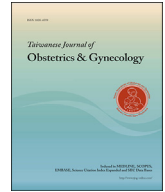




Contents lists available at ScienceDirect

Taiwanese Journal of Obstetrics & Gynecology

journal homepage: www.tjog-online.com

Original Article

Protective effects of quercetin on uterine receptivity markers and blastocyst implantation rate in diabetic pregnant mice

Ayeh Bolouki ^a, Fatemeh Zal ^{a,b,*}, Zohreh Mostafavi-pour ^a, Azizollah Bakhtari ^c^a Biochemistry Department, Medical School, Shiraz University of Medical Sciences, Shiraz, Iran^b Infertility Research Center, Shiraz University of Medical Sciences, Shiraz, Iran^c Reproductive Biology Department, School of Advanced Medical Sciences and Technologies, Shiraz University of Medical Sciences, Shiraz, Iran

ARTICLE INFO

Article history:

Accepted 21 July 2020

Keywords:

Diabetes

Phytoestrogen

Quercetin

Uterine receptivity markers

Blastocysts implantation sites

ABSTRACT

Objective: Diabetic women have different reproductive problems. In pregnant diabetic women, high rates of perinatal mortality, spontaneous abortion and congenital anomalies are observed. We hypothesized that quercetin, as an antidiabetic and phytoestrogen, might have protective effects on the embryo implantation in pregnant diabetic mice. We investigated the ameliorative effects of quercetin on the levels of serum estrogen and progesterone, rate of blastocyst implantation, and uterine receptivity markers in diabetic mice.

Materials and methods: Diabetic and healthy female mice were treated with quercetin (30 mg/kg/day) four weeks before pregnancy. Plasma sex-steroid levels were determined on day 4 of pregnancy. Also, uteri were harvested for investigation of protein and mRNA expression changes. In another set of our study, implantation rate was determined on day 5 of pregnancy.

Results: Our results indicated that quercetin was significantly reduced blood glucose levels in diabetic mice. The number of implantation sites as well as serum estradiol level was reduced in diabetic mice, and then treatment with quercetin significantly increased both. On the other hand, *insulin like growth factor1*, *integrin $\alpha\beta3$* , and *cyclooxygenase2* mRNA expression in the uterus of diabetic mice were significantly reduced, and quercetin treatment augmented the expression level of these genes. Besides, the level of inactive β -catenin protein level in the uterus of diabetic mice was higher than normal group; treatment with quercetin reduced the level of inactive β -catenin protein as compared to diabetic mice.

Conclusion: We conclude that administration of quercetin before pregnancy can probably alleviate reproductive problems in diabetic women likely via its estrogenic and antihyperglycemic effects.

© 2020 Taiwan Association of Obstetrics & Gynecology. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Implantation is the early stage of pregnancy at which the blastocyst adheres to the wall of uterus. For successful blastocyst implantation, a series of molecular and structural changes should occur in the uterus early in pregnancy in order to be able to receive the conceptus [1]. The early step of blastocyst implantation is highly sensitive to metabolic disturbances. Therefore, the investigation of the embryo-maternal crosstalk around the time of implantation could be an important approach in the understanding of

the adverse effect of a metabolic disturbance that could exert on implantation [2].

Diabetes mellitus is one of the most common endocrine disorders that associated with metabolic disturbances. During pregnancy, diabetes causes reproductive abnormalities. In pregnant diabetic individuals, high rates of perinatal mortality, spontaneous abortion and congenital anomalies were observed [3]. Studies indicated that the risk of embryo loss after implantation in women with diabetes was nine-times higher than in healthy women [4]. Besides, studies with diabetic animal models demonstrated uterine atrophy [5] and alterations of the hypothalamic-hypophyseal ovarian axis [6]. Implantation may also be influenced by an increase of stress oxidative associated with diabetic pathology [7]. Most diabetic animal studies focused on embryo development after implantation and during organogenesis. Besides, in spite of important development in treatment of diabetic women, rate of

* Corresponding author. Shiraz University of Medical Sciences, Medical School, Shiraz, Iran. Fax: +98 711 32303029.

E-mail address: fatemehzal@yahoo.com (F. Zal).

reproductive abnormalities is still high in diabetic pregnant women.

Implantation is driven by the coordinated action of 17- β estradiol (E) and progesterone (P) [8,9]. In mice during days 1–2 of pregnancy, estrogen is the predominant hormone secreted by the ovary and results in the proliferation of uterine cells. After that, with the increase of progesterone secretion from the newly formed corpora lutea, the uterine epithelium switches from a proliferative to a differentiated state, and becomes receptive to embryo attachment. This estrogen secretion by ovary in early gestation is an essential requirement for initiation of the implantation process [9]. Animal models of diabetes had shown changes in the hormonal secretion at the time of implantation and also modifications in the kinetic and gene expression of estrogen receptors in the uterine tissue [10]. Recent developments in molecular methods were associated with the discovery of various molecules involved in the embryo implantation such as steroid hormones, growth factors, integrins and prostaglandins [1]. Among different type of growth factors, insulin like growth factor-1 (IGF1) has an important role in growth, apoptosis, metabolism, and development of uterus around the time of embryo implantation [11]. The other uterine development marker that we investigated in this study is integrin $\alpha\beta3$. Integrins are a class of cell adhesion molecules. Heterodimers of α and β integrins could serve as receptors for extracellular matrix ligands and transduce signals from soluble ligands [12]. Integrin $\alpha\beta3$ expression in the uterus is upregulated at the time of implantation. Because integrin $\alpha\beta3$ upregulation coincides with the implantation time, this has led to the conclusion that this integrin could serve as a marker of uterine receptivity [13].

As another important pathway in uterine receptivity development, Wnt signaling plays a pivotal role in the development and functioning of the endometrium and implantation of blastocysts [14].

The other aspect of structural changes in the preimplantation uterus is apoptosis. During the initial steps of implantation, the mouse uterine epithelium of the implantation chamber undergoes apoptosis in response to the interacting blastocyst (day 4.5 of pregnancy in mouse) [15]. With progressing implantation, regression of the decidual cells allows a restricted and coordinated invasion of trophoblast cells into the maternal compartment. Cell proliferation and apoptosis occur simultaneously in tissues undergoing remodeling and both processes are tightly regulated in the uterus during implantation of embryos [16].

Quercetin, a flavonoid present in food sources such as apples, berries and onions has been reported to possess many health effects on multiple organ systems [17]. Quercetin is known as a strong antioxidant, anti-inflammatory, anti-carcinogenic, anti-viral, cardioprotective as well as estrogenic, anti-estrogenic [18,19] and antihyperglycemic agent [20]. Quercetin reduced blood glucose levels and ameliorated different complications of diabetic animal models [21]. Quercetin has also been found to stimulate both types of estrogen receptor alpha (ER α) and beta (ER β) [22]. Therefore it has been proposed that quercetin could be used as a phytoestrogen agent.

Regarding the deleterious effect of diabetes on the estrogen level and blastocyst implantation, we investigated the ameliorative effects of quercetin, as an antidiabetic and phytoestrogen agent, on the blastocyst implantation in an animal model of diabetic pregnancy.

Materials and methods

Induction of diabetic state and animal treatment

Wild-type BALB/c female mice, weighing 30 ± 5 g, were purchased from comparative and experimental medicine center of

Shiraz University of Medical Sciences. All procedures performed in studies involving animals were in accordance with the ethical standards the use of laboratory animals adopted by Shiraz University of Medical Sciences. Mice were given free access to food and water, and maintained in a 12 h light/dark cycle (lights on at 06:00 h).

Seventy five female mice were randomly distributed into five groups of fifteen, which received the following treatments for 4 weeks:

1-Normal, 2- diabetic group, 3- diabetic group with quercetin at 30 mg/kg/day, 4- quercetin at 30 mg/kg/day and 5- vehicle group, 0.3% ethanol controls.

Diabetic state was induced by intraperitoneal injection of 175 mg/kg streptozotocin (Sigma, USA). Non-diabetic animals received citrate buffer alone. Only diabetic animals with a fasting glucose level of ≥ 250 mg/dl were used for the various studies. In this study, quercetin (Sigma, USA) was dissolved in 0.3% ethanol prior to gavage and working concentration of quercetin was 3.75 mg/ml. After inducing diabetes, they were treated daily with quercetin during four weeks. At the end of four weeks of quercetin treatments, young virgin females (2–3 months old) were mated naturally with untreated young males. The animals were checked each morning and when a vaginal plug was seen, that day was designated as gestation day 0.5. All the animals were sacrificed with cervical dislocation on Day 4.5 of pregnancy. Blood samples were collected at sacrificing time by cutting the tip of the animal's tail. In the diabetic group, animals with glycaemia below 200 mg/dl were excluded from the experiment. Uteri were carefully dissected and placed in ice-cold PBS in petri dishes. Using a dissecting microscope, we carefully flushed the uteri to the exit of the blastocysts and then uterine tissues immediately were frozen in liquid nitrogen and stored at -80°C until further use.

Determination of implantation sites number

In another set of our experiment, mice were sacrificed on Day 5.5 of pregnancy, and 0.1–0.2 ml of 1% Evans Blue (Sigma, USA) in normal saline was injected intravenously via the tail vein to stain areas of high vascularity including sites of embryo implantation. Images of the implantation sites (blue stained spots) and inter-implantation sites (unstained spaces between two blue-stained spots) in the uterus were captured, and the number of implantation sites was counted.

Measurement of 17 β -estradiol and progesterone levels by ELISA

Serum sex-steroid levels were determined from the serum of blood collected on Day 4.5 of pregnancy (prior to sacrifice). The blood was collected into separator tubes, allowed to clot for 30 min at room temperature, followed by centrifugation at $3000 \times g$ for 15 min. Serum was aliquoted and stored at -20°C . Levels of estradiol and progesterone were analyzed by enzyme-linked immunosorbent assay (ELISA) commercial kit (MyBiosource, San Diego, USA). Analyses were done in triplicate. ELISA was performed according to the manufacturer's guidelines. Absorbance was read by use of a microplate reader (Eppendorf, North America) at a wavelength of 450 nm. A set of standard dilution of known concentrations were used to construct a standard curve prior to measuring hormone levels extrapolated from the curve.

Quantification of mRNA levels by real time PCR

Total RNA was isolated from mice uteri by the Biozol reagent (BioFlux, Japan) according to the manufacturer's guidelines. The purity and concentration of RNA were assessed by determining

260/280 UV absorption ratios. Reverse transcription into cDNA was performed by QuantiTect Reverse Transcription Kit (Qiagen, Germany) which includes a step for the elimination of genomic DNA. The primers used are shown in Table 1. Real-time RT-PCR was performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, USA). The PCR reaction was prepared in a final volume of 25 μ l consisting of 12.5 μ l of RealQ Plus 2x Master Mix Green Low ROX (Ampliqon, Denmark), 1 μ l of each primer (10 pmol/ μ l), and 1 μ l of the cDNA template. *gapdh* was used as reference or house-keeping gene as their expression in the uterus is reported to be the most stable [23]. Samples were analyzed using $2^{-\Delta\Delta Ct}$ method with three replicates per group.

Quantification of protein expression levels by Western blotting

Whole uterine tissues were snap frozen in liquid nitrogen and then stored at -80°C . Protein isolation and Western blot analyses were performed with 20 μ g protein extracted from uterus. Following extraction with RIPA solution (NaCl 150 mM, Nonidet P-40 (1%), Sodium deoxycholate (DOC) (0.5%), SDS (0.1%), Tris 50 mM, pH 7.4, 2.5 μ l protease inhibitor cocktail and 3 μ l phosphatase inhibitor cocktail per 50 mg tissue), equal amount of protein was mixed with a loading dye, boiled 5 min and separated with SDS-PAGE 12%. Protein was then transferred onto nitrocellulose membrane, blocked with 5% BSA for 2 h at room temperature. The membrane was then exposed to ser33/37/thr41 phospho-beta catenin (inactive β -catenin) primary antibody (Cell Signaling technology, USA) at a dilution of 1:250 in TBS containing 1% BSA and Tween-20 overnight. The blots were rinsed three times in TBS-T, 5 min each. The membranes were incubated with anti-rabbit horseradish peroxidase (HRP) conjugated secondary antibody (Cell Signaling technology, USA) at a dilution of 1:2000, for 1 h and then subjected to ECL Western Blotting Substrate Kit (Abcam, USA) to visualize the protein bands. Photos of the blots were captured by ChemiDoc™ MP Imaging System (Bio-Rad, USA) and density of each band was determined by Image Analysis Software (Bio-Rad, USA). The ratio of each target band/GAPDH was calculated and considered as the expression level of the target. The average ratio for each band/GAPDH was obtained from three different membranes. Each membrane was from different animals receiving similar treatment.

Caspase3 activity assay

Uterine cell lysates were extracted, and protein concentration was measured by Bradford protein assay. Caspase3 activity was detected using caspase3 colorimetric detection kit (Abcam, USA) by measuring the absorbance of free p-nitroaniline (pNA) generated by cleavage of Ac-DEVD-pNA as a colorimetric substrate according to the manufacturer's instructions. Briefly, 50 μ g of protein for each uterine cell lysate in triplicate in a 96-well plate were incubated with caspase3 substrate for 2 h at 37°C . Then, the caspase3 activity was measured by microplate reader (Eppendorf, North America) at 405 nm absorbance. Activity was expressed as U/ μ g cytoplasmic protein. One unit of caspase3 activity corresponds to the amount of enzyme needed to convert one picomole of substrate per minute at 37°C .

Table 1
Primers used in Real-time PCR.

Gene name	Forward sequence	Reverse sequence	Length of amplicon (bp)
IGF1	TCCTGTGTTCTTCTATGTCC	CCTGCTGTATTCTCTTCTAT	145
Integrin α v	TCAAGGAGGATTCAGCATT	CCACAGAGTAACCCAAATAAC	215
Integrin β 3	CCTTGCTACTCTGCTCATCTG	GCTCTGGCTCGTCTCTCC	154
COX2	TGCCTGGTCTGATGATGATGC	TGAGTATGAGTCTGCTGTTTGG	126
GAPDH	TGTTTCCTCGTCCCGTAGA	ATCTCCACTTGGCACTGC	106

Statistical analysis

Statistical analysis was performed by using one-way ANOVA test (PRISM software version 6.0; GraphPad, San Diego, CA). $P < 0.05$ was considered as significant. Differences between experimental groups were determined by the Tukey's test and all values obtained were >0.8 which indicate adequate sample size. All data are representative of at least three individual experiments performed in triplicate. As all the results of the normal and vehicle groups were similar approximately, only results related to the normal group were shown in the tables and figures.

Results

Quercetin treatment increased estradiol level in diabetic pregnant mice

In this study, weekly monitoring of blood glucose levels indicated that levels of blood glucose were significantly reduced in diabetic mice that received 30 mg/kg/day quercetin in comparison to diabetic mice (Table 2, $P < 0.05$). As shown in Table 2, serum estradiol level in diabetic mice was significantly lower than that in normal group ($P < 0.05$). On the other hand, in diabetic mice receiving 30 mg/kg/day quercetin serum estradiol level was approximately 1.5-fold higher than that in the diabetic group. However, serum progesterone level was remained unchanged in all groups, but the estradiol/progesterone ratio was lower in diabetic mice and increased following quercetin administration.

Quercetin was able to protect blastocysts implantation in diabetic pregnant mice

Table 2 shows the effect of diabetes and administration of 30 mg/kg/day quercetin on the number of embryo implantation sites. As our results indicated, number of implantation sites was reduced by 40% in diabetic group in comparison to the normal group ($P < 0.05$). However, in diabetic mice treated with quercetin the number of implantation sites were significantly increased in comparison to the diabetic group ($P < 0.05$). In contrast, quercetin treatment in normal mice slightly, but statistically significantly reduced the number of implantation sites (Fig. 1 and Table 2).

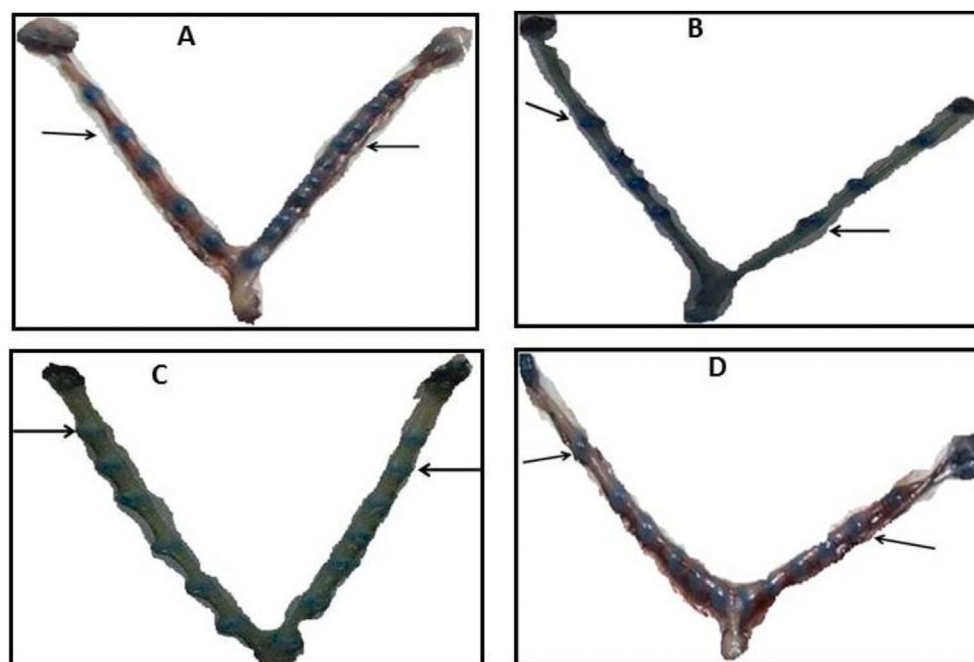
Quercetin treatment increased expression of IGF1, integrin α v β 3 and COX2 in uterine tissue of diabetic pregnant mice at implantation time

Transcript abundance of three markers of uterine receptivity, including IGF1, integrin α v β 3 and COX2 was assessed for each group at the time of embryo implantation in uteri with semi quantitative (Fig. 2) and quantitative (Fig. 3A–D) RT-PCR. Results showed that the level of *igf1* mRNA was down-regulated by more than 80% in the diabetic mice. Quercetin treatment corrected the negative effects of diabetes on the expression of this gene at the time of embryo implantation in diabetic mice. Besides, the level of *igf1* mRNA in the

Table 2

Blood glucose concentration, estradiol and progesterone levels and number of embryo implantation sites in diabetic mice and following administration of quercetin.

Group	blood glucose concentration (mg/dl)	17.βestradiol (μl/ml)	Progesterone (μl/ml)	Estradiol/progesterone ratio	NO. of implantation	P value
Normal	200 ± 7.85	82.18 ± 15.66	165.2 ± 11.43	0.49	14.37 ± 0.79	<0.05
Diabetic	383 ± 11.89 ^a	53.85 ± 11.33 ^a	162.9 ± 14.76	0.33	5.89 ± 0.8 ^a	<0.05
Diabetic + quercetin	218 ± 10.16 ^b	83.98 ± 12.45 ^b	163.7 ± 13.67	0.51	14 ± 0.58 ^b	<0.05
Quercetin	201 ± 3.91	110.5 ± 16.45 ^a	160.8 ± 16.56	0.68	10.75 ± 0.8 ^a	<0.05

^a p < 0.05 compared to Normal group. Data were expressed as mean ± S.D. with n = 8 mice per group.^b p < 0.05 compared to Diabetic group. Data were expressed as mean ± S.D. with n = 8 mice per gr.**Fig. 1.** Representative images showing embryo implantation site in mice. Blue-stained granules indicate sites of embryo implantation. Arrows showing implantation sites. A- Normal, B- Diabetic, C- Diabetic + quercetin, D- Quercetin.

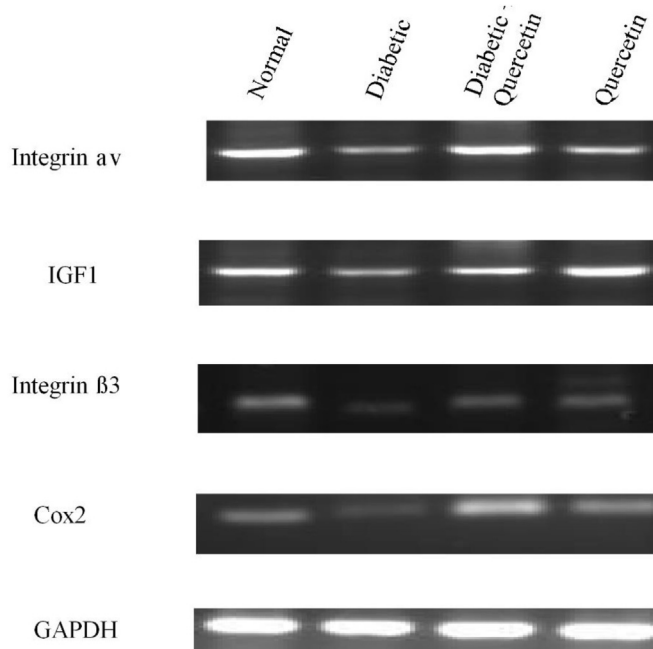
uterus of normal mice was markedly increased following quercetin treatment as shown in Fig. 3A ($P < 0.05$).

Fig. 3B and C present the results of quantitative RT-PCR for αv and $\beta 3$ integrin subunits expression in normal and diabetic groups, as well as those administered with 30 mg/kg/day quercetin. Data exhibited a reduction of about 50% and 65% in the αv and $\beta 3$ subunits of integrin mRNA expression, respectively, in diabetic group compared with the normal group ($P < 0.05$). However, in diabetic mice treated with quercetin, mRNA expression levels of these subunits of integrin in uterus were markedly increased ($P < 0.05$) as compared to the diabetic mice. Moreover, in normal mice receiving quercetin treatment, *integrin αv* mRNA level in uterus was significantly lower than that in the normal group ($P < 0.05$).

Our results in Fig. 3D indicated that the level of *cox2* mRNA in the uterus was reduced more than 50% in diabetic mice in comparison to the normal ones ($P < 0.05$). Data showed an approximate 2.5- fold enhancement in the *cox2* mRNA expression in diabetic group receiving quercetin treatment compared to the diabetic group ($P < 0.05$).

Quercetin treatment increased active form of β -catenin in uterine tissue of diabetic pregnant mice at implantation time

We performed Western blot analysis to evaluate the Ser33/37/Thr41phospho-beta catenin (inactive β -catenin) protein level in the uterus of pregnant mice at the time of embryo implantation in

**Fig. 2.** Semi quantitative PCR.

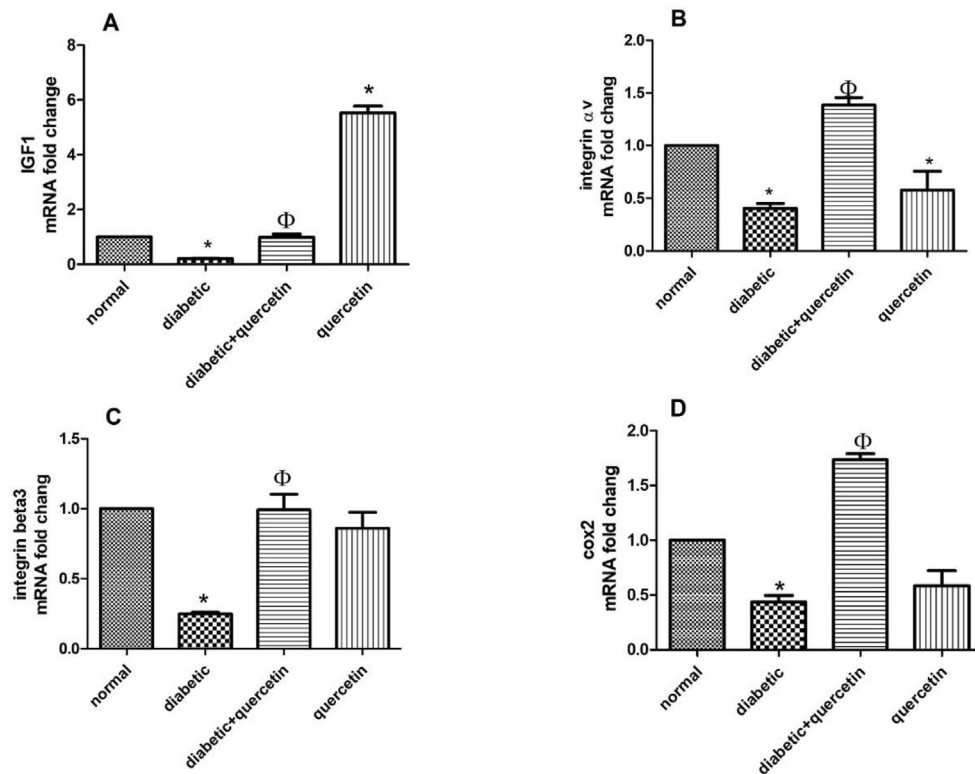


Fig. 3. qRT-PCR analysis of the expression of the A- IGF1, B- integrin αv , C- integrin $\beta 3$ and D- COX2 genes in the mice uterus. *p < 0.05 compared to Normal group and Φ p < 0.05 compared to Diabetic group (one-way ANOVA followed by Tukey post hoc test). Data are presented as Mean \pm SEM.

normal and diabetic mice with/without quercetin treatment. As shown in Fig. 4, results indicated that inactive β -catenin protein level in uterus was increased in diabetic mice as compared to the normal group ($P < 0.05$). Further observed was a significant lower inactive β -catenin protein expression level in the uterus of diabetic mice which received 30 mg/kg/day quercetin in comparison to diabetic mice ($P < 0.05$).

Quercetin treatment reduced apoptosis in uterine tissue of diabetic pregnant mice at implantation time

In this study, we measured Caspase3 activity, as a marker of the extrinsic and intrinsic signaling pathways of apoptosis, in the

uterine tissue. Results indicated that Caspase3 activity had an approximate 8-fold increase in diabetic mice as compared to the normal mice ($P < 0.05$) and was reduced in diabetic mice following quercetin treatment as compared to diabetic mice (Fig. 5, $P < 0.05$).

Discussion

We investigated the impact of quercetin administration, as a phytoestrogen and antidiabetic agent, on uterine receptivity and blastocyst implantation in diabetic animal models. We showed that 30 mg/kg/day quercetin administration before pregnancy reduced blood glucose levels, increased serum 17β -estradiol, and improved uterine receptivity and blastocyst implantation in the diabetic

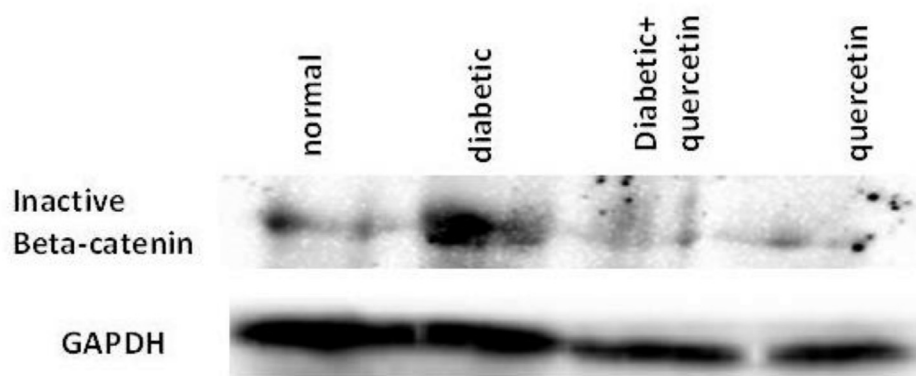


Fig. 4. Expression pattern of ser33/37/thr41 phospho-beta catenin (inactive β -catenin) protein in uterus from pregnant mice at the time of embryo implantation. Anti ser33/37/thr41 phospho-beta catenin (inactive β -catenin) antibody used in immunoblotting assays against extracts from uterine tissues, recognized a protein band of ~ 92 kDa for Ser33/37/Thr41 phospho-beta catenin (inactive β -catenin) as expected. The antibody against gapdh revealed the expected 37 kDa band of the GAPDH protein.

pregnant mice. The results of the presented study were summarized in Fig. 6. In our study, diabetic mice showed a significant increase in blood glucose level compared with the normal mice. Administration of quercetin in diabetic mice caused a reduction in the level of blood glucose which may be due to the anti-hyperglycemic effect of quercetin. This research further measured the circulating estradiol and progesterone levels as two important hormones for uterine proliferation, differentiation, decidualization and blastocyst implantation in mice [8,9]. We reported that in diabetic mice, estradiol production by ovary was inhibited; hence the fact that the diabetic group had lower 17 β -estradiol levels in the implantation period. Serum progesterone levels were not significantly reduced; therefore the estradiol/progesterone ratio was decreased in pregnant diabetic mice. This inhibitory effect is associated with a reduction in implantation rate in diabetic mice. Hormonal imbalance at the implantation site changed uterine receptivity to pre-implantation blastocysts. The decrease in estradiol/progesterone ratio observed in the pregnant diabetic mice was able to reduce the implantation rate of blastocysts. In the present study, we found that the administration of quercetin in diabetic mice normalized estradiol/progesterone ratio. Indeed, serum 17 β -estradiol levels were increased in pregnant diabetic mice receiving quercetin. Furthermore, the implantation rate of blastocysts was increased more than 75% in pregnant diabetic mice that received quercetin. In fact, we think that quercetin is able to improve the implantation of blastocyst by normalizing the estradiol/progesterone ratio. However, in the normal group, administration of quercetin before pregnancy was observed to slightly reduce the number of implantation blastocysts. Moreover, its administration was able to interfere with steroid levels, where serum 17 β -estradiol level increased, which is consistent with the previous findings [24]. In fact, it seems that quercetin was able to disrupt the implantation of blastocysts by interfering with the sex-steroid level, where serum estradiol level increased. Several studies have investigated the effects of quercetin on sex hormone levels and some mechanisms have been suggested. It indicated that quercetin was found to change estrogen metabolism in human liver cells in a way that it

increases estradiol level and reduces other forms of estrogens [25]. Huma Shahzad et al. (2017) indicated that quercetin administration at high doses (25–100 mg/kg/day) augmented serum estrogen levels in early pregnancy [26]. In our previous study, we also have shown that the number and volume of growing follicles and corpora lutea were significantly decreased in diabetic mice, and quercetin treatment increased the volume and number of healthy follicles. Actually, in the previous study, we have shown that impaired follicular growth and development caused by hyperglycemia in diabetic mice can be alleviated through quercetin treatment [27]. In this study, we indicated that quercetin may improve hormonal balance at the implantation time by improving follicles growth in the diabetic pregnant mice. Quercetin is a polyphenolic compound that has estrogen-like effects and can enhance estrogen synthetase (aromatase) activity [28,29]. Quercetin has also been shown to cause morphological and proliferative changes in uterine tissue. Shahzad et al. showed that quercetin stimulates cell proliferation through its effect on estrogen receptors, but the main mechanism is yet to be elucidated [30].

The present research provided the first demonstration that dietary quercetin can improve the deleterious impacts of diabetes on the uterine receptivity and implantation rate. Indeed, it was shown that quercetin administration could affect the uterine receptivity and blastocyst implantation in diabetic mice by changes in the expression of uterine receptivity molecules i.e. *igf1*, *integrin $\alpha v \beta 3$* and *cyclooxygenase2 (cox2)* during pre-implantation period. Expression of *igf1*, which increases during embryo implantation, is dependent on estrogen. Growth factors such as *igf1* are integral components in the growth response of the rodent uterus to estrogen [11,31]. Immunohistochemical analysis during the pre-implantation period demonstrated that on days 4 into pregnancy, *igf1* increased in the uterus of the mice. Indeed, *igf1* is a paracrine mediator of stromal-epithelial cross-talk involved in epithelial proliferation during pre-implantation period, resulting in embryo implantation [11]. In the present study, *igf1* mRNA expression in uterus was reduced in diabetic mice. However, treatment with quercetin in diabetic mice augmented *igf1* expression. Expression of *igf1* in the implantation period was further observed to be markedly increased in normal mice receiving quercetin. In addition to *igf1*, expression of *integrin $\alpha v \beta 3$* in uterus was also reduced in diabetic mice. *Integrin $\alpha v \beta 3$* is an essential molecule for blastocyst implantation and increasing its expression in the uterus coincides with the period of the uterine receptivity [32]. *Integrin $\alpha v \beta 3$* was found to be highly expressed in the uterine luminal and glandular epithelia in normal fertile woman, and its absence was reported to interfere with embryo attachment [33]. Therefore, reduced *integrin $\alpha v \beta 3$* expression in the uterus of diabetic mice can cause interfere with embryo adhesion to the luminal endometrium, subsequently causing implantation failure in the diabetic animal models [13]. In the present research, *integrin $\alpha v \beta 3$* expression at implantation time was observed to be increased in diabetic mice that received quercetin.

Reduced *cox2* expression in the uterus of diabetic mice at the implantation time may also be cause of failure in implantation. *Cox2* gene expression during the adhesion phase is critical for implantation. Recent evidence has reported that steroid hormones could upregulate *cox2* gene expression in the uterus, which plays an important role in embryo implantation and decidualization through prostaglandins synthesis [34]. Disturbances in PG synthesis before or during the time of implantation causes complete inhibition, a delay in implantation or a reduction in the number of implantation sites [35]. Expression of *cox2* in early pregnancy was found to be increased in diabetic mice receiving quercetin.

The present study demonstrated canonical Wnt/beta-catenin signaling pathway to be inhibited in the pregnant diabetic mice

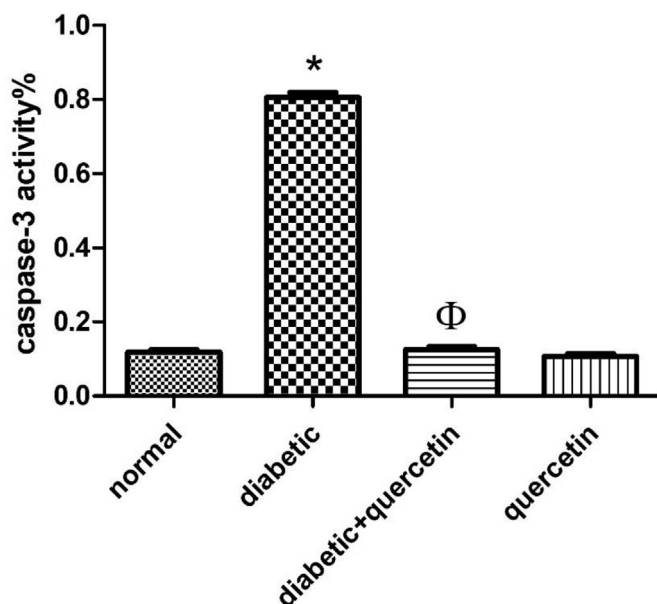


Fig. 5. Apoptosis assay with measurement of caspase-3 enzyme activity in the mice uterus. * $p < 0.05$ compared to Normal group and $\Phi p < 0.05$ compared to Diabetic group (one-way ANOVA followed by Tukey post hoc test). Data are presented as Mean \pm SEM.

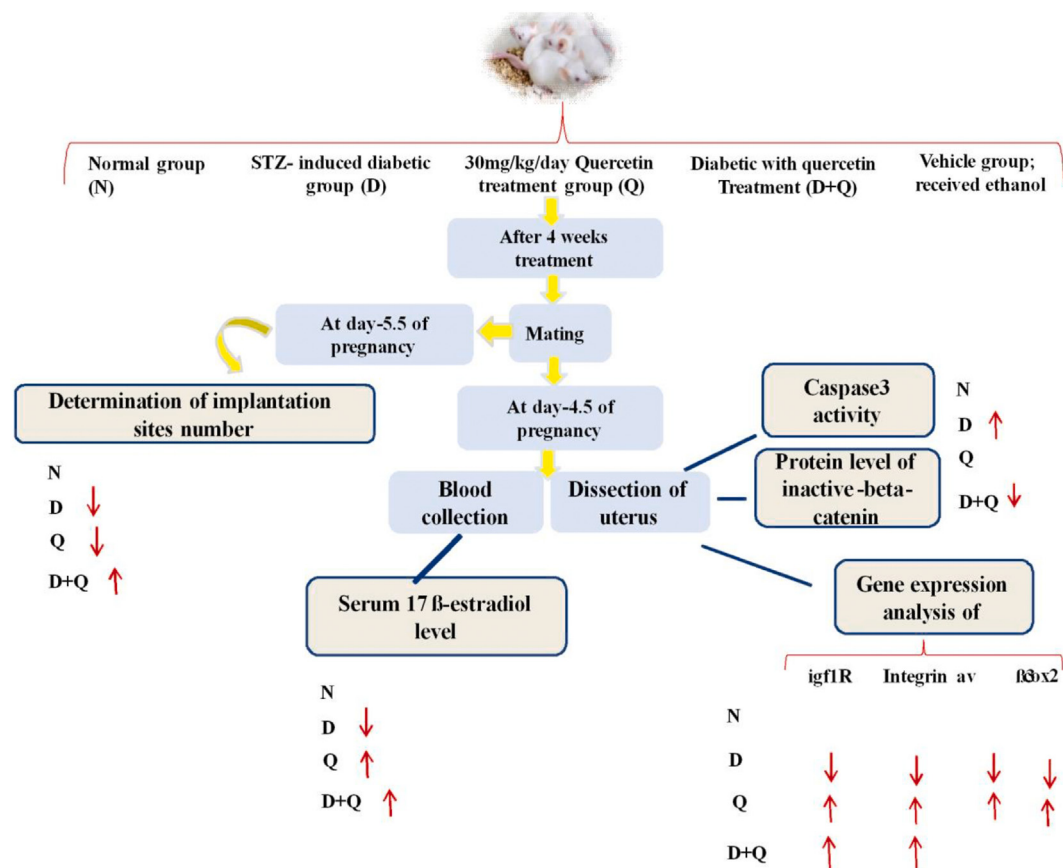


Fig. 6. The summary diagram of results of this study. N) normal group, D) diabetic group, Q) quercetin group and D + Q) diabetic group with quercetin treatment.

at the time of implantation as indicated by the increase in Ser33/37/Thr41phospho-beta-catenin (inactive β -catenin) protein levels. The canonical Wnt/ β -catenin signaling pathway contributes to the cell proliferation, differentiation, and epithelial-mesenchymal communication in uterus during implantation period [36]. It has been shown that *igf1* and *cox2* are the target genes in the Wnt/ β -catenin signaling pathway [37]. In our research, inactive β -catenin level was reduced in pregnant diabetic mice receiving quercetin. Wnt signaling plays a role in mediating estrogen actions in the mouse uterus [38]. This study demonstrated the activation of canonical Wnt signaling pathway in response to the administration of quercetin in pregnant diabetic mice as indicated by the decrease in inactive β -catenin protein level.

In the present study, Caspase3 activity was increased in the uterus of diabetic mice in pre-implantation period, while treatment with quercetin reduced the activity of Caspase3 enzyme. It has been shown that the apoptotic degeneration of the uterine epithelium in the implantation period is mediated by TNF receptor1 followed by Caspase3 [39]. Certain other studies have further indicated that in the mouse uteri, apoptotic epithelial cell death increases when the levels of 17 β -estradiol are reduced and blocked by estrogen treatment [40]. In our study, treatment with quercetin, as a phytoestrogen, was reduced Caspase3 activity in the pre-implantation period in the uterus of diabetic mice.

In sum, quercetin administration was able to increase estradiol level in pregnant diabetic mice and normalize the balance of estradiol/progesterone ratio at implantation time. Additionally, we demonstrated that quercetin diets can improve blastocysts implantation in pregnant diabetic mice. Quercetin probably ameliorates uterine receptivity in pregnant diabetic mice by promoting

cell proliferation through increasing *igf1* gene expression and promoting the adhesion phase of implantation by augmenting *integrin $\alpha v \beta 3$* and *cox2* genes expression. Moreover, a quercetin diet promotes canonical Wnt/ β -catenin signaling pathway in the uterus of pregnant diabetic mice by an unknown manner.

In conclusion, administration of quercetin prior to pregnancy can probably alleviate the reproductive problems in diabetic women, likely via its estrogenic and antihyperglycemic effects.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards the use of laboratory animals adopted by Shiraz University of Medical Sciences.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgment

This paper has been extracted from the PhD thesis of Ayeh Bolouki and was supported by Grant Number 95-11335 from Vice-chancellor for Research Affairs of Shiraz University of Medical Sciences.

References

- [1] Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA, et al. Embryo implantation. *Dev Biol* 2000;223:217–37.

- [2] Jawerbaum A, Gonzalez E. The role of alterations in arachidonic acid metabolism and nitric oxide homeostasis in rat models of diabetes during early pregnancy. *Curr Pharmaceut Des* 2005;11:1327–42.
- [3] Lepercq J. French multicentric survey of outcome of pregnancy in women with pregestational diabetes. *Diabetes Care* 2003;26:2990.
- [4] Moley KH. Hyperglycemia and apoptosis: mechanisms for congenital malformations and pregnancy loss in diabetic women. *Trends Endocrinol Metabol* 2001;12:78–82.
- [5] Garris DR. Diabetes-associated alterations in uterine structure in the C57BL/KsJ Mouse: relationship to changes in estradiol accumulation, circulating ovarian steroid levels, and age. *Anat Rec* 1985;211:414–9.
- [6] Chan O, Chan S, Inouye K, Vranic M, Matthews SG. Molecular regulation of the hypothalamo-pituitary-adrenal axis in streptozotocin-induced diabetes: effects of insulin treatment. *Endocrinology* 2001;142:4872–9.
- [7] Wentzel P, Eriksson UJ. Embryopathy and diabetes. Karger Publishers; 2020. p. 132–44.
- [8] Franco HL, Jeong J-W, Tsai SY, Lydon JP, DeMayo FJ. In vivo analysis of progesterone receptor action in the uterus during embryo implantation. *Semin Cell Dev Biol* 2008;178–86. Elsevier.
- [9] Robertshaw I, Bian F, Das SK. Mechanisms of uterine estrogen signaling during early pregnancy in mice: an update. *J Mol Endocrinol* 2016;56:R127–38.
- [10] Mauvais-Jarvis F. Estrogen and androgen receptors: regulators of fuel homeostasis and emerging targets for diabetes and obesity. *Trends Endocrinol Metabol* 2011;22:24–33.
- [11] Murphy LJ, Ghahary A. Uterine insulin-like growth factor-1: regulation of expression and its role in estrogen-induced uterine proliferation. *Endocr Rev* 1990;11:443–53.
- [12] Humphries MJ, Mostafavi-Pour Z, Morgan MR, Deakin NO, Messent AJ, Bass MD. Integrin-syndecan cooperation governs the assembly of signalling complexes during cell spreading. *Novartis Found Symp* 2005;269:178–88.
- [13] Illera MJ, Lorenzo P, Gui Y-T, Beyler SA, Apparao K, Lessey BA. A role for $\alpha\beta 3$ integrin during implantation in the rabbit model. *Biol Reprod* 2003;68:766–71.
- [14] Chen Q, Zhang Y, Lu J, Wang Q, Wang S, Cao Y, et al. Embryo–uterine cross-talk during implantation: the role of Wnt signaling. *Mol Hum Reprod* 2009;15:215–21.
- [15] Parr EL, Tung H, Parr MB. Apoptosis as the mode of uterine epithelial cell death during embryo implantation in mice and rats. *Biol Reprod* 1987;36:211–25.
- [16] Kokawa K, Shikone T, Nakano R. Apoptosis in human chorionic villi and decidua during normal embryonic development and spontaneous abortion in the first trimester. *Placenta* 1998;19:21–6.
- [17] Formica J, Regelson W. Review of the biology of quercetin and related bioflavonoids. *Food Chem Toxicol* 1995;33:1061–80.
- [18] Moutsatsou P. The spectrum of phytoestrogens in nature: our knowledge is expanding. *Hormones-Athens* 2007;6:173.
- [19] Neisy A, Zal F, Seghatoleslam A, Alaei S. Amelioration by quercetin of insulin resistance and uterine GLUT4 and ER gene expression in rats with polycystic ovary syndrome (PCOS). *Reprod Fertil Dev* 2019;31:315–23.
- [20] Alam MM, Meerza D, Naseem I. Protective effect of quercetin on hyperglycemia, oxidative stress and DNA damage in alloxan induced type 2 diabetic mice. *Life Sci* 2014;109:8–14.
- [21] Hatware K, Annapurna A. The effect of quercetin on blood glucose levels of normal and streptozotocin induced diabetic (type i & type ii) rats. *Int J Pharmaceut Chem Biol Sci* 2014;4:613–9.
- [22] van der Woude H, ter Veld MG, Jacobs N, van der Saag PT, Murk AJ, Rietjens IM. The stimulation of cell proliferation by quercetin is mediated by the estrogen receptor. *Mol Nutr Food Res* 2005;49:763–71.
- [23] Lin P, Lan X, Chen F, Yang Y, Jin Y, Wang A. Reference gene selection for real-time quantitative PCR analysis of the mouse uterus in the peri-implantation period. *PLoS One* 2013;8:e62462.
- [24] Yigitaslan S, Erol K, Özatik FY, Özatik O, Şahin S, Çengelli C. Estrogen-like activity of quercetin in female rats. *Erciyes. Med J* 2016;38.
- [25] Weber A, Jäger R, Börner A, Klinger G, Vollan R, Matthey K, et al. Can grapefruit juice influence ethinylestradiol bioavailability? *Contraception* 1996;53(1):41–7.
- [26] Shahzad H, Giribabu N, Karim K, Kassim N, Muniandy S, Kumar K, et al. Quercetin interferes with the fluid volume and receptivity development of the uterus in rats during the peri-implantation period. *Reprod Toxicol* 2017;71:42–54.
- [27] Bolouki A, Zal F, Bordbar H. Ameliorative effects of quercetin on folliculogenesis in diabetic mice: a stereological study. *Gynecol Endocrinol* 2019;142:186–92.
- [28] Krazeisen A, Breitling R, Möller G, Adamski J. Phytoestrogens inhibit human 17 β -hydroxysteroid dehydrogenase type 5. *Mol Cell Endocrinol* 2001;171:151–62.
- [29] Lacey M, Bohday J, Fonseka SM, Ullah AI, Whitehead SA. Dose–response effects of phytoestrogens on the activity and expression of 3 β -hydroxysteroid dehydrogenase and aromatase in human granulosa-luteal cells. *J Steroid Biochem* 2005;96(3–4):279–86.
- [30] Shahzad H, Giribabu N, Sekaran M, Salleh N. Quercetin induces dose-dependent differential morphological and proliferative changes in rat uteri in the presence and in the absence of estrogen. *J Med Food* 2015;18:1307–16.
- [31] Kobayashi R, Terakawa J, Omatsu T, Hengjan Y, Mizutani T, Ohmori Y, et al. The Window of implantation is closed by Estrogen via insulin-like growth factor 1 pathway. *J Reproduction Infertil* 2017;18:231.
- [32] Lessey B. Endometrial integrins and the establishment of uterine receptivity. *Hum Reprod* 1998;13:247–58.
- [33] Sutherland A, Calarco P, Damsky C. Developmental regulation of integrin expression at the time of implantation in the mouse embryo. *Development* 1993;119:1175–86.
- [34] Paria B, Lim H, Das S, Reese J, Dey S. Molecular signaling in uterine receptivity for implantation. *Semin Cell Dev Biol* 2000;67–76. Elsevier.
- [35] Lim H, Gupta RA, Ma W-G, Paria BC, Moller DE, Morrow JD, et al. Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPAR δ . *Genes Dev* 1999;13:1561–74.
- [36] Tepekoy F, Akkoyunlu G, Demir R. The role of Wnt signaling members in the uterus and embryo during pre-implantation and implantation. *J Assist Reprod Genet* 2015;32:337–46.
- [37] Teo J-L, Kahn M. The Wnt signaling pathway in cellular proliferation and differentiation: a tale of two coactivators. *Adv Drug Deliv Rev* 2010;62:1149–55.
- [38] van der Horst PH, Wang Y, van der Zee M, Burger CW, Blok LJ. Interaction between sex hormones and WNT/ β -catenin signal transduction in endometrial physiology and disease. *Mol Cell Endocrinol* 2012;358:176–84.
- [39] oswig A, Gabriel H-D, Kibschull M, Winterhager E. Apoptosis in uterine epithelium and decidua in response to implantation: evidence for two different pathways. *Reprod Biol Endocrinol* 2003;1:44.
- [40] Kurita T, Wang Y, Donjacour A, Zhao C, Lydon J, Malley B, et al. Paracrine regulation of apoptosis by steroid hormones in the male and female reproductive system. *Cell Death Differ* 2001;8:192.