryo quality (16, 32, 33). This is in line with previously shown data on the importance of the mTOR activator MHY1485 and the AKT activators (PTEN inhibitor and PI3K stimulator) in promoting the ovarian follicle development and oocyte maturation in mice (29). Similarly, another study also reported the use of MHY1485 for the promotion of follicle growth suggesting it as a therapeutic agent for premature ovarian insufficiency syndrome (34).

Previously, it has been reported that ROS is a commonly linked to the impairment of embryo development. The successive release of ROS generally promotes mitochondrial membrane permeabilization and collapsing condition in mitochondrial membrane potential (MMP) as well as induction of apoptosis through the release of certain pro-apoptotic factors including caspases (35-37). The activation and the cleavage of caspase 3 is an indicator for the final stage of apoptosis (38). In the present study, mTOR activation using MHY1485 was associated with attenuation of ROS in day-8 embryos. Similar results were previously shown in oocytes and embryos displaying high ROS levels and low developmental competence, confirming our above mentioned results (17, 33). In addition, the apoptosis marker caspase-3 tested at both transcriptional and protein levels displayed a slight decrease under the pressure of MHY1485, albeit did not reach the significant difference as compared to control. The results that are in line with previous studies on the positive role of mTOR activation in reducing apoptosis in different cell types, while the activity decreased in response to stress-inducing disease condition (39, 40).

Moving forward, we showed a down-regulation in the protein level of PI3K, the up-stream effector in the mTOR pathway, under the condition of mTOR activation. The PI3K and the downstream effectors AKT and mTOR play critical roles in the control of different cellular processesincluding apoptosis, differentiation, development, and survival, the effects that were detected in oocytes, embryos, ovarian follicles, and stem cells (14, 16, 17, 29, 33, 41-43). Previously, we have found a clear up-regulation in the protein and mRNA of the genes involved in the PI3K/AKT/mTOR pathway, as well as lower incidence of ROS and oxidative stress markers in day-8 blastocyst generated under the treatment of antioxidants such as nicotinamide and melatonin (16, 43). These are in line with our current data on the increase in the expression of PI3K and the attenuation of ROS and apoptosis in embryos under mTOR activator treatment.

5. Conclusions

In conclusion, the current study reports that mTOR activation post-fertilization using the specific activator MHY1485 can decrease apoptosis and improve the developmental potential and survival of bovine pre-implantation embryos which paves the way for more in-depth studies to investigate the impact of mTOR signaling throughout the various stages of embryo development. In addition to the possible application in assisted reproductive technologies for the purpose of enhancing women fertility.

6. Conflicts of interest

There are no conflicts to declare.

7. Acknowledgments

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