Journal of Gynecology Obstetrics and Human Reproduction 48 (2019) 193–199

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Rapamycin administration during normal and diabetic pregnancy effects the mTOR and angiogenesis signaling in the rat placenta

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ARTICLE INFO

Article history:
Received 17 August 2018
Received in revised form 4 December 2018
Accepted 12 December 2018
Available online 12 December 2018

Keywords:
mTOR
Rapamycin
Diabetes
Placenta
Angiogenesis

ABSTRACT

Objective: The mammalian target of rapamycin (mTOR) signaling pathway has newly been recommended to be a nutrient sensor in the placenta. It is speculated that mTORC1 may be activated in diabetes, associated with increased placental nutrient availability. Thus, we aimed to investigate the mTOR signaling pathway both in diabetic and non-diabetic placenta and searched for the alterations of angiogenic factors VEGF, VEGFR1 and VEGFR2.
Methods: Streptozotocin (STZ) was administered by intravenous injection in doses of 60 mg/kg body weight and STZ injected rats were exposed to Everolimus (Rapamycin analog) and sacrificed at gestational days 14 and 20. mTORC1 and mTORC2 target proteins and angiogenic factors were analyzed at protein and mRNA levels in the placenta. Soluble VEGF A and İnsulin protein levels were determined in blood serum.
Results: Placenta and embryo weights were altered after STZ and/or Rapamycin administration. mTOR pathway inhibition was confirmed by decreased p70S6K (Thr389) phosphorylation levels. We found that maternal diabetic environment led to an increase in Akt phosphorylation at 14th and decrease at 20th gestational days. Serum levels of Insulin in 14 th and 20 th days of gestation were decreased in Rapamycin and diabetic groups. On the other side serum levels of Soluble VEGF were increased in 14 th and decreased in 20 th days of pregnancy.
Conclusion: According to our results, it might be suggested that angiogenesis related proteins will be related with placental growth regulation and mTOR may be a candidate pathway mediating the process in normal and diabetic pregnancy.

Introduction

The mammalian target of rapamycin (mTOR) signaling pathway has newly been recommended to be a nutrient sensor in the placenta, controlling fetal develop by regulating placental function and nutrient transport [1].

mTOR, a serine/threonine kinase, is a member of the phosphatidylinositol kinase-related kinase superfamily [2] functions as an signal transduction element by integrating extracellular and intracellular signals originating from growth factors, hormones, and nutrients. Therefore, mTOR seems to have a key role in the regulation of cellular response to nutrients. mTOR exists in 2 complexes: mTORComplex1 (mTORC1), which is rapamycin-sensitive and phosphorylates P70S6K1 and initiation factor 4E binding proteins (4E-BPs), and mTORComplex2 (mTORC2), which is rapamycin-insensitive and phosphorylates protein kinase B (PKB, also known as Akt) [3]. The phosphorylation of p70S6K1 and 4E-BP1 enhance the protein synthesis capacity of the cell thereby promoting cell growth [4].

Previous studies indicate that mTOR is essential for early mouse embryonic development and embryonic stem cells. It is shown that the particularly abnormal development of the extra-embryonic ectoderm and the shortened ectoplacental cone displayed by the mutant embryos suggests that mTOR is required for trophoblast development [5].

Placental mTORC1 activation is diminishing in human IUGR, and perhaps it can be activated in diabetes, related with augmented placental nutrient availability. Presently mTORC1 is well-known as a modulator of amino acid transfer across the placenta [6].

However, very little is known regarding the role of mTOR-mediated placental angiogenesis during intrauterine development

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http://dx.doi.org/10.1016/j.jogoh.2018.12.003
2468-7847/

in a diabetic milieu. Diabetes is known to affect both maternal and fetal health adversely unless the glycemic control is efficiently regulated. Placenta is a unique organ which mediates proper embryonic development by regulating multiple processes. Therefore regular placental development is also essential for a regular embryonic development. Thus, we aimed to investigate the mTOR signaling pathway in pregnant rats both in diabetic and nondiabetic females and searched for the alterations of angiogenic factors VEGF, VEGFR1 and VEGFR2. STZ injected female pregnant rats were exposed to Everolimus (Rapamycin analog) and sacrificed at gestational days 14 and 20. mTORC1 and mTORC2 targets and angiogenic factors were analyzed at protein and mRNA levels.

Materials and methods

Animals and experimental groups

Female Wistar rats (n = 5 for each day and group; in total 40 animals) (Rattus norvegicus) (200–250 g) were used for the experiments. All procedures in this study were approved by the Akdeniz University Animal Experiments Local Ethical Committee. The animals were kept under standard conditions, maintained on a cycle of 12 h light/12 h darkness with free access to food and water. The rats had been kept in cages overnight, two female per male. After mating, the presence was designated as day 0 of pregnancy.

In order to compose the diabetic group, pregnant females were injected with a single dose of 60 mg/kg Streptozotocin (STZ, N-Methyl Nitrosocarbamoyl-D-glucosaminel, Sigma; Saint Louis, MO) intraperitonally at gestational day 7 (GD7). After 48 h of STZ injection, blood glucose levels were measured from tail vein and 200 mg/dl or more accepted as diabetic. 0.05 mg/kg/day Rapamycin (Everolimus, Selleckchem; Houston,TX) were administered by oral gavage beginning from GD12 until the day of sacrification of both diabetic and nondiabetic female rats. All groups were sacrified at day 14 and 20.

The placentas and embryos were weighted after sacrification. Placentas were stored at −196 °C for further Western blot and PCR analysis (n = 12 placentas for PCR and Western Blot. Blood serum of each group blood was separated by centrifugation and sera were stored at −80 °C for ELISA analyses.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and Western blotting

Rat placental lysates were prepared and total cellular protein was separated by SDS polyacrylamide gel electrophoresis as previously described by Ozmen et al [7].

Briefly, membranes were incubated with VEGF (S.Cruz; Dallas, Tx), VEGFR1 (Abcam; Cambridge, UK), VEGFR2 (Abcam; Cambridge, UK), p-p70S6K (Thr389) (Cell Signaling; Danvers MA), p70S6K (Cell Signaling; Danvers MA), p-4EBP1 (Thr37/46) (Cell Signaling; Danvers MA), 4EBP1 (Cell Signaling; Danvers MA), p-Akt (Ser473) (Cell Signaling; Danvers MA), Akt (Cell Signaling; Danvers MA), p-mTOR(Ser2448) (Cell Signaling; Danvers MA), mTOR (Cell Signaling; Danvers MA) and Beta Actin (Abcam; Cambridge, UK) antibodies + 4 °C overnight. After washing with Tris buffered Saline with Tween 20 (TBS-T; 0.05 M Tris, 0.15 M NaCl, 0.001% Tween 20), membranes were incubated for 2 h at room temperature (RT) with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (BioRad; Hercules,CA) and goat anti-mouse IgG (BioRad; Hercules, CA) antibodies. After washing with TBS-T, antibodies were detected by chemiluminescence-based SuperSignal CL HRP Substrate System (Pierce; Waltham, MA). Membranes were exposed to Hyperfilm (Amersham; Pittsburgh, PA), which was subsequently analyzed by using Alpha DigiDoc 1000 gel documentation unit (Alpha Innotech Corporation, CA, USA). All results were normalized to beta-actin.

RNA isolation and cDNA synthesis

Total RNA was isolated from rat placentas using Trizol Reagent (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. RNA pellets were dissolved in RNase-free water and quantified using UV spectrophotometer. Preparation of cDNA was carried out from 2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen; Carlsbad, CA) according to manufacturer's instructions.

Quantitative Real time (q)-PCR analysis

The expression of VEGF, VEGFR1 and VEGFR2 and β-actin was determined by q-PCR using QuantiFast SYBR Green PCR Kit (Qiagen; Germantown, MD). 125 μL of SYBR Green (2x) Master Mix was combined with 8.5 μL of nuclease-free water,10 μM forward/ reverse primers (1 μL each) and 2 μL of cDNA in the PCR strip tubes. Amplification used 35 cycles of PCR in Applied Biosystems StepONEplus Real Time PCR System, with the following program: initial denaturation at 95 °C for 10 min, followed by 35 cycles at 95 °C for 15 s, annealing for 30 s and extension at 72 °C for 30 s with a final melting curve at 95 °C 1 min and 55 °C 1 min. All samples were run in dublicate and the average used for each sample. Expression of the target mRNAs was normalized to β-actin levels and the $2^{-\Delta\Delta Ct}$ values were used to evaluate relative expression levels. Primer sequences are as follows:

VEGF *f*:5′-TCTTCAAGCCGTCCTGTGTG-3′, *r*:5′-GCCTTGGCT TGTCACATCTG-3′, VEGFR1 *f*:5′-CGGACTGTGGCTGTGAAGAT-3′, *r*:5′−CCTTCCACATCGCTCACACT-3′, VEGFR2 *f*:5′-GCATGGCATCGTG TACATCA-3, *r*:5′-TTCTCCGGCAGATAGCTCAA-3′, Beta Actin *f*:5′-CATGAAGATCCTGACCGAGC-3′, *r*:5′-CAGACAGCACTGTGTTGGCA-3′

Enzyme linked immunosorbent assay (ELISA) analyses

Commercial ELISA kits were used per manufacturer's instructions. Soluble VEGF (sVEGF-A) (R&D; Mineapolis,MA) and Insulin (Millipore; Billerica, MA) concentrations was determined in rat sera. Briefly, the appropriate amount of samples were pipetted into pre-coated 96 well plates and incubated for 2 h at RT. After washing, 200 μL HRP-conjugated antibody was added and incubated for 2 h. A 200 μL aliquot of substrate solution was then added and incubated in the dark for 30 min. After stopping reaction, the plate was read at 450 nm using a spectrophotometer.

Statistical analysis

Placenta and embryo weights of the Control, Rapamycin, Diabetes, Diabetes + Rapamycin groups were measured and the One Way Anova was performed by using the SigmaStat 3.5 software. A value of $p \leq 0.001$ was considered as statistically significant. Western blot band densities were measured with the Alpha DigiDoc 1000 gel documentation unit. Densitometric data obtained from Western blot and $2^{-\Delta\Delta Ct}$ values obtained from Q-PCR analysis were subjected to statistical analysis by using the SigmaStat 3.5 soft-ware. One Way Anova was performed and $p \leq 0.05$ was considered as statistically significant. For ELISA analysis, protein concentrations were evaluated and $p \leq 0.001$ was considered as statistically significant.

Results

Rapamycin administration altered placental and embryonal weights

Placenta and embryo weights were altered after STZ and/or Rapamycin administration. In diabetic groups, placenta ($p \leq 0{,}05$) and embryo ($p \leq 0{,}001$) weights were decreased at GD14 compared

to control. Besides, it was seen that Rapamycin administration led to increased embryonal weight ($p \leq 0{,}05$) and increased placental weight ($p \leq 0{,}001$) compared to control group. Rapamycin administration in diabetic group also led to increase in placental weight ($p \leq 0{,}05$) and embryonal weight ($p \leq 0{,}001$) (Fig. 1). But at GD20, placental weight ($p \leq 0{,}05$) was increased but embryonal weight ($p \leq 0{,}001$) was decreased in diabetic group compared to control. Rapamycin administration resulted with decreased embryonal weight ($p \leq 0{,}001$) both in diabetic and non-diabetic groups. But placental weight was only decreased in non-diabetic–Rapamycin administered group ($p \leq 0{,}05$) (Fig. 2). With compare to control group, in STZ and/or Rapamycin administration groups' number of pups per labor was not affected. There is no difference in the number of embryos within the groups.

Rapamycin did not affect insulin concentration

Insulin concentrations were significantly decreased in all STZ injected groups compared to control ($p \leq 0{,}001$). Rapamycin did not significantly affect insulin concentrations in non-diabetic and diabetic females compared to control, but tend to lower the concentrations (Fig. 3a). Our ELISA results showed that, soluble VEGF (sVEGF) concentrations in rat sera affected both from diabetic conditions and Rapamycin administration. sVEGF levels were increased at GD14 in diabetes and rapamycin administered diabetic groups, and decreased at GD20 in all groups compared to control (Fig. 3b).

mTORC1 was inhibited by rapamycin administration

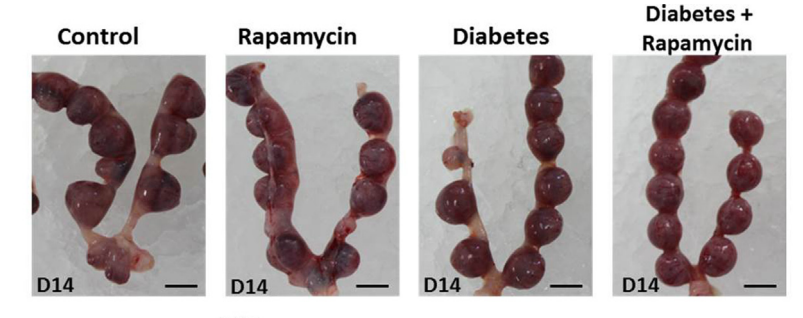
mTOR pathway inhibition was confirmed by decreased p70S6K (Thr389) phosphorylation levels (Fig. 4). After normalization with β- actin, it was observed that 0.05 mg/kg/day Rapamycin administration resulted with decreased p-p70S6K (Thr389) levels both at GD14 and GD20 in diabetic and non-diabetic rat placentas. On the other hand, 4EBP1 (Thr37/46) and mTOR (Ser2448) phosphorylation levels were not altered by Rapamycin administration. However, Akt (Ser473) phosphorylation was decreased after mTOR inhibition at GD20 in non-diabetic group. According to the control group, we found that p-Akt protein level is increased in diabetes group (Fig. 4)

mTORC1 inhibition altered angiogenic factor expression

It was observed that VEGF has a different expression pattern in mid-gestation and term in diabetes. In diabetic groups at GD14, VEGF expression was decreased but on contrary it was increased at GD20 compared to control. After mTOR inhibition, VEGF expression was significantly decreased at GD14 but not altered at GD20 (Fig. 5). When VEGF Western Blotresults were normalized β- actin, it is observed that VEGF was decreased in Rapamycin and diabetes groups, and not changed in D20 groups.

VEGFR1 expression was increased both at GD14 and GD20 in diabetes. However, in Rapamycin administrated group, VEGFR1 expression was first increased at GD14 then decreased at GD20 in non-diabetic placentas compared to control. Additionally, in Rapamycin administered diabetic groups, VEGFR1 expression was similar to control. VEGFR2 expression pattern was also variable between groups and gestational age. At GD14, VEGFR2 expression was significantly decreased in diabetic placentas. On the other hand, Rapamycin administration led to a significant increase both in diabetic and non-diabetic placentas. On contrary at GD20, VEGFR2 expression was significantly increased in diabetes, but significantly decreased in all Rapamycin administered groups (Fig. 5).

According to our Q-PCR results VEGF mRNA levels were similar between all groups. VEGFR1 mRNA was significantly decreased at GD20 in Diabetes + Rapamycin group. VEGFR2 mRNA levels were significantly decreased at GD14 in diabetic and Rapamycin administered groups (Fig. 6).



	GD14 Placenta	GD14 Embryo
Control (n:42)	0,141	0,164
Rapamycin (n:36)	0,179	0,189
Dibetes (n:44)	0,135	0,127
Diabetes+Rapamycin (n:33)	0,156	0,17

Fig. 1. Placental and embryonal weights at Gestational Day 14. Placental and embryonal weights were increased after Rapamycin treatment in non-diabetic rats compared to control. In diabetic group, both weights were decreased compared to control. After Rapamycin treatment placental and embryonal weight were increased in Diabetes + Rapamycin group compared diabetes. *$p \leq 0.05$, **$p \leq 0.001$.

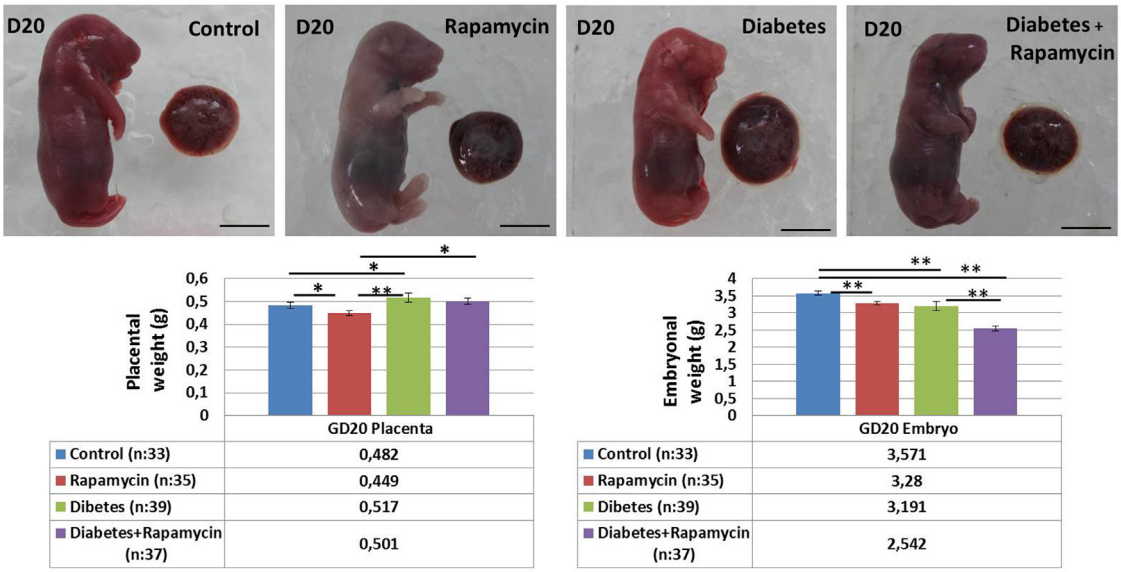


Fig. 2. Placental and embryonal weights at Gestational Day 20. Placental weights were decreased after Rapamycin treatment in non-diabetic rats compared to control. In diabetic group, placental weights were increased compared to control. Rapamycin treatment also led to a decrease in embryonal weight both in non-diabetic and Diabetes + Rapamycin groups compared to control and diabetes, respectively. And in diabetic group, embryonal weights were decreased compared to control. $^{*}p \leq 0.05$, $^{**}p \leq 0.001$.

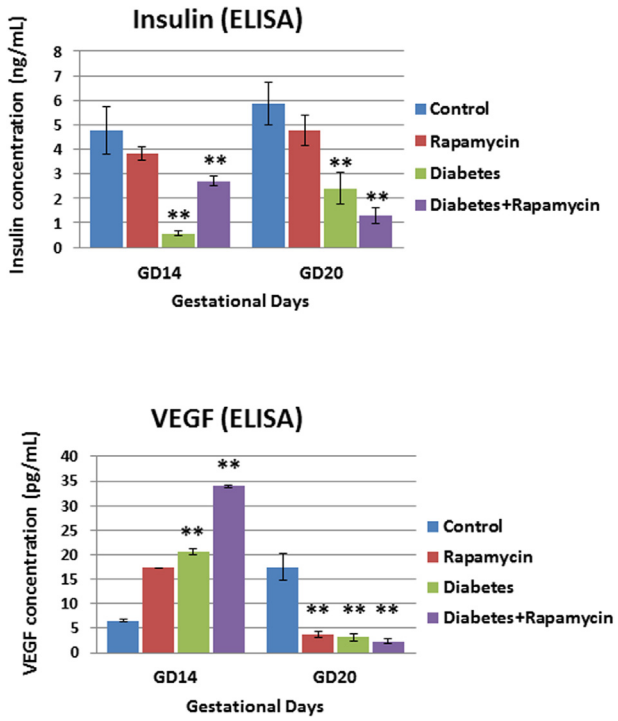


Fig. 3. a Insulin concentrations in rat sera. Insulin concentrations were significantly decreased in all STZ injected groups compared to control. Rapamycin did not significantly affect insulin concentrations. **3b.** VEGF concentrations in rat sera. sVEGF levels were increased at GD14, and decreased at GD20 in all groups compared to control. $^{**}p \leq 0.001$.

Discussion

In this study, mTOR pathway activity was evaluated in rat placentas during pregnancy. We have shown that mTOR pathway was successfully inhibited by 005 mg/kg Rapamycin (Everolimus) indicated by decreased p70S6K phosphorylation levels at gestational day 14 and 20.

There are several studies including Rapamycin administration in animal models. Rapamycin analogs are used in variable doses starting from 0.1 mg/kg up to 5 mg/kg, either as single or repeated courses depending on the aim of the study [8,9]. To the best of our knowledge, this is the first report that examines Rapamycin effect on placental development in terms of placental angiogenesis during diabetic and non-diabetic pregnancies. There are only few studies of which Rapamycin administration (1 mg/kg) was performed during pregnancy [10,11]. In these studies, $Tsc^{-/-}$ knockout animal models were used and mTOR was inhibited by 1 mg/kg Rapamycin as a putative therapeutic model for tuberous sclerosis complex. In another study [12], behavioral effects of a single prenatal rapamycin (1 mg/kg) treatment were evaluated.

It was reported by The U.S. Food and Drug Administration [13], in prescribing information for Everolimus usage that the dose in the pre- and post-natal development study in rats that caused reduction in body weights and survival of offspring was 0.1 mg/kg (0.6 mg/m^2) (http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/022334s6lbl.pdf) [13]. Therefore we decided to use 0.05 mg/kg Everolimus as a lower dose not to cause fetal toxicity.

According to our results, placental and embryonal weights were affected by Rapamycin and/or STZ administration. Although the diabetic placental weights were decreased at GD14 in accordance with previous paper [14], interestingly it was increased after mTOR inhibition both at diabetic and non-diabetic groups. In all groups embryonic weights were in a positive correlation with placental weights at GD14.

On contrary, in diabetes and rapamycin administered diabetes groups placental and embryonal weights were not correlated at term. Besides, mTOR inhibition did not similarly effect placental and fetal development. In rapamycin administered group, mTOR inhibition decreased both placental and embryonal weight at GD20. On the other hand in diabetic group, hyperglycemic conditions resulted with decreased embryonal weight but increased placental weight. Moreover, in diabetic groups, embryonal weight was prominently decreased after mTOR inhibition but not placental weight.

According to our results, mTOR phosphorylation itself was not affected by either STZ or Rapamycin administration. But Akt

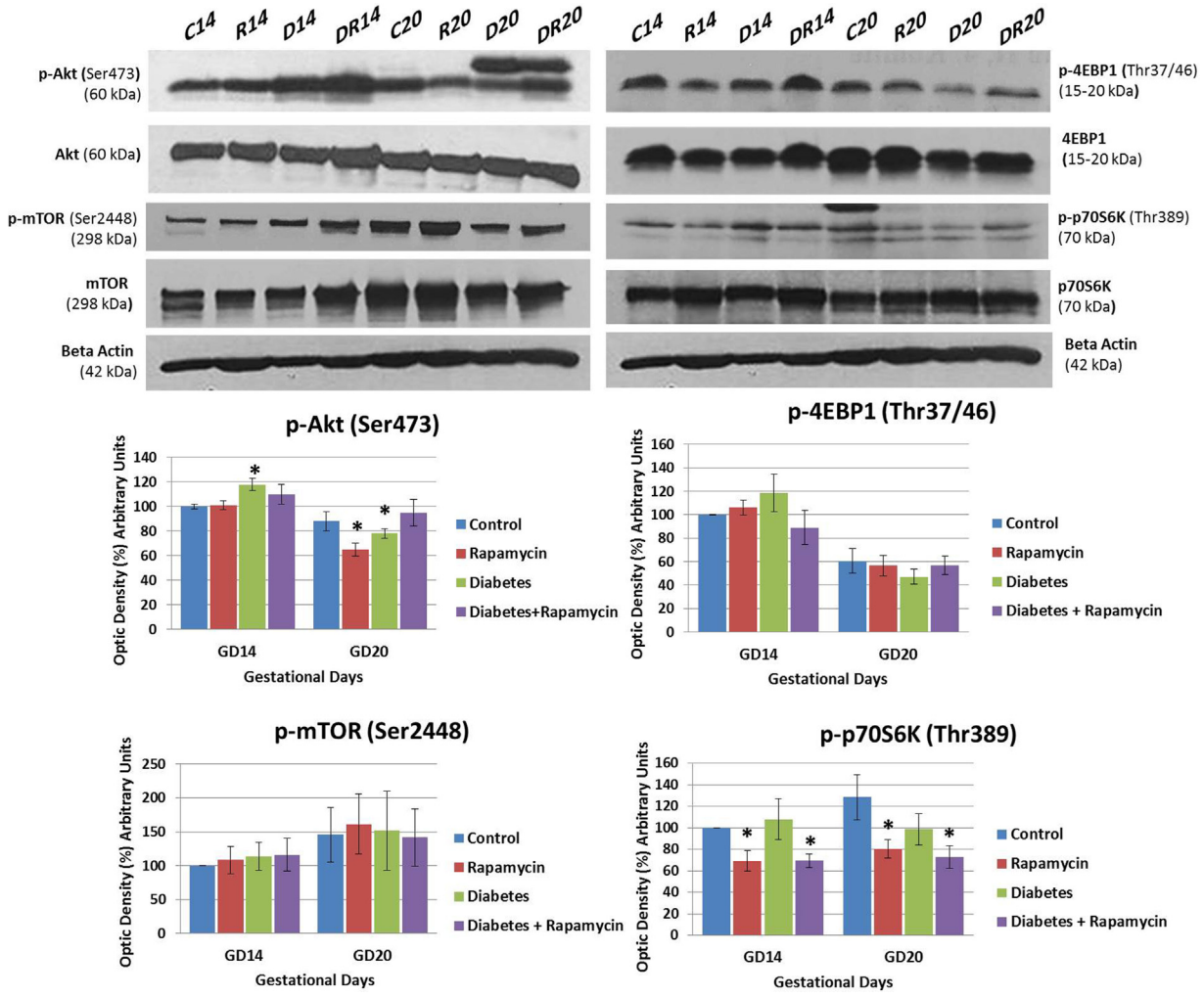


Fig. 4. Western Blot results of mTOR pathway proteins. p70S6K (Thr389) phosphorylation was significantly decreased after Rapamycin administration both at GD14 and GD20 in diabetic and non-diabetic rat placentas compared to control. On the other hand, 4EBP1 (Thr37/46) and mTOR (Ser2448) phosphorylation levels were not altered by Rapamycin administration. Akt (Ser473) phosphorylation was first increased at GD14 than decreased at GD20 in diabetic group compared to control. Besides, Akt (Ser473) phosphorylation was decreased after mTOR inhibition at GD20 in non-diabetic group compared to control. C: Control, D: Diabetes, R: Rapamycin, DR: Diabetes + Rapamycin, $^{*}p \leq 0.05$.

phosphorylation was decreased at term both in diabetic and non-diabetic mTOR inhibited group. On contrary, at GD14 Akt phosphorylation was increased in diabetic placentas. It was previously reported by Yu et al. [15], that high glucose decreased Akt phosphorylation in a dose-dependent manner [15]. In accordance with the mentioned reports, hyperglycemic conditions in our study could have been resulted with decreased Akt phosphorylation at term.

Everolimus administration also resulted with decreased Akt phosphorylation at GD20. It was previously reported that although Rapamycin is known to inhibit mTORC1 targets; p70S6K and 4EBP1, it has also been shown that prolonged Rapamycin administration resulted with decreased mTORC2 activation [16]. Therefore, in our study prolonged Everolimus administration until term, may result with both mTORC1 and mTORC2 inhibition.

It was previously proposed [17] that the placenta functions as a nutrient sensor, regulating the flux of nutrients to the fetus by integrating signals on maternal nutrient availability and fetal demand. It was also reported that in the placenta of dams fed with the High Fat diet, the expression of phosphorylated 4EBP1 (T37/46 &S65) is significantly higher and phosphorylation of rpS6 (S235/236) trended toward an increase suggesting that placental mTORC1 signaling is involved in sensing the nutritional/metabolic/endocrine status of the mother [18]. In our study, we observed partially similar results. At GD14, phosphorylation of Akt (Ser473) and p706SK (Thr389) was increased and phosphorylation of 4EBP1 (Thr37/46) was tend to increase in diabetic placentas. The increase in p706SK (Thr389) phosphorylation was recovered by Rapamycin inhibition. Thus, it will be proposed that mTOR pathway may mediate hyperglycemia associated alteration in diabetic rat placentas in mid-gestation. However, at term the results were not clear enough to support such possibility. At GD20, phosphorylation of Akt (Ser473) was significantly decreased while p706SK (Thr389) phosphorylation was slightly decreased in diabetic group placentas. Moreover, the decrease of p706SK (Thr389) phosphorylation became significant with Rapamycin administration in Diabetic group. Therefore, at term placentas, our results are not strong enough to support a positive correlation between hyperglycemia and mTOR pathway activity but, our results do not indicate an mTOR independent affect, either. Moreover there may be a negative correlation between diabetes and mTOR pathway activity depending on several putative variables like the duration of hyperglycemia, severity of hyperglycemia, maternal and/or fetal metabolic fluctuations.

Anti-angiogenic effect of mTOR pathway inhibition was reported in several studies [19]. According to our results,

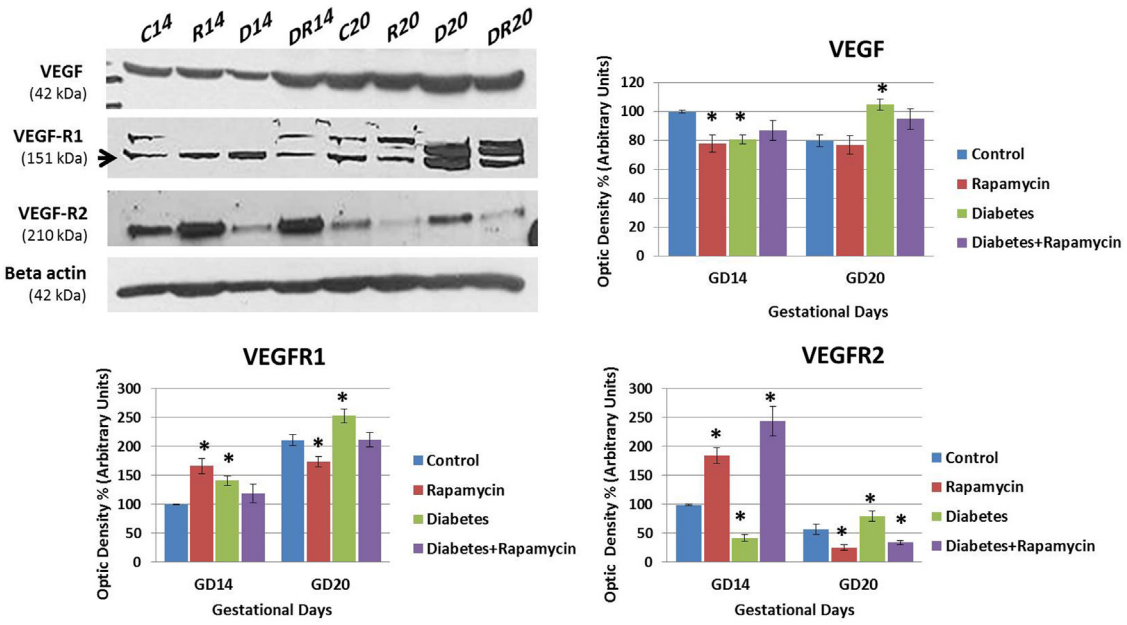


Fig. 5. Western Blot results of angiogenic factors. VEGF expression was significantly decreased at GD14 in Rapamycin and Diabetes groups. At GD20, there was an increase in VEGF expression in diabetic rat placentas. VEGFR1 expression was increased in Rapamycin treated non-diabetic group and STZ injected diabetic group at GD14. On contrary at GD20, Rapamycin treatment decreased VEGFR1 expression in non-diabetic group. In diabetic group VEGFR1 expression remained higher than control at GD20. In Rapamycin treated groups VEGFR2 expression was increased at GD14 and decreased GD20 compared to control and diabetes. C: Control, D: Diabetes, R: Rapamycin, DR: Diabetes + Rapamycin, $^{*}p \leq 0.05$.

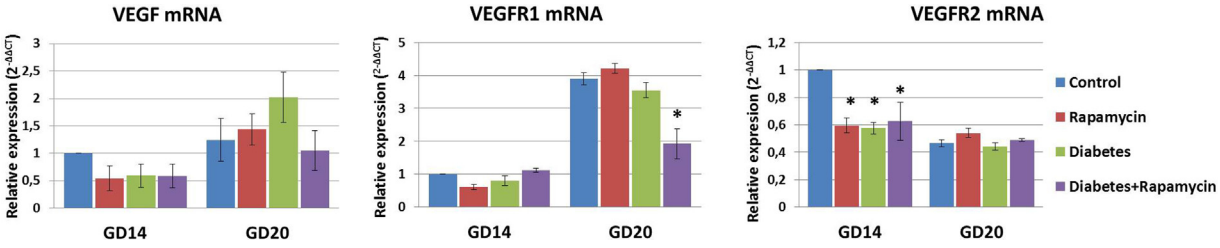


Fig. 6. mRNA levels of angiogenic factors. VEGF mRNA levels were similar between all groups. VEGFR1 mRNA was significantly decreased at GD20 in Diabetes + Rapamycin group. VEGFR2 mRNA levels were significantly decreased at GD14 in Rapamycin administered diabetic and non-diabetic groups. GD: Gestational Day, $^{*}p \leq 0.05$.

decreased VEGF and VEGFR1&2 expressions were observed in rat placentas of diabetic and non-diabetic groups after mTOR pathway inhibition depending on gestational age. VEGF expression was decreased at mid-gestation in non-diabetic group but un-changed at term. On contrary after Rapamycin administration, VEGFR1 and VEGFR2 expression was decreased at term in diabetic and non-diabetic group rat placentas, but increased at GD14. According to our results, VEGF Receptor expression seems relatively more affected from diabetes, Rapamycin administration, and gestational age rather than VEGF itself. It is now well known that as a consequence of interaction with VEGF, VEGFR2 has a higher tyrosine kinase activity compared to VEGFR1 [20]. Activation of VEGR2 by VEGF, triggers several intracellular signaling pathways which have roles in proliferation, migration, survival etc . . . [21]. According to our results, VEGF and VEGFR2 expressions in diabetic conditions are well correlated which may indicate a regulatory role in placental growth regulation. An important finding of our results is that in Rapamycin administered diabetic and non-diabetic groups VEGFR2 expression pattern is positively correlated with alterations in placental weight during gestation. Therefore it might be suggested that placental angiogenesis will be closely related with placental growth regulation in diabetes and mTOR may be an important candidate pathway mediating the process.

Besides providing data about placental angiogenesis mechanisms, our results may also suggest pre-clinical data about Rapamycin usage during pregnancy. mTOR inhibitors are clinically used as immunosuppressive agents. Immunosuppressive drugs can cross the placental barrier and enter the fetal circulation [22]. With the widespread use of sirolimus and everolimus as primary immunosuppressant in organ transplant recipients [23], a question rose if these drugs may be teratogenic, and may be contraindicated for pregnancy [24].

The FDA currently includes mTOR inhibitors in Pregnancy Category C (fetal risk cannot be ruled out); therefore, in patients treated with mTOR inhibitors, pregnancy is discouraged. The risk of teratogenicity in humans may be a major concern, but no reports about fetal malformation in humans are available [25]. Although in several papers everolimus therapy seems promising [25–27], more data are needed to determinate whether the use of mTOR-inihibitors is safe during pregnancy.

In conclusion, our study reveals that 0.05 mg/kg Rapamycin administration during pregnancy decreased placental and fetal weight at term. Diabetic microenvironment also resulted with alterations in placental and embryonal weights. Our hypothesis was that mTOR pathway will be a mediator for hyperglycemia related effects on placental angiogenesis mechanisms. The results of our study indicates a putative relationship between placental

angiogenesis and mTOR pathway but more detailed studies are necessary to be able to explain the whole processes in detail.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgement

This study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) with 113S463 project number.

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