Research Article

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The effect of rapamycin treatment on mouse ovarian follicle development in dehydroepiandrosterone-induced polycystic ovary syndrome mouse model

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Abstract

Polycystic ovary syndrome (PCOS) is a complex reproductive and endocrine disorder affecting 5–10% of women of reproductive age, but the pathophysiology of PCOS still remains unknown. Here, the aim of our study was to analyze the effects of rapamycin treatment that may regulate impaired hormonal levels and folliculogenesis in dehydroepiandrosterone (DHEA)-treated PCOS mouse. We hypothesized that rapamycin may ameliorate the negative effects of PCOS in DHEA-induced PCOS mouse model. The target of rapamycin (TOR) gene product is a serine/threonine kinase that has been implicated in the control of cell growth, proliferation and autophagy, and rapamycin is a potent inhibitor of mTORC1 pathway. In this study, for the first time, mTORC1 and activation products are presented at protein and mRNA levels after rapamycin treatment in DHEA-induced PCOS mouse ovary. We showed that rapamycin treatment may regulate follicular development, hormonal levels and provide ovulation in DHEA-induced PCOS mouse. Additionally, we assessed decreased primordial follicle reserve, increased number of primary and secondary follicles, corpus luteum structure forms again after 10 days of rapamycin treatment. This study presented here suggests rapamycin treatment regulates hormonal phenotype and folliculogenesis in the ovary and also mTOR signalling pathway in granulosa cells of DHEA-induced PCOS mouse ovary which may have potential to attenuate understanding the mechanism of dominant follicle selection and anovulatory infertility.

Introduction

Polycystic ovary syndrome (PCOS) was first defined in 1935 by Stein and Leventhal as amenorrhoea, obesity and hirsutism triangle (Andrews, 1952). Even though PCOS prevalence changes based on different diagnostic criteria which affects 5–10% women of reproductive age, it is the most common reproductive endocrinopathy for women (Asuncion *et al.*, 2000). PCOS is a complex syndrome with endocrine, reproductive and metabolic characteristics (Dumesic *et al.*, 2015). The most common endocrine feature in women with PCOS is hyperandrogenism. Several studies have shown that androgen excess plays a key role in POCS pathogenesis (Abbott and Dumesic, 2019, Cox *et al.*, 2020).

In the morphological evaluation of PCOS, there is cortical thickening in the ovary, there are many small capsular follicular cysts (FCs), and the inner theca cells of these cysts are luteinized. Hyperplasia of stromal cells is located between these FCs in the ovary. In addition, presence of immature multiple follicles is taken to indicate cessation of folliculogenesis (Ghafurniyan *et al.*, 2015). PCOS presents intrinsic abnormality of ovarian theca cell steroidogenesis (Gilling-Smith *et al.*, 1997). Theca cells in PCOS women are more sensitive to LH stimulation than healthy women (Rosenfield and Ehrmann, 2016). Therefore, they produce excess androgen in PCOS ovary. In the PCOS mouse model, it is possible to see several salient features such as hyperandrogenism, insulin resistance, abnormal development of follicles in the ovary, and anovulation. In addition, in previous studies, infertility, increased number of atretic follicles in the ovaries and FC formation were observed in mice induced with dehidroepiandrostenedion (DHEA) (Solano *et al.*, 2011, Yaba and Demir, 2012, Li *et al.*, 2016).

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase which has very crucial roles in cells. mTOR has the ability to form two different complexes with different roles in cell metabolism. These two complexes are called TORC1 (mTOR, Raptor and GβL (LST8) (Jacinto *et al.*, 2004, Kim and Chen, 2004) and TORC2 (mTOR, Rictor, mammalian stress-activated

protein kinase interacting protein 1 (mSIN1) and GβL (LST8) (Sarbassov *et al.*, 2004, Wullschleger *et al.*, 2005, Frias *et al.*, 2006, Sarbassov *et al.*, 2006). Phosphorylation of serine 2448 (S2448) participates in TORC1 complex and phosphorylation of S2481 participates in TORC2 complex. The active form of TORC1 is sensitive to rapamycin treatment (Copp *et al.*, 2009). Phosphorylation of S2448 participates in TORC1 complex and increases proliferation of granulosa cells by follicle stimulating hormone (FSH)-mediated pathway and phosphorylation p70S6K kinase increases two-fold in granulosa cells that are treated by FSH (Kayampilly and Menon, 2007). It has been showed that rapamycin treatment decreases LH levels in female rats and control the onset of puberty (Roa *et al.*, 2009). We presented before cytoplasmic mTOR localization in granulosa cells and a S2448 phosphorylated form of mTOR (P-mTOR) expressed at the contractile ring near mitotic spindles during cytokinesis (Yaba *et al.*, 2008). We also found that when mTOR activity is inhibited by rapamycin *in vitro*, the proliferation of granulosa cells and follicle growth decrease depending on the dose. Thus, we suggested that stress and nourishment-dependent defects can directly affect follicle development in a mouse ovary (Yaba *et al.*, 2008). The development of a healthy follicle depends on proliferation of granulosa cells and the secretion of ovarian steroid hormones. When mTOR signal transduction pathway is inhibited by rapamycin in mouse granulosa cells, granulosa cell proliferation and consequently follicle size decreases (Yu *et al.*, 2011). We also showed mTOR signal proteins in DHEA-induced PCOS mouse model and we suggested that mTOR signalling may be important to understand the pathogenesis of PCOS in a mouse model (Yaba and Demir, 2012). It is well known that high levels of LH in PCOS ovary contribute to the high levels of androgens, and this along with low levels of FSH contributes to poor oocyte maturation, follicle development and an inability for ovulation. Therefore, in this study, we hypothesized that inhibition of mTOR expression with rapamycin treatment could have regulative potential for reproductive hormones and folliculogenesis in PCOS mouse model. We aimed to show ovulation through regulating hormone levels and mTORC1 expression in PCOS mouse ovary after 10-days and 20-days rapamycin treatment. We proposed that rapamycin treatment may ameliorate dominant follicle selection and anovulatory infertility in PCOS by regulating folliculogenesis and hormonal phenotypes depending on time and dose in PCOS mouse ovary.

Material and methods

Animal model and groups

All animals used in this study were obtained from Yeditepe University Faculty of Medicine Experimental Research Center (YUDETAM) and all the experimental procedures have been approved by Yeditepe University Ethical Committee with the relevant permit numbers 01-09-2014, 411. All procedures carried out in this study were conducted according to the Yeditepe University Experimental Animals Ethics Directive which has been prepared according to the rules and principles in Universal Declaration of Animal Rights, European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (European Treaty Series-No.123), National Research Council of USA, United Nations' Convention on International Trade in Endangered Species of Wild Fauna and Flora and Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention). This manuscript complies

with the ARRIVE guidelines (Percie du Sert *et al.*, 2020). All procedures were carried out under the supervision of veterinarians. Female prepuberal (25 days old) Balb/C mice were fed with normal food and water. Food and water were available ad libitum. The temperature was maintained at 21–23°C.

The hyperandrogenized environment of PCOS was induced by DHEA administration (Luchetti *et al.*, 2004, Elia *et al.*, 2006, Sander *et al.*, 2006). Subsequently, time-dependent rapamycin injection was performed into female Balb/C mice with PCOS in order to determine the therapeutic role of rapamycin in DHEA-induced PCOS pathophysiology. The experimental design is illustrated in Figure 1.

The PCOS mouse model obtained was the same method we used before (Yaba and Demir, 2012). Briefly, female prepuberal (25 days old) Balb/C strain mice were daily injected (sc) with DHEA (Sigma Aldrich) (6 mg/100 g body weight, dissolved in 0.01 ml dimethylsulfoxide (DMSO) (Santa Cruz Biotechnology)) and mixed with 0.09 ml sesame oil (Sigma Aldrich) for 20 consecutive days (DHEA-induced PCOS group (P)) (Aragno *et al.*, 1997, Aragno *et al.*, 2002, Yaba and Demir, 2012). Then, rapamycin (LC Laboratories) (5 mg/kg body weight, dissolved in DMSO and mixed with sesame oil (1:40)) treatment was performed with female Balb/C mice with PCOS for 10 (R10) and 20 (R20) days as two different groups. The control groups consisted of 10 untreated female mice 45, 55 and 65 days old (Control group (C)) and 4 untreated 25 days old female mice (Postnatal 25 days group (PN25)). The vehicle groups included 10 mice injected with 0.01 ml DMSO and mixed with 0.09 ml sesame oil daily for 20 consecutive days (PCOS vehicle (PV)) and also rapamycin vehicle groups (RV) were daily injected with DMSO and sesame oil (1:40) for 10 (RV10) and 20 days (RV20). At the end of the experimental procedure, blood sample was taken with cardiac puncture after anaesthesia with ketamine. Afterwards, cervical dislocation was applied before dissection. Right ovaries were removed and fixed with formalin solution for 6 hours for morphological evaluation, the left ovaries were stored at −80 °C for WB and qRT-PCR.

Blood sampling for 17β-oestradiol, progesterone and luteinizing hormone

Whole blood was obtained from animals by cardiac puncture. Blood samples were centrifuged at 4500 rpm for 15 min at 4°C; serum was stored at −80°C until assayed. The levels of 17β-Estradiol (Abcam), Progesterone (Sigma, St. Louis, MO) and Luteinizing Hormone (Biomatik) were determined for each serum sample by applying the ELISA technique as described by the manufacture of the ELISA kit. Synergy™ HT Multimod Microplate Spectrophotometer was used at 450 nm wavelengths (PN25, n = 5), (C, n = 10), (P, n = 10), (PV, n = 5), (R10, n = 10), (R20, n = 10).

Morphological evaluation of ovary

Ovarian tissue samples from each group (n = 10) were fixed in 10% formaldehyde for 6 hours. Afterwards, samples were dehydrated in each alcohol series for 24 hours (70%, 80%, 90% and 100%), cleared in xylene and embedded in paraffin. Serial tissue sections were taken from 5 μm and stained with Hematoxylin and Eosin (H&E) staining and examined under light microscope (Nikon Eclipse i5, Tokyo, Japan).

Morphological classification of follicles and follicle counting

The follicular organization in the mouse ovary begins after birth and the classification of follicles is based on the number of granulosa cell
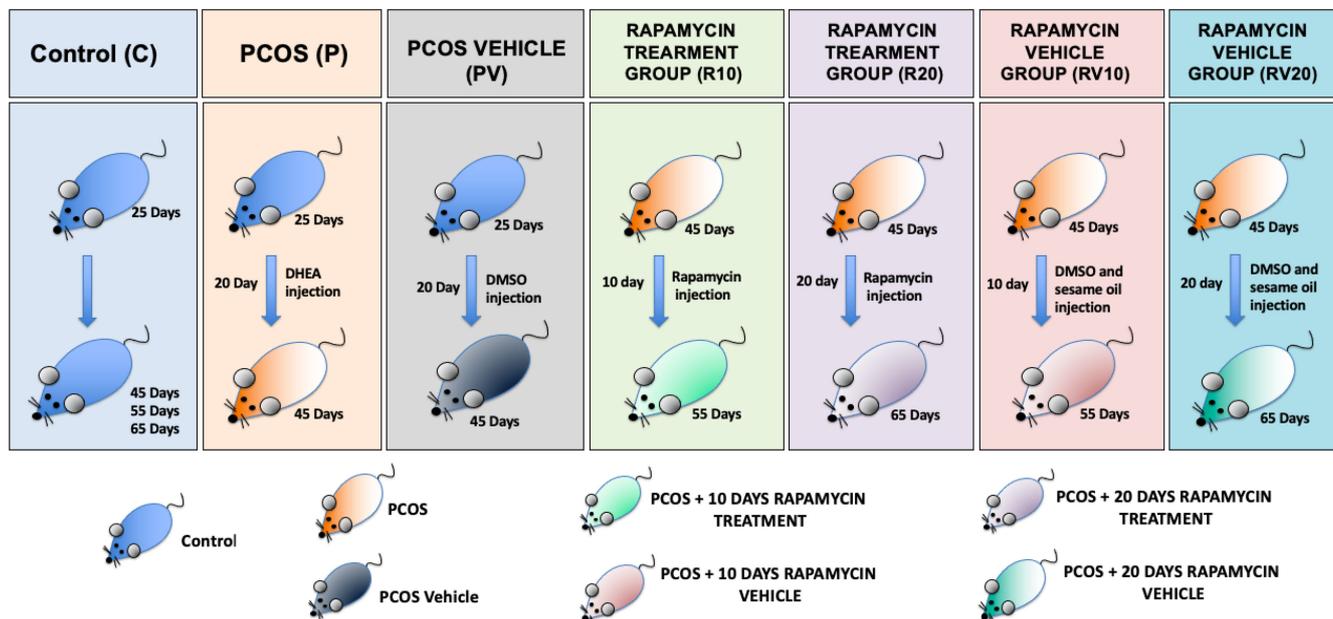
Figure 1. Schematic representation of the experimental procedure. In this figure, the experimental procedure is given schematically. Balb/C mice were included in the experiment when they were 25 days old. DHEA (Dehydroepiandostenedione) was given to 25-day-old mice for 20 days to form a PCOS model group (PCOS). As the Vehicle group, the solvent dissolved in DHEA (DMSO, dimethylsulfoxide) for 25 days was injected into the PCOS Vehicle (PV) group for 25 days. PCOS treatment groups, rapamycin (R-10 and R-20) injections were applied to the groups in which we created a DHEA-induced PCOS model and then treated for 10- and 20-days with 5 mg/kg rapamycin administration. As a rapamycin Vehicle (RV) group, the PCOS model, in which rapamycin was dissolved, was injected for 10- and 20-days (RV10 and RV20) in order to control 10- and 20-days. As the control group, 25-day-old Balb/C mice without any treatment were used (C: 25-day control group).

layers surrounding the oocyte. Primordial follicles are defined as surrounded by single-layer, flat granulosa cells surrounding the oocyte. Primary follicles are defined by oocytes surrounded by single-layered cuboidal granulosa cells. If there are two or more ordered granulosa cells around the oocyte, this follicle is called the secondary follicle and antrum space is not seen in this stage. In the early antral follicle, there are usually 1 or more antral cavity; in the antral follicle, there is a single large antral cavity. In the Graafian follicle, the oocyte is surrounded by cumulus cells and is place eccentrically in the follicle (Yaba *et al.*, 2015). In order to calculate the number of follicles accurately and not to count the same follicle again, only follicles whose nuclei were visible were counted from the follicles in the sections after staining. The resulting number was divided by the total number of sections. Thus, the number of follicles in the ovaries in all groups was determined (The number of ovaries taken from all groups for follicle counting; n = 10).

TUNEL assay

Parafin-embedded ovarian tissues were serially sectioned (5 µm) and mounted on glass slides. TUNEL assay was performed using *In Situ* cell detection kit (Roche Diagnostics Corp., Indianapolis, IN) as per the manufacturer's instructions and analyzed by Axio Zoom V16 (Zeiss) fluorescence microscope (The number of ovaries taken from all groups for TUNEL assay; n = 5).

Total RNA isolation and reverse transcription

Total RNA was obtained from control and PCOS ovary tissues by using TRIzol (GIBCO/BRL) according to the manufacturer's instructions. RNA samples were quantified spectrophotometrically. Reverse transcription reactions with oligo d(T) primers and the Omniscript kit (Qiagen) were performed with 2 µg of total

RNA from each tissue according to the manufacturer's instructions.

Quantitative real-time PCR

Gene expression studies by quantitative real-time PCR (qRT-PCR) were carried out on an iCycler (Bio-Rad, CA, USA). Tissue cDNA was assayed in duplicate, and each experiment was repeated three times. qRT-PCR was performed as described previously (Yaba *et al.*, 2015). Briefly, qRT-PCR was assessed in a 25-µl final reaction that contained 12.5 µl SYBR Green supermix (Thermo Scientific), 0.4 mM of each primer, and 1 µl of template. qRT-PCR was carried out using the following parameters: one cycle of 94 °C for 5 min (denaturation), followed by 35 cycles (amplification) at 94 °C for 30 s, 59 °C for 1 min, and 72 °C for 45 s. Melt curve analyses were run with each series to confirm the specificity of the amplified products. In order to confirm the amplification specific for mRNA, no reverse transcriptase controls were also performed for each sample. The standard curves were prepared for *mTOR, Raptor, Rictor, LST8 (GβL), p70S6K, PKCalpha,* and *Beta-actin*. All expression data were normalized by dividing the amount of target gene by the amount of *Beta-actin* used as control. All primers are shown in Table 1. (The number of ovaries taken from all groups for qRT-PCR; n = 5).

Western blot

Total protein from the ovary tissues (n = 10) from each group were extracted using 100 µl Radioimmunoprecipitation assay buffer (RIPA buffer) lysis solution (Santa Cruz; sc-24948) and 10 mg/ml Protease Inhibitor Cocktail was added into each sample and homogenized for lysis. Protein concentration was measured by Qubit Protein Assay Kit (ThermoFisher Scientific; Q33211). Immunoblotting was performed as described before (Yaba and Demir, 2012). Briefly, 2,5 µl of Loading

Table 1. List of primers

	Size	Forward	Reverse
mTOR	150 bp	TTGGAGTGGCTGGGTGCTGA	AAGGGCTGAACTTGCTGGAA
Raptor	148 bp	GCCATCACAGATACCATCGC	CTGCTTACTGGGGTGCAGTT
Rictor	110 bp	GAGAACGTCCCGCTCGATCT	TGGCCCAGCTTTCTCATATT
GβL	138 bp	GACTAAGGCAGAGTGCAGAG	AAAAGCGCACCGTGTGGTCA
p70S6K	300 bp	CTTGGCGAATTAAGGGCTGC	GCATAGGCCAGTTCTACAAT
PKCalpha	300 bp	GTCCTGCACCGGTTGGCGAA	GACCCACAGTGATCACAGAA
β-actin	398 bp	GATGACGATATCGCTGCGCTG	GTACGACCAGAGGCATACAGG

Buffer (LDS, 4X), 1 µl of reducing agent (10X), 1 µl of molecular grade water and 5 µl of protein lysate was mixed in a tube. The samples were incubated at 70ºC for 10 min for denaturation. For blotting, proteins were transferred to polyvinylidene difluoride (PVDF) membrane from gel using iBlot (ThermoFisher Scientific; IB401001). Blocking was performed with Tris Buffered Saline Tween-20 (TBS-T) containing 5% non-fat dry milk at room temperature for 2h. Afterwards, the membranes were incubated with rabbit polyclonal primary antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4ºC, and washed three times with TBS-T. mTOR (cell signalling #2983), phospho-mTOR (Ser2448) (cell signalling #5536), p70S6K (cell signalling #2708), phospho-p70S6K (cell signalling #9234), Proliferating cell nuclear antigen (PCNA) (cell signalling #13110S) were diluted as 1:1000 in 5% bovine serum albumin, Caspase-3 (cell signalling #9665S) was diluted as 1:1000 in 5% non-fat dry milk and β-actin (cell signalling #5125) was diluted as 1:5000 in 5% non-fat dry milk. After primary antibody incubation, membranes were washed 5 minutes for six times with TBS-T and incubated in IgG HRP conjugated anti-rabbit secondary antibody (1:5000 in 5% non-fat dry milk) at room temperature for 2 h. The membranes were washed 5 minutes for six times with TBS-T. Signal was detected by chemiluminescence (Amersham #RPN2109) and visualized with A charge-coupled device (CCD) camera and software (BioRad). All experiments were repeated at least three times, and the intensity of the signals was analyzed using NIH ImageJ analysis programme. β-actin was used as an internal control (The number of ovaries taken from all groups for western blot; n = 5).

Statistical analysis

Data is represented as the mean± standard error of the mean (SEM) from at least three replicates per experiment. Data is presented as mean ± SEM and statistical analysis was performed using GraphPad Prism Software. ELISA, western blot and qRT-PCR results from each group were compared by two way-ANOVA. Follicle counting results from each group were compared by two way-ANOVA. Normally distributed data was analyzed by Student's t-test to compare two groups and ANOVA with Tukey's post-hoc test to compare multiple groups. Differences were considered significant when $p < 0.05$.

Results

Effects of rapamycin treatment on serum 17β-oestradiol, progesterone and luteinizing hormone levels

To determine the hormone levels before and after rapamycin treatment in DHEA-induced PCOS pathogenesis in mice, serum 17β-Estradiol (E2), Progesterone (P) and LH were assessed by ELISA (Figure 2A, B, C). C25 group (untreated female 25 days old Balb/C mice), C45 group (no treatment-45 days old Balb/C mice) and rapamycin treatment for 10- and 20-days group. We detected increased E2 levels in DHEA-induced PCOS when compared to the control group. There was no significant difference in E2 levels after rapamycin treatment compared with PCOS group (Figure 2A). P level increased in the DHEA-induced PCOS group and was higher than in other groups. Subsequent to PCOS pathogenesis, 10- and 20-days rapamycin treatment significantly decreased serum P levels compared with PCOS group. In these results, it was determined that the increased serum P level in the PCOS group decreased after rapamycin treatment and approached the control group (Figure 2B). On the other hand, serum LH level of PCOS group decreased in DHEA-induced mice compared to the other groups. The rapamycin treatment resulted in increased LH levels compared to the PCOS group. Thus, rapamycin brought LH levels closer to the control group values (Figure 2C).

Rapamycin treatment alters developing follicle numbers and activates ovulation in PCOS mouse ovary

To determine the cyst pathology and amendatory effect of rapamycin in DHEA-induced PCOS mouse ovary, all of the experimental groups, control, PCOS and 10- and 20-days rapamycin treated, and vehicle groups were evaluated morphologically. We observed that control group ovaries present folliculogenesis characterized by the development of successive waves of different stages of ovarian follicles during the ovarian cycle and corpus luteum (CL) observed in the mouse ovarian tissue after ovulation (Figure 3A and 3B). The PCOS ovary exhibited a significant increase of FCs identifying characteristic fluid-filled antrum, they especially remained at the secondary and Graafian follicle stage (Figure 3C and 3D). We detected increased number of primordial follicle and atretic follicle in PCOS group. Rapamycin treatment groups (10- days and 20-days) showed less primordial follicle, and a higher number of primary follicle than PCOS group.

The 10-day rapamycin treatment group after DHEA treatment presented ovarian follicles with different stages of development and a CL structure was seen (Figure 3E and 3F). The 20-day rapamycin treatment group after DHEA treatment presented normal follicle development with different stages of developing follicles and especially some of the secondary follicles were very large with a small antrum (Figure 3G and 3H).

No significantly different morphological findings were obtained from the vehicle group compared to the control group. See Figure 3 for a better representation of the morphological differences in all experimental groups. Based on general morphological evaluation, CL
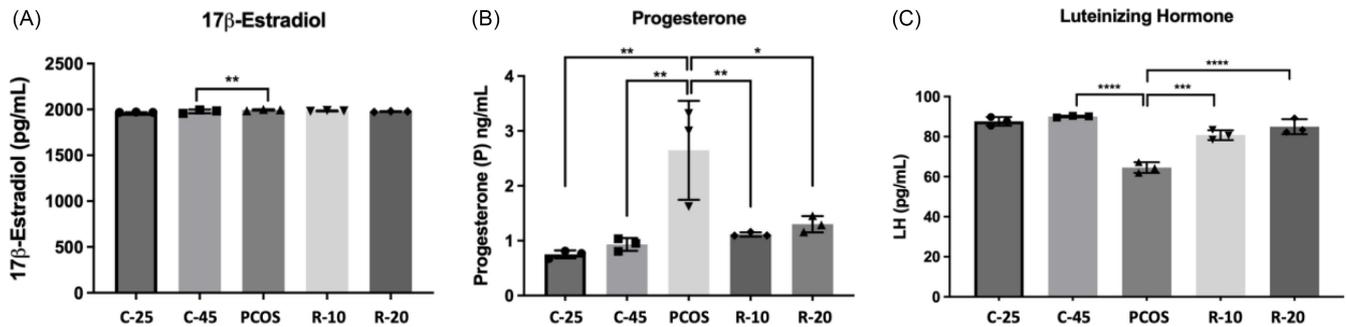
Figure 2. Serum 17β-oestradiol (E2) (A), progesterone (P) (B) and luteinizing hormone (LH) (C) levels were evaluated with using ELISA. Female mice of 25 day old (postnatal 25-day old, C25), no treatment (C45, 45-day old-control group), PCOS group (25 days old Balb/C mice + 20 days DHEA treatment), rapamycin Injection for 10 days group (R-10, 25 days old Balb/C mice + 20 days DHEA treatment + 10 days rapamycin administration), rapamycin Injection for 20 days group (R-20, 25 days old Balb/C mice + 20 days DHEA treatment + 20 days rapamycin administration). After treatment with DHEA, E2 level increased significantly compared to the control group (** $p < 0.01$). There was no significant difference between the other groups. When the P level was examined, a significant increase was seen in the PCOS group compared to the control group (*** $p < 0.001$). After rapamycin treatment in PCOS mice, P level in R10 (*** $p < 0.001$) and R20 (** $p < 0.01$) group approached the control group while it decreased significantly compared to the PCOS group. There was a significant decrease in LH concentration in the PCOS group compared to the control group (*** $p < 0.001$). In the R-10 and R-20 groups, the LH level increased significantly when approaching the control group compared to the PCOS group (*** $p < 0.001$).

structure that formed after ovulation was observed in the control group (Figure 3A and 3B). In PCOS group, as ovulation is suppressed, follicles at different developmental stages and non-ovulated FCs were observed (Figure 3C and 3D). CL was seen only in the group that was treated with rapamycin for 10 days (3E and F). In the group that was treated with rapamycin for 20 days non-ovulated FCs and CL not observed (3G and H). This observation tells us that 10-days rapamycin treatment after PCOS supports a dynamic follicle development and ovulation in ovary.

Follicle counting was performed to evaluate the numbers of follicles in each experimental group (Figure 4). The primordial follicle number increased significantly in PCOS group compared with control group. The reason for the increase in the number of primordial follicles in the PCOS group is the decrease in follicular activation as the ovarian cycle is disrupted. However, it was observed that the number of primordial follicles decreased in the rapamycin treatment for 10-days and 20-days group compared with PCOS group. According to this result, primordial follicle activation in 10-days and 20-day rapamycin-treated group occurred at a rate close to the control group. Primary follicle increased significantly in the rapamycin treatment for 20-days group compared to the PCOS group. There was no significant difference between the groups in the number of secondary follicles. When other groups were compared with the control group, CL was not observed in PCOS group and the 20-day rapamycin-treated group. Interestingly, CL was observed in the 10-day rapamycin-treated group after PCOS but the number of CL was less than the control ovary. We detected that 10-day rapamycin treatment may regulate follicle development and ovulation. The atretic follicle number increased significantly in PCOS and rapamycin treatment for 10-days group compared with control group. In contrast, a reduction was seen in the rapamycin treatment for 20-days group compared to the PCOS group (Figure 4). Results obtained from follicle counting support our ELISA and morphological evaluation findings.

Rapamycin treatment decreases apoptosis in DHEA-induced PCOS mouse ovary

To investigate whether the apoptotic changes observed in granulosa and theca cells, TUNEL-positive cells were detected in all the experimental groups. In the control group ovary, especially in the antral follicles, some of the granulosa cells neighbouring antrum were stained positive with TUNEL (green fluorescent) (Figure 5A). In primary and secondary follicles that are in early stages of development, negligible amounts of apoptotic cells were observed (Figure 5A). Numerous non-ovulated and large follicles were detected in ovarian tissues retrieved from PCOS mouse ovary. When these follicles were examined under higher magnification, antral and mural granulosa cells were detected TUNEL positive (Figure 5B). In the 10-day rapamycin-treated group, follicles were healthy and a few apoptotic cells were detected. We determined TUNEL-positive cells only in some stromal areas. (Figure 5C). The 20-day rapamycin-treated group showed a lot of TUNEL-positive granulosa cells. We used a thymus section for the positive control (Figure 5C insert).

Comparison of mTOR signal pathway expression in PCOS ovary and rapamycin-treated mouse ovary

Total mouse ovaries were evaluated from each experimental group. It was determined that key components of *mTOR* signal pathway were expressed in the control ovary, PCOS mouse ovary, 10-day and 20-day rapamycin-treated ovaries and vehicle groups (Figure 6). Although some expressional differences were determined in the *mTOR, Raptor, Rictor, GβL, p70S6K* and *PKCalpha* expression, there was no statistical significance determined at mRNA level.

Detection of mTOR signal proteins in PCOS and rapamycin-treated mouse ovary

To study the underlying mTORC1 signal mechanism (Figure 7A), mTOR expression increased significantly in the PCOS group compared to the control group. In the rapamycin treatment for 10- and 20-days groups, mTOR expression significantly decreased compared to the PCOS group (Figure 7B). p-mTOR (S2448) expression was found to be significantly decreased in the PCOS group compared to the control group. However, it was observed that p-mTOR (S2448) expression level increased in the rapamycin treatment for 10- and 20-days groups (Figure 7C). The expression of p70S6K in PCOS and rapamycin-treated groups was almost similar. The p70S6K expression level was significantly higher in the control group (Figure 7D). In contrast, expression of phosphorylated p70S6K was significantly lower in the PCOS group compared to the control, rapamycin treatment for 10- and 20-days groups. Expression of phosphorylated p70S6K increased
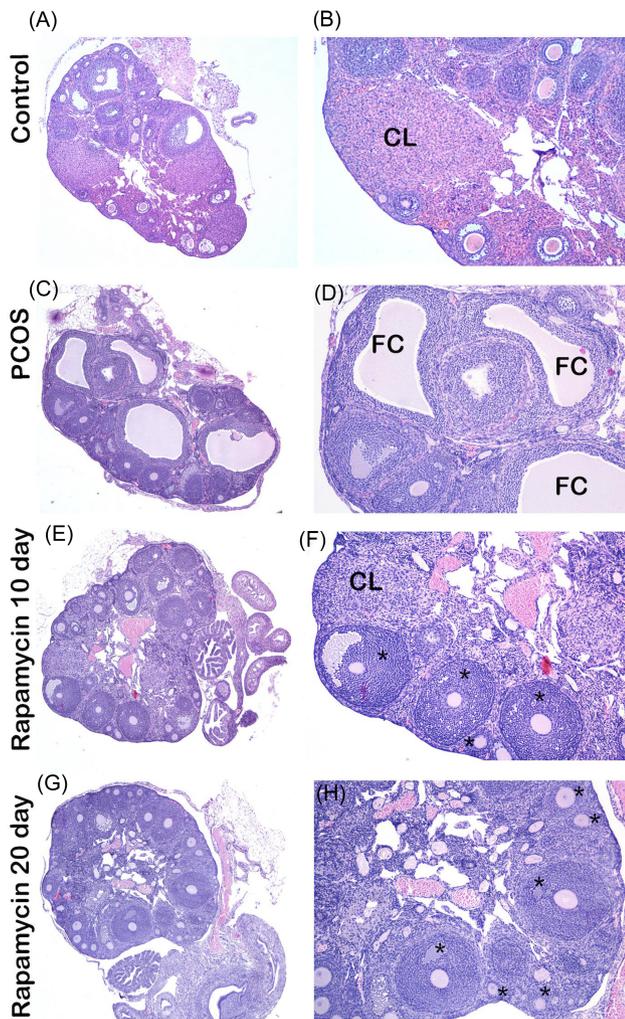
Figure 3. Morphological evaluation. Morphological evaluation of control ovaries, PCOS ovaries, 10-days and 20-days-treated rapamycin mouse ovaries were performed with Hematoxylin and Eosin staining. (A) Control group ovary. (B) Corpus Luteum (CL) structure was seen in the control group ovary. (C) In the DHEA-induced PCOS mouse ovary, FCs detected at different stages were observed. (D) The figure shows FC structures. (E) The figure shows the R-10 group ovary treated with rapamycin for 10 days. (F) Ovarian tissue revealed follicles in different developmental stages and CL. (G) The ovarian treated with 20 days of rapamycin and (H) follicles in different follicular stages, together with the FC structure. (Stars indicate secondary follicles). Scale bars, 500 μm (A, C, E, and G) and 100 μm (B, D, F, and H).

Discussion

PCOS is characterized by the presence of at least two pathophysiological conditions of the following chronic anovulation, hyperandrogenism and polycystic ovarian morphology. Although genetic and environmental factors contribute to the origin and development of this disease, the aetiology of PCOS is still unclear (Dou *et al.*, 2018, Anagnostis *et al.*, 2019). DHEA, as the most abundant steroid hormone in the circulation, has been used to stimulate the PCOS mouse model for almost a decade (Luchetti *et al.*, 2004). We have previously shown that the mTOR signal pathway has a role during pathogenesis of DHEA-induced PCOS in mouse ovary (Yaba and Demir, 2012). Siddappa et al. have shown that when the kinase activity of mTOR is inhibited, the follicles in ovary develop until pre-ovulatory stage, indicating that mTOR has a functional role in the development of follicle and CL (Siddappa *et al.*, 2014). In the present study, we performed the DHEA-induced PCOS mouse model to mimic anovulatory PCOS and then aimed to eliminate negative effects of PCOS with rapamycin treatment.

Morphological findings obtained from experimental groups were verified with ELISA results. We observed that the PCOS mouse model increased E2 and P levels due to high levels of circulating androgens similar to our previous study (Yaba and Demir, 2012). We considered that high P levels determined in the 20-day rapamycin-treated group could be related to the expansion of theca cells to stromal regions due to hypertrophy. PCOS patients had increased levels of LH and so excessive increase in LH levels in the PCOS group were expected. However, in the present study, we showed a decreased LH level in PCOS group when compared with the control group and an elevated LH level observed after rapamycin treatment. In a study with female rats, Roa et al. showed that the LH level decreased after rapamycin treatment and when rapamycin treatment was applied chronically (30 days), LH level increased (Roa *et al.*, 2009). In a recent study, Guo *et al.* used 21-day-old female C57BL/6J mice for daily s.c. 6 mg/100g DHEA treatment to induce hyperandrogenic PCOS mouse model and they investigated the effect of rapamycin treatment on ovarian activity and metabolic changes (Guo *et al.*, 2021). They showed that rapamycin treatment worsened the development of reproductive dysfunction and increased elevated testosterone, with decreased levels of LH and LHr and increased levels of FSH and FSHr (Guo *et al.*, 2021). Differences between literature and our findings may be attributed to the dose and period of rapamycin treatment in PCOS mouse ovary.

A characteristic of ovarian morphology in the PCOS group appeared in some FCs through abnormal ovarian follicle development, impaired selection of the dominant follicle, cystogenesis and subsequently anovulation (Yaba and Demir, 2012). We observed that numerous non-ovulated FCs remained at the secondary and Graafian follicle stage in the DHEA-induced mouse ovary. Additionally, we suggested that large follicles in the PCOS group that cannot ovulate due to hormonal suppression include more apoptotic cells and poor follicle quality (and oocyte quality) (Yaba and Demir, 2012). Here, we showed ovulation and CL structure in the DHEA-induced PCOS mouse ovary after the 10-day rapamycin treatment. In the 20-day rapamycin treatment group, CL was not seen and secondary follicles were observed at a rather large diameter, indicating ovulation was suppressed by the 20-days treatment with rapamycin. However, while rapamycin treatment regulates follicle development in the PCOS mouse ovary, different time periods and doses have to be used to observe the regulative effect on folliculogenesis.

significantly in the rapamycin treatment groups compared to the other groups. When rapamycin treatment was continued for up to 20 days, phosphorylated p70S6K expression was determined to be excessively high (Figure 7E). When PCNA levels were compared, a significant decrease was seen in the PCOS group compared to the control and rapamycin treatment for 20-days group (7F). Similar to the PCOS group, a significant decreased was observed in the rapamycin treatment for 10-days group compared to the control. PCNA expression level increased in the rapamycin treatment for 20-days group (Figure 7F). Caspase-3 expression decreased in PCOS group compared to the control group. While caspase-3 level decreased in 10-days rapamycin treatment group compared to the control and PCOS group, Caspase-3 significantly increased in 20-days rapamycin treatment group (Figure 7G).

Figure 4. Follicle counting. In the control, PCOS group, R-10 and R-20 group ovarian tissues, follicle count was performed based on the organization of the granulosa cells surrounding the oocyte. Considering the results, the number of primordial follicles increased significantly in the PCOS group compared to the control group (*** $p < 0.001$). After 10-days of treatment with R-10, the number of primordial follicles decreased significantly compared to the PCOS group and approached the control group (*** $p < 0.001$). The number of primary follicles increased significantly after 20-days of R-20 compared to the PCOS group (*** $p < 0.001$). While the number of atretic follicles increased significantly in the PCOS group compared to the control group, it decreased significantly after R-20 (**** $p < 0.0001$).
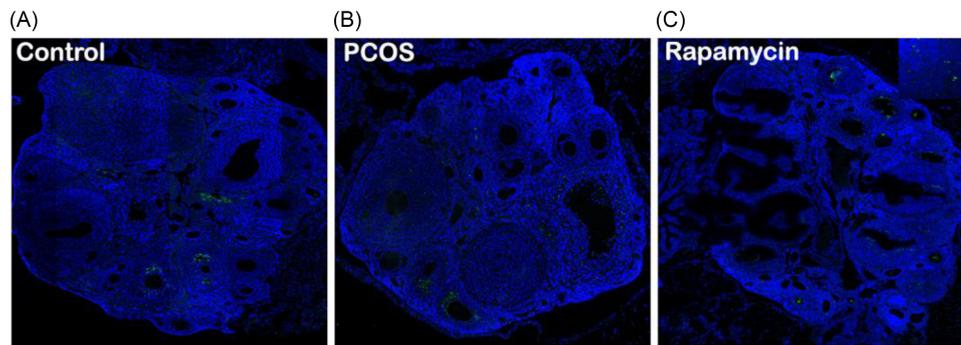
Figure 5. Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) reactivity. Ovarian sections from each group were stained by TUNEL. Ovaries of PCOS mouse ovary exhibit more apoptotic granulosa cells than control as assessed by TUNEL assay. **A**, control ovary. Example of a Graafian follicle from control ovary with a few TUNEL-positive granulosa cells. **B**, PCOS ovary. **C**, 10-days rapamycin-treated ovary. Thymus tissue section was used as a positive control (insert). Scale bar, 100 μm.
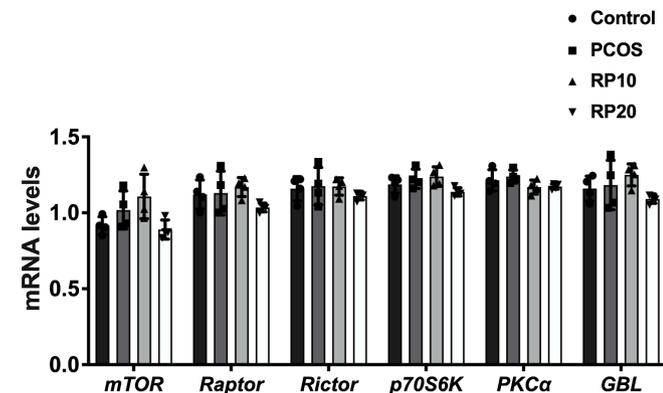
Figure 6. mTOR signal pathway evaluation by qRT-PCR. Ovaries from each experimental group expressed the key components of mTORC signalling pathway. qRT-PCR analysis showed that key components of both mTORC1 and mTORC2 (*mTOR, Raptor, Rictor, GβL*) and downstream genes (*p70S6K and PKCalpha*) were expressed in control ovary, PCOS ovary, 10- and 20-days rapamycin-treated groups (R10 and R20, respectively). There is no statistical significance determined at mRNA level.

In the study by Dou *et al*, they showed that rapamycin administration can prolong ovarian life in mice. However, some adverse effects on reproductive function, such as reduced ovary size, irregular oestrus cycle, and inhibition of follicle development were also observed (Dou *et al*., 2017). Based on this, we demonstrated the positive effects of the 10-day rapamycin treatment both hormonally and morphologically on the PCOS model mouse ovaries. However, when the treatment period was extended, we showed that the effect of rapamycin on the ovary was reduced compared to the 10-day treatment group and 20 days of rapamycin treatment also suppressed CL formation.

Recent studies have implicated that the mTOR signalling pathway has an important role during ovarian follicle development (Sanchez-Garrido and Tena-Sempere, 2013, Castellano and Tena-Sempere, 2016). The downstream pathway was examined with the knockout of mTOR specific to the oocyte stage and the roles of mTOR-dependent pathways in the control of oocyte, and follicle development were examined in primordial follicle and growing oocytes (Guo *et al*., 2018). In a study conducted on the PCOS mouse model in 2021, they showed that the downstream targets of mTOR, rpS6 and p-rpS6 were significantly reduced after 14 days and 4 mg/kg of rapamycin treatment (Guo *et al*., 2018). We determined that mTOR protein levels decreased in 10- and 20-days 5 mg/kg rapamycin-treated group but p-mTOR increased in the 10- and 20-days rapamycin-treated group. Therefore, our findings indicated that 5 mg/kg rapamycin treatment for 10 days is more effective in eliminating the negative effects of PCOS than the 20-days rapamycin-treated group. In our study, we showed that mTORC1 signal proteins with rapamycin treatment in PCOS mouse ovary and when we evaluated the p70S6K, protein levels increased in the 20-days rapamycin-treated group. However, phosphorylation of p70S6K protein level increased in the 10- and 20-days rapamycin treatment. When we evaluate our work, we have shown that the effect of rapamycin in the PCOS model may vary depending on the treatment time and dose. In addition to that, in terms of PCNA expression for cell proliferation in the 20-days rapamycin-treated mouse ovary was higher compared to the PCOS group. Caspase activity was lower in the 10-days rapamycin treatment group. However, the Caspase activity in the 20-days rapamycin treatment group was closer to the control group. These results show that rapamycin treatment triggers and maintains a healthy follicle development in PCOS ovaries (Figure 8). In our study, we suggest that 10-days rapamycin treatment (5 mg/kg)
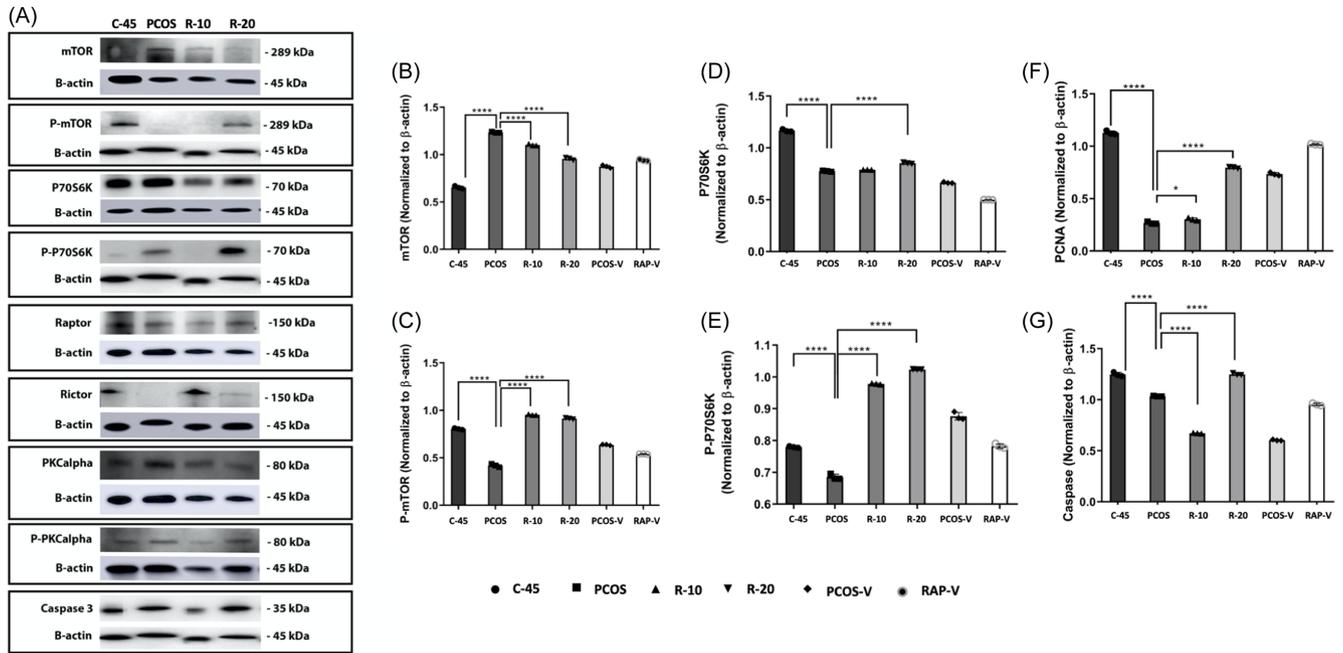
Figure 7. mTOR signal pathway evaluation by western blot. Protein expression levels were compared in control, PCOS, R-10 and R-20 ovary (A). mTOR expression increased in PCOS group. 10- and 20-days rapamycin treatment decrease the expression of mTOR (B) and increase phosphorylation (at Ser2448) of mTOR (C) (**** $p < 0.0001$). Increased phosphorylation of mTOR target phosphorylation of p70S6K expression in the 20-days treatment group (D and E) (**** $p < 0.0001$). PCNA expression increased (F) (* $p < 0.1$) and Caspase3 expression decreased (G) (**** $p < 0.0001$) after 10-days and 20-days rapamycin treatment. Beta-actin was used as control for protein loading. The signal intensities were measured NIH ImageJ programme. Similar results were obtained in three additional experiments.
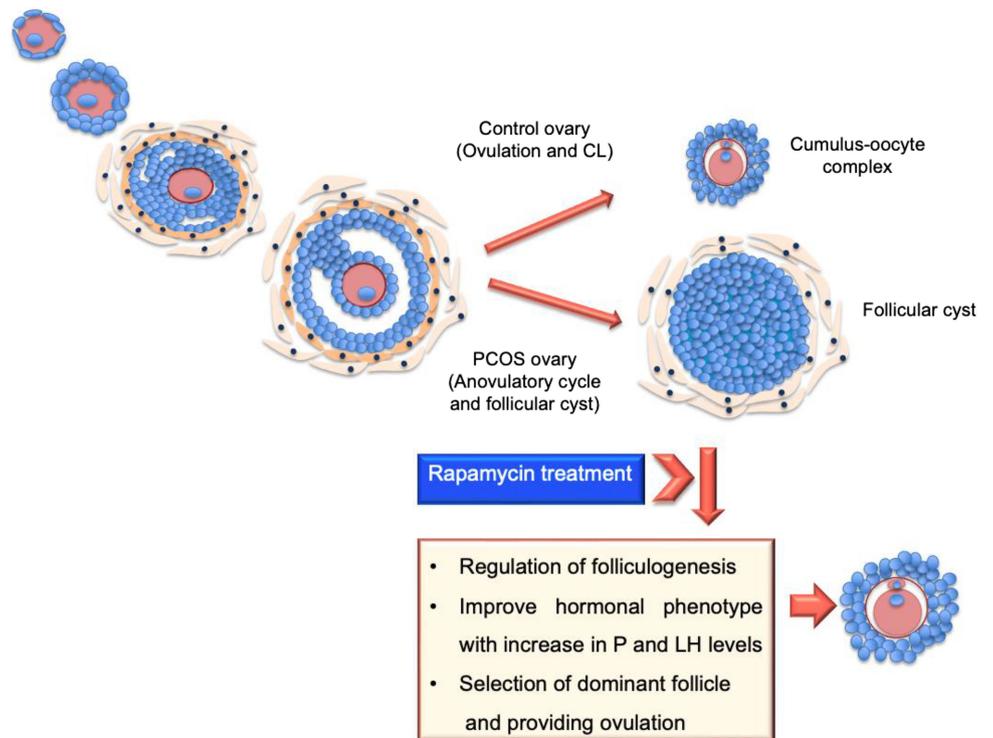
Figure 8. The effect of rapamycin treatment on mouse ovarian follicle development and ovulation in DHEA-induced polycystic ovary syndrome mouse model.

modulates hormonal regulation, follicle development and ovulation in PCOS mouse ovary. We believe that our study will contribute to our understanding the effect of rapamycin treatment on follicle development and anovulatory infertility in PCOS mouse model.

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