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The expression of Forkhead transcription factors in decidua and placenta in patients with missed abortion

K. Ozbilgin[1], B. Kahraman[1], A. Turan[2], C. Atay[3], S. Vatansever[1], S. İnan[1], T. Özçakır[4]

[1] Department of Histology and Embryology, School of Medicine Celal Bayar University, Manisa
[2] Departments of Gynecology and Obstetrics, Ercis State Hospital, Van
[3] Department of Computer Technologies, Izmir University of Economics, Balçova, Izmir
[4] Department of Gynecology and Obstetrics, School of Medicine Celal Bayar University, Manisa (Turkey)

Summary

Background: Forkhead transcription factors 3a (FOXO3a) has pleiotropic biological functions in the female reproductive tract. FOXO3a has a function in decidualization, in placental development, and also in inhibition of apoptosis. This study aims to investigate a possible role of FOXO3a in missed abortion. *Materials and Methods*: Decidual and placental tissue samples were obtained from the women with unwanted pregnancy as the control group and with missed abortion as the patient group. Immunohistochemistry technique was utilized to compare FOXO3a expression of the decidual cells in uterine decidual stroma and cytotrophoblast-syncytiotrophoblast cells in placental villous stroma. Immunohistochemistry was evaluated semi-quantitatively utilizing the H-score technique. *Results*: It was demonstrated that H-Scores of FOXO3a expression in both uterine decidual stroma were increased in the missed abortion group (255.83±12.41) than in the normal pregnancy group (133.33±17.43). It was also shown that there was no difference between non-decidual area of the endometrium of the normal pregnancy and the missed abortion group (30.33±4.32; 39.66±14.30, respectively) and placental villous stroma (13.00±1.89; 13.00±1.67, respectively). However, the immunoreactivity of cytotrophoblast and syncytiotrophoblast cells significantly increased in the missed abortion group (18.83±1.47; 322.00±6.06, respectively) than in the normal pregnancy group (11.00±1.26; 254.00±8.17, respectively) ($p < 0.05$). *Conclusion*: These data support the hypothesis that increased FOXO3a expression in missed abortion may prevent the discharge of dead fetus to maintain decidualization, prevention of oxidative stress, immunomodulation, and inhibition of apoptosis.

Key words: Forkhead transcription factors 3a; Decidua; Placenta; Missed abortion.

Introduction

Missed abortion is a pregnancy in which there is a fetal demise (usually for a number of weeks), but with no uterine activity to expel the products of conception. The prevalence of missed abortion is about 2% in singleton pregnancies at 10–14 weeks of gestation [1]. Although studies have mainly focused on diagnosis and treatment of missed abortions, attention has not been paid to the underlying causal factor of missed abortion [2].

It is known that both intertwined and coordinated endometrial and embryonic adequate expression is required for a normal implantation and gestation [3]. The number of differentially expressed genes reveals the extent of changes within the endometrium during conception. One of the them is the FOXO subfamily of Forkhead transcription factors (FOXO) proteins comprises three functionally related members, FOXO1, FOXO3a, and FOXO4, which are identified as the important downstream molecules of phosphoinositide-3-OH kinase (PI3K)/Akt pathway, and that are known to play important roles in adaptation to cellular stress, immunomodulation, and the regulation of apoptosis [4].

FOXO3 has been causally linked to multiple cellular processes, which are activated during human parturition [4, 5]. FOXO3 induces matrix metalloproteinase (MMP)-2 and -9 which actively participates the remodeling process. MMP-2 and MMP-9 have the ability to break down several proteins of the extracellular matrix (ECM) to maintain tightly controlled trophoblastic invasion and remodeling of the endometrium [6].

Decidualization is a series of proliferation and differentiation process of the endometrial stromal cells into the decidual cells. Decidual cells are thought to be involved in embryo implantation and in the maintenance of pregnancy through the regulation of trophoblastic invasion, the development of the blastocyst, hormonal secretions, and the provision of protection for the embryo from maternal immune rejection. The decidualization process is poorly understood at the molecular level; a key stimulus is progesterone action on estrogen-primed endometrial stromal cells, which leads to dramatic transcriptional reprogramming [7]. Kajihara *et al.* reported that FOXO transcription factors upon endometrial decidualization favor tissue preservation and in-

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Table 1. — *H-score of FOXO3a expression on decidua and placental villi on normal pregnancy and missed abortion.*

	Normal pregnancy	Missed Abortion
Decidual cells	133.33±17.43*	255.83±12.41*
Non-decidual cells	30.33±4.32	39.66±14.30
Syncytiotrophoblasts	11.00±1.26	18.83±1.47
Cytotrophoblasts	254.00±8.17	322.00±6.06
Stroma	13.00±1.89	13.00±1.67

*p < 0.05.

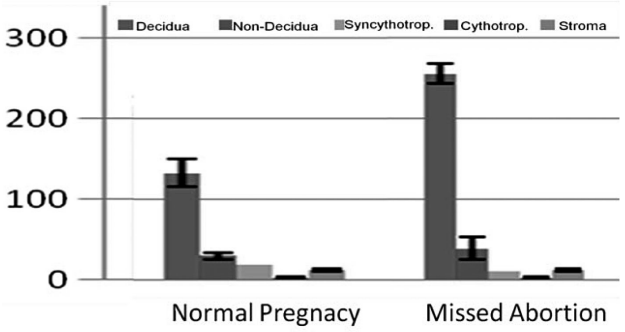

Figure 1. — H-Score of FOXO3a expression is increased in decidual cells, cytotrophoblasts, and syncytiotrophoblast cells.

tegrity over apoptotic clearance of defective cells when faced with prolonged oxidative insult during pregnancy [8].

During normal pregnancy, major fluctuations in oxygen concentrations occur at the feto-maternal interface; the dramatic changes in oxygen tension at the utero-placental interface lead to oxidative stress and induce a burst of intracellular reactive oxygen species [9]. The overexpression of a constitutive active FOXO3a mutant in decasualizing cells causes apoptosis, and the silencing of endogenous FOXO3a confers resistance to oxidative apoptosis in undifferentiated cells [8].

Thus, the aim of this work was to analyze the localization and distribution of FOXO3a expression in decidual and placental tissue both in normal pregnancy and missed abortion, to evaluate pathogenesis of missed abortion.

Materials and Methods

The endometrial tissue samples of 15 unwanted pregnancies (five to ten weeks gestational age) and 19 missed abortions (six to 11 weeks gestational age) were obtained with informed consent and in accordance with the requirements of the Ethics Committee of Celal Bayar University. The mean age of women was 27.53 years; the range 21-37 years for normal pregnancy group and mean 28.74 years; range 18-41 years for the missed abortion group.

The abortions were diagnosed by transvaginal ultrasound and were confirmed by repeat ultrasound prior to the dilation and curettage procedure. Chorionic villi and maternal decidua were separated and cleaned. Placental and decidual tissues were fixed in 10% buffered formalin solution and embedded in paraffin. The blocks were cut in four to five mm thick serial sections. The first tissue sections were stained with FOXO3a primary antibody by means of immunohistochemical technique.

Immunohistochemistry

Formalin-fixed, paraffin-embedded sections were used for immunohistochemical staining. The tissue samples were stored at 60°C overnight and were then dewaxed by xylene for 30 minutes. After the dehydration of the sections with ethanol, they were washed with distilled water. Then, they were treated with 2% trypsin at 37°C for 15 minutes and incubated in 3% H_2O_2 solution for 15 minutes to inhibit endogenous peroxidase activity. Then, the sections were incubated with anti-FOXO3A antibody in a 1/100 dilution for 18 hours at +4°C. They were given an additional three five-minute washes in PBS, followed by incubation with biotinylated IgG and administration of streptavidin peroxidase. After washing the secondary antibody with PBS three times for five minutes, the sections were stained with a substrate system containing diaminobenzidine to detect the immunoreactivity, and then were stained with Mayer's hematoxylin for counterstaining. They were covered with mounting medium and observed with light microscopy.

Immunostaining for FOXO3a was evaluated semi-quantitatively by means of FOXO3a analysis. Immunostaining intensity was categorized into the following scores: 0 (no staining), 1 (weak, but detectable, staining), 2 (moderate staining), and 3 (intense staining). A HSCORE value was derived for each specimen by calculating the sum of the percentage of cells for fibroblast and decidual cells in uterine decidual stroma; and fibroblasts and mesenchymal cells in placental villous stroma that stained at each intensity category multiplied by its respective score, by means of the formula H-score=$\sum$Pi (i+1), where i =intensity of staining with a value of 1, 2 or 3 (weak, moderate or strong, respectively) and Pi is the percentage of stained epithelial cells for each intensity, varying from 0 to 100%. For each slide, five different fields were evaluated microscopically at x 200 magnification. H-score evaluation was performed independently by at least two investigators blinded to the source of the samples, as well as to each other's results; the average score of both were utilized.

Statistical analysis

Immunohistochemical values (mean ± SD) data are summarized in Table 1. The comparisons between the two groups were performed by means of Mann-Whitney U test. A p-value < 0.05 was considered significant. The statistical analysis was performed via SPSS statistical software, version 10.0. Error bars of H-Score of HBEGF expression is shown in Figure 1.

Results

In the examination of the deciduas of the samples stained with FOXO3A antibody, the decidua of the group of missed was determined to have a denser immunoreactivity (255.83±12.41) than the control group (133.33±17.43) and a statistical difference between them (p <0.05) was found. When examining the non-decidual regions, the immunoreactivity of both groups was observed to be relatively weaker than decidual region, and a statistically significant difference was not determined between the non-decidua of the control group (30.33±4.32) and the missed group (39.66±14.30) (Table 1, Figures 1, 2).

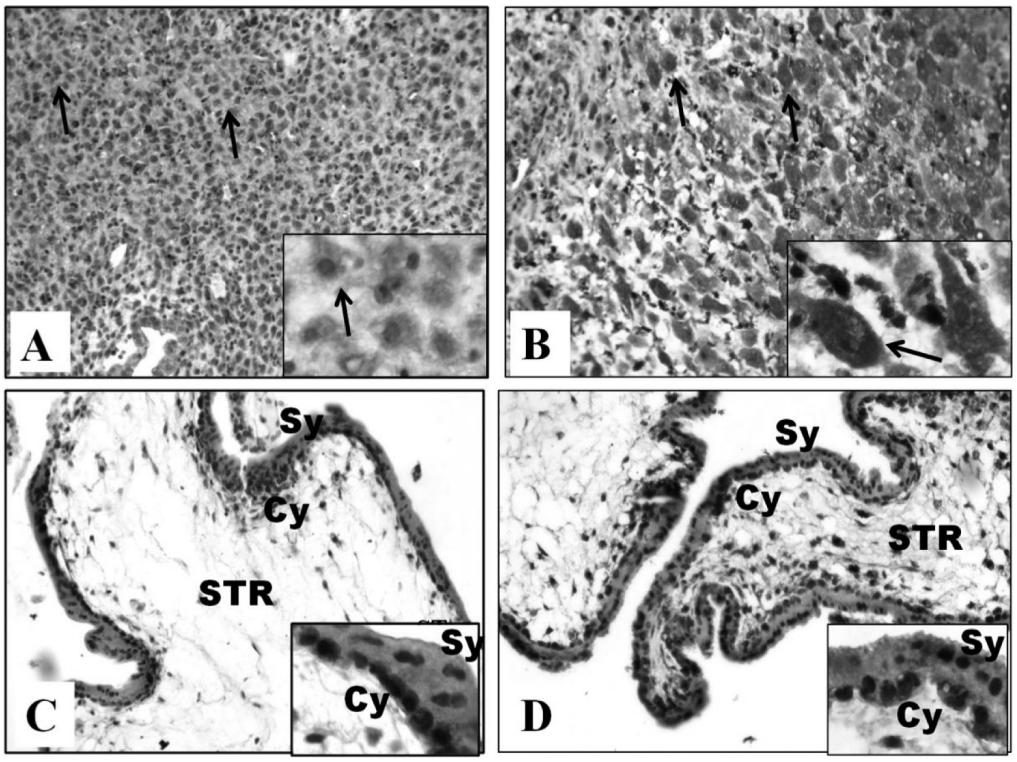

Figure 2. — Immunohistochemical localization of FoxO3 in human decidua (A,B) and placental villi (C, D) on the normal pregnancy (A, C) and missed abortion (B, D). Strong FoxO3 staining has been determined in the decidual cells (arrows) on missed abortion group (B). FoxO3 expression is primarily localized in the cytotrophoblasts and the staining intensity is higher in the missed abortion group than normal pregnancy. The original magnification was x200, and magnification at insert was x400 for syncytiotrophoblast cells (Sy) and stroma (STR).

Moreover, in the examination of FOXO3A immunoreactivities of syncytiotrophoblast and cytotrophoblast forming chorionic villi, the syncytiotrophoblast immunoreactivity of the missed group (18.83±1.47) was observed to be stronger than the control group (11.00±1.26). When examining the cytotrophoblasts, unlike syncytiotrophoblasts, the FOXO3A immunoreactivity was determined to be rather stronger, and the missed group was observed to have a stronger immunoreactivity than the control group (respectively, 322.00±6.06; 254.00±8.17), and a statistically significant difference ($p < 0.05$) between them was as also determined (Table 1, Figures 1, 2).

In the comparison of the immunoreactivity of the stromas of the control group and the missed abortion group, it has been determined that both groups have showed a weak immunoreactivity (13.00±1.89; 13.00±1.67) and there has not been a statistically significant difference between them (Table 1) (Figures 1, 2).

Discussion

This is the first study to report on the immunolocalization of FOXO3 in human decidua and placenta of missed abortion. The authors observed that FOXO3 is found in decidual and placental cells of the normal pregnancy and missed abortion groups. FOXO3 expressions are higher in decidual cells, cytotrophoblast, and syncytiotrophoblast in missed abortion group compared to normal pregnancy. The localization of FOXO3 in decidua and placenta of both groups suggests that it has several potential functions during pregnancy and missed abortion pathogenesis.

The authors observed that strong FOXO3a immunoreactivity both in decidua and in missed abortion and normal pregnant endometrium and have supposed that FOXO3a has an important role in maintaining the decidualization during pregnancy. FOXO proteins are not constitutively expressed in human endometrium. Kajihara *et al.* also reported that prolonged exposure to H_2O_2 induced FOXO3a expression in undifferentiated cells [8]. Christian *et al.* have shown that Forkhead homologue in rhabdomyosarcoma is induced in decidualizing endometrium and participates in PKA signal transduction through its ability to interact and transcriptionally cooperate with C/EBP β [10]. The decidua, that is, undergoes noticeable differentiation into the deciduum, a specialized and neovascularized tissue that encapsulates the developing embryo to provide nutrients and control trophoblastic invasion [11].

It is known that FOXO proteins regulate ECM remodelling enzymes and also FOXO3 induces MMP-2 and MMP-9 enzymatic activities [5]. The present authors also considered that FOXO3a may be responsible for pregnancy process and for maintaining decidual remodeling via MMP-2 and MMP-9. Fontana *et al.* evaluated in vivo and in vitro decidual MMP-2 and -9 activities on 10th day of gestation in CF-1 mouse and suggested specific roles for MMP-2 and MMP-9 in decidual tissues [12]. Matsumoto *et al.* reported

that the activity of MMP-2 and MMP-9 might increase during decidualization without a corresponding increase of the expression of these genes [13]. Nissi *et al.* also reported that increase in serum levels of MMP-2/TIMP-2 complex and MMP-9 as well as their inhibitors TIMP-1 and TIMP-2 could reflect the altered architecture of the extracellular matrix during pregnancy [14].

MMP-2 and MMP-9 are not essential for the decidualization, but also they are essential for invasion of EVT cells into endometrial stroma. Invasion of trophoblastic cells into the maternal endometrium is an important step in human embryo implantation and placentation. Cohem *et al.* reported that this process requires MMP-2 and -9 [15], which are considered key enzymes in degradation of basement membrane, that mainly consist of type IV collagen [16]. Staun-Ram *et al.* reported that MMP-2 immunoreactivity has been detected in decidual cells but MMP-9 is dominant on the trophoblasts of six to eight weeks of gestation and claimed that MMP-2 is the key regulator of trophoblast invasion in early pregnancy expression and in importance of MMP-2 and -9 in human trophoblast invasion [16].

The present authors have found that FOXO3a immunoreactivity is higher in missed abortion decidua than control group decidua and have supposed that FOXO3a has played an important role in missed abortion pathogenesis. High FOXO3A immunoreactivity in the missed abortion cases of this study may be in order to provide the increase in resistance of decidual cells to mild oxidative stress beside the decidualization. It is known that oxidative stress induces FOXO3a and apoptosis in undifferentiated but not in decidualized HESCs. They have claimed that the FOXO3a expression in the missed abortion group during endometrial decidualization favors tissue preservation and integrity over apoptotic clearance of defective cells when faced with prolonged oxidative insult during pregnancy. Despite the low levels of oxidative stress, the cells might continue to survive due to the effect of FOXO3 on cell survival at the same time, and this may obstruct the excretion of embryo. It is known that excessive oxidative stress in endometrial tissue brings about pregnancy losses and fetal growth retardation through pre-eclampsia by causing cell death [17], and such cases are given antioxidants during pregnancy [8]. Therefore, randomized studies have shown that giving vitamins C and E reduces the incidence of pre-eclampsia in hazardous preeclampsia cases [18].

The increase of FOXO3 immunoreactivity in the missed abortion group may be related with immunoregulation, and thus it hinders the prevention of discharge of the embryo. FOXO3 is a trigger for apoptosis through upregulation of genes necessary for cell death [19]. It is known that FasL expression has been reported in human endometrium as well as in the immune-privileged tissues, including testis, cornea, trophoblast, and cancer cells [20]. Fas-FasL system plays an important role in the mechanism underlying this immune-privileged status [21]. Fas-L and C-FLIP are expressed in decidual cells during pregnancy, and this increase is not linked to the elimination of decidual cells but it could be associated with the elimination of activated T cells in order to provide maternal immunotolerance [22].

In the examination of the placental samples of the control group and missed abortion group, the immunoreactivity of FOXO3 has been observed to be rather low in both groups but it has also been determined to increase slightly in the missed abortion group. Syncytiotrophoblasts are known to be in directly contact with maternal blood and to have a critical role in fulfilling the placental functions. It has been reported that FOXO3A plays an important role in the complications of pregnancy, and the increase in syncytiotrophoblasts causes intrauterine growth restriction and preeclampsia by bringing about apoptosis [23]. The disturbances in syncytiotrophoblast are known to obstruct the transmission of nutrients from mother to fetus [24], and the activation of FOXO proteins are also known to disrupt glucose metabolism [25]. Therefore, the moderate expression of FOXO3A in syncytiotrophoblasts has been suggested to inhibit apoptosis and to have a role in the continuation of pregnancy. Furthermore, the overexpression of FOXO3 in missed abortion has a critical role in the pathogenesis missed abortion and leads to the continuation of pregnancy by increased decidualization and prevents apotosis in both decidual and syncytiotrophoblast cells.

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Address reprint requests to:
K. OZBILGIN, M.D.
Celal Bayar University
School of Medicine
Department of Histology & Embryology
Dekanlık Binası, Uncubozköy
Manisa, Turkey 45020
e-mail: kemalozbilgin@yahoo.com