Expression of peroxisome proliferator activation receptors (PPARs) and TNFα in placenta tissues in unexplained recurrent pregnancy loss: An immunohistochemical study

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Summary. Introduction. PPAR expression in placenta tissues regulates proinflammatory cytokine production and preserves the quiescence of the uterus during pregnancy. PPAR-γ regulates inflammatory response during gestation while PPAR-δ and TNFα play a central role at implantation, decidualization and placentation. However, their expression levels affect normal pregnancy and may cause gestational complications and miscarriage. The aim of this report is to investigate the relationship of these molecules with unexplained recurrent miscarriage.

Materials-methods. The miscarriage group was obtained from 12 women, between the ages of 35 to 42 years, who miscarried during the 1st trimester of gestation and controls consisted of 12 healthy women, between the ages of 27 to 39 years, who had electively terminated their pregnancies, during the 1st trimester of gestation. The abortion material was processed and specimens taken were studied using immunohisto-chemical methods. Specimens were taken from decidua basalis and decidua parietalis. Monoclonal antibodies were used against PPAR-γ (Peroxisome Proliferator Activation Receptor γ), PPAR-δ and TNFα (Tumor Necrosis Factor alpha). The results were statistically analyzed with Mann-Whitney test.

Results. Our research identified PPAR-γ expression in decidua basalis and decidua parietalis from control group and decidua basalis from miscarriage group. PPAR-δ expression was also identified in both deciduas from both groups. Statistically, no significant change in PPAR-γ and PPAR-δ expression was observed between recurrent miscarriage group and controls. On the contrary, a statistically significant upregulation of TNFα was identified in both deciduas between miscarriage group and controls (p<0.05).

Conclusions. Our evidence did not support a possible role of PPARs expression in recurrent pregnancy loss. However, a potential involvement of TNFα in the syndrome was reported. Further research should be performed due to insufficient bibliographic data.

Key words: Recurrent miscarriage, PPAR-γ, PPAR-δ, TNFα

Introduction

Recurrent pregnancy loss is a clinical entity defined as three or more consecutive miscarriages prior to 25th week of pregnancy (Toth et al., 2010). Recurrent miscarriage affects 1% of population (Practice Committee of the American Society of reproductive medicine, 2012). The etiology of the disease covers a wide spectrum of genetic, endocrine, anatomic, immunological, environmental, thrombophilic and metabolic factors (Banerjee et al., 2013). Although much research has already been done, approximately 50%-75% of unexplained recurrent miscarriage cases are characterized as idiopathic. (Practice Committee of the American Society of reproductive medicine, 2012). The immunological changes during pregnancy are the main

field of investigation and the newest research reported indications of their involvement in this clinical entity (Makhseed et al., 2000).

Tumor Necrosis Factor alpha (TNFα) is a proinflammatory cytokine expressed from activated macrophages during Th-1 inflammatory response. In human pregnancy, TNFα seems to regulate trophoblast proliferation and differentiation, tissue remodeling, cell adhesion, the apoptosis of villous trophoblasts and trophoblast hormone production (Lea et al., 1997). However, TNF-α is considered highly abortogenic in great concentrations (Lea et al., 1997; Makhseed et al., 2000). Th1 cytokines, such as TNFα and IFN gamma, may directly damage the conceptus by apoptosis of trophoblast cells, by inhibiting the secretion of the growth-stimulating GM-CSF from the uterine epithelium and also through the upregulation of procoagulant fg12, a prothrombinase (Raghupathy and Kalinka, 2008). Moreover, TNFα and IFN-γ inhibit embryonic and fetal development as well as the proliferation of human trophoblast lines (Haimovici et al., 1991) as both these cytokines are considered cytotoxic to embryonic fibroblast like cells (Suffys et al., 1988). It is also reported that IL-2, TNFα and IFN-γ, terminate normal pregnancy, when injected (Chaouat et al., 1990). Lymphocytes from women with a history of recurrent miscarriage secreted significantly greater levels of the pro-inflammatory cytokines IL-2, IFN-Gamma, and TNFα than their counterparts with normal pregnancies (Raghupathy and Kalinka, 2008) Consequently, a successful pregnancy may be the effect of a tight regulation of TNFα expression in the reproductive tract (Lea et al., 1997).

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear transcription factors which belong to the steroid receptor superfamily but are not activated by steroid hormones. Three PPAR isotypes have been identified and include PPAR-α, PPAR-δ and PPAR-γ (Mansour et al., 2009). PPAR-γ ligands have been demonstrated to inhibit the secretion of IL-6, IL-8, and TNFα in amnion and chorion (Lappas et al., 2006), highlighting the role of PPARs in the regulation of the inflammatory response in human gestational tissues and cells (Lappas et al., 2002; Ackerman et al., 2005). These anti-inflammatory actions of PPARs are achieved by inhibiting the production of the COX-2 and sPLA2 in fetal membranes and by changing adiponectin and leptin levels (Ackerman et al., 2005). The production of prostaglandins by the endometrium, the myometrium, and the fetal membranes induces the contraction of the myometrium during labor (Ackerman et al., 2005). PPAR-γ level of expression remains stable throughout gestation, except for the period just before labor, when its expression in fetal membranes declines (Borel et al., 2008). Furthermore, in gestational tissues, PPAR-γ has been localized to the amnion, choriodecidual, and placental membranes (Marvin et al., 2000; Waite et al., 2000; Dunn-Albanese et al., 2004) and has been found to play an important role in trophoblast differentiation and

placental vascularization (Marceau et al., 2012).

The aim of our research is to study the hypothesis that PPAR-δ and PPAR-γ are expressed in human deciduas and are involved in the pathophysiology of unexplained recurrent pregnancy loss of first trimester. Additionally, our report will try to study further the potential relationship of TNFα expression and multiple abortions.

Materials and methods

The miscarriage group was obtained from 12 women, between the ages of 35 to 42 years, who miscarried during the 1st trimester of gestation, and controls consisted of 12 healthy women, between the ages of 27 to 39 years, who had electively terminated their pregnancies, during the 1st trimester of gestation. All samples were collected after obtaining informed written consent from all patients. All 12 women from the miscarriage group had a history of at least three prior first trimester miscarriages of unexplained etiology (normal parental karyotypes, intrauterine structural study, luteal phase endometrial biopsy, hormone concentrations and negative cervical cultures, lupus anticoagulant and antibodies to cardiolipin and phosphatidyl serine).

Pathology-examination

Tissues

Tissues were collected immediately after miscarriage or elective abortion and washed with distilled water for removal of mucus and blood. Then, tissues were studied under a microscope, so that specimens from deciduas, chorionic villous and parts of the embryo could be distinguished and examined for formation abnormalities or placental lesions. Specimens with formation abnormalities or placental lesions were excluded from the study. Specimens were collected from distinguished deciduas, chorionic villous and parts of the embryo. Afterwards, they were stabilized in aqueous solution that consisted of neutral formalin 10% v/v for 12-24 hours. Following this, they were placed in an automatic machine for further processing, including fixation, dehydration, clarification with xylene and paraffin embedding. Paraffin-embedded blocks of specimens were cut in 3 mm sections, covered with tape and transferred to positive charged and properly prepared glass plates, which were kept in an oven, at 37- 40°C for 30-45 min. Finally, specimens were stained with haematoxylin-eosin solution (Harris). The stained specimens were examined with a microscope and the most suitable of them were selected for immunohisto-chemical study.

Immunohistochemistry

In all specimens, decidua basalis was identified

using the antibody cytokeratin (CK7), which is positive in trophoblastic cells. For discrimination between decidual and trophoblastic cells at the fetomaternal interface, duplicate sections were stained with a monoclonal antibody against prolactin, for the visualization of decidual cells. The unstained specimens were further processed using an automatic machine (Bond Max) that carried out the appropriate procedures. First, deparaffinization was performed in xylene. Afterwards, specimens were immersed in absolute alcohol, in degressive densities 100%, 96% and 70% v/v consecutively. Finally, they were rinsed with distilled water. Antigen retrieval was performed by incubation at various temperatures, depending on the antibody that was examined each time. Following this, specimens were first rinsed with PBS buffer, then incubated in H_2O_2 for 5 min to quench endogenous peroxidase activity and finally rinsed again with PBS buffer. Thereafter, specimens were covered with a solution of the primary tonic monoclonal antibody, one of the three used in our study. These antibodies as mentioned above are: PPAR-γ (Anti-PPAR-γ antibody [8D1H8F4], Abcam Inc., Cambridge, USA), PPAR-δ (PPAR delta antibody [N2C3] GeneTex International Cooperation, Texas, USA), TNFα (anti TNFα antibody, Abcam Inc., Cambridge, USA). Eventually, they were washed using WAS solution.

For the detection of immunohistochemical staining, specimens were firstly immersed in Post-Primary solution. After being washed, they were immersed in polymere solution and then in chromogen-diaminobenzidine (DAB) solution. Finally, they were stained with Haematoxylin-Eosin. After their automatic procession, specimens were rinsed in tap water and dehydrated with escalating densities of ethanol solution and xylene (70, 96 and 100% v/v consecutively). Then, they were covered with tape, placed in glass plates and

immersed in Canada balsam. The previously reported immunohistochemical staining procedure was repeated twice for each of the 3 antibodies that were examined. The monoclonal antibodies PPAR-γ, PPAR-δ and TNFα were already set and ready to be used. Microscopic evaluation was performed on the cells of the intermediate trophoblast on decidua basalis and decidua parietalis of recurrent miscarriage group and elective abortion group. Evaluation was performed in a blind way with positive and negative controls. Specimens were examined using an optical microscope (Zeiss) and photographs were taken using a camera (Contax), attached to the microscope. Three photographs were taken from each specimen. In total, 48 specimens (24 from decidua basalis and 24 from decidua parietalis) were examined. Intensity of staining was evaluated with a qualitative method as negative (-), weak (+), moderate (++) and strong (+++) by two independent reviewers. Finally, the results were statistically analyzed and checked for their significance using the Mann-Whitney test.

Results

Considering immunohistochemical staining for PPAR-δ in control group, 2 out of 12 sections (16.7%) of decidua basalis were found negative(-), while the other 10 sections were found positive (Fig. 1a). Specifically, 3 out of 12 sections (25%) presented weak (+) staining, 3 sections (25%) moderate (++) and the other 4 (33.3%) strong (+++). On the contrary, in miscarriage group, PPAR-δ expression in decidua basalis was found positive in all sections (n=12) with 2 of them (16.7%) weakly stained (+), 8 sections (66.7%) of moderate (++) staining and the other 2 (16.7%) strongly (+++) stained (Fig. 1b). Observing sections from decidua parietalis in control group, staining for
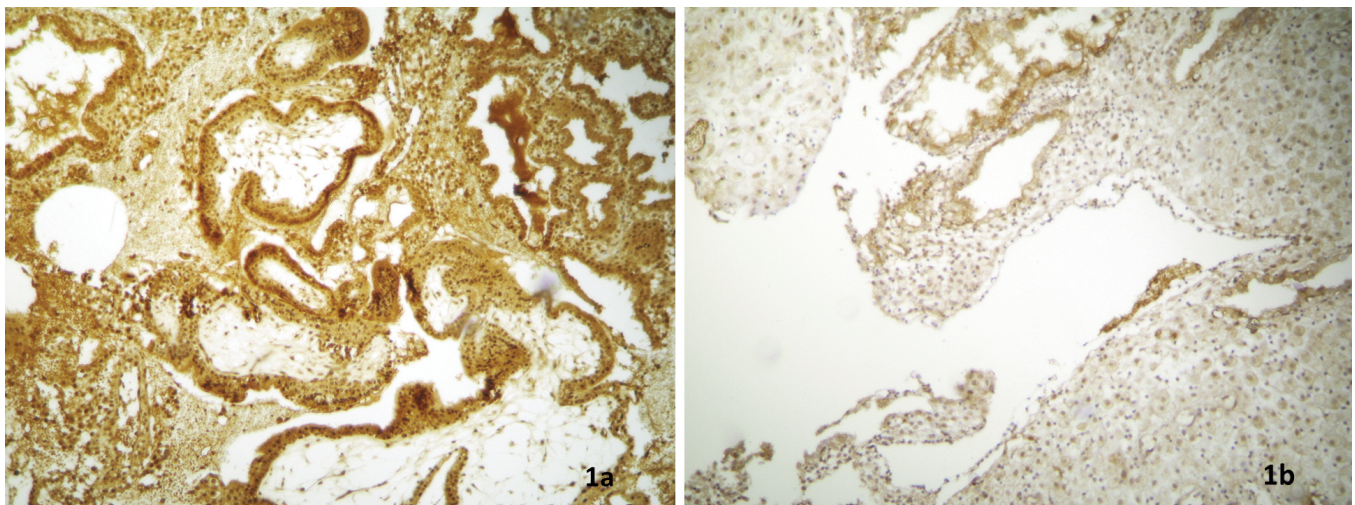

Fig. 1. Staining for PPAR- δ in Decidua Basalis. **a.** Control Group. Strong (+++) intensity. **b.** Miscarriage Group. Moderate(++) intensity. x 40

PPAR-δ expression was found positive in most sections (91.7%). Analytically, 3 sections (25%) presented weak (+) expression, 7 sections (58.3%) presented moderate (++) and 1 section strong (+++) expression of PPAR-δ (Fig. 2a). In decidua parietalis of women from the miscarriage group, 5 sections (41.7%) were found negative (-) and 7 out of 12 positive. From the stained sections, 4 of them (33.3%) presented weak (+) staining, 1 (8.3%) moderate (++) and the other 2 (16.7%) strong (+++) staining (Fig. 2b). No statistically significant difference in PPAR-δ expression was discovered in deciduas of either group (Table 1).

Regarding immunohistochemical staining for PPAR-γ in decidua basalis of women from control group, 7 out of 12 sections (58.3%) were found negative (-) and 5

positive, with 4 (33.3%) weakly (+) stained and 1 section (8.3%) strongly(+++) stained (Fig. 3a). In decidua basalis from miscarriage group, PPAR-γ expression was found negative in 10 sections (83.3%) and weakly (+) positive in 2 of them (16.7%) (Fig. 3b). Furthermore, in decidua parietalis from miscarriage group, PPAR-γ expression was negative in all sections (n=12) (Fig. 4b). On the contrary, in decidua parietalis of control group, 9 out of 12 sections (75%) were found negative (-) and 3 positive (Fig. 4a). Specifically, 2 of them (16.7%) presented weak (+) staining for PPAR-γ and one of them (8.3%) strong (+++) staining. Consequently, no statistically significant difference in PPAR-γ expression was discovered in deciduas of either group (Table 1).

Table 1. Intensity of staining for PPAR-δ, PPAR-γ and TNFα in decidual tissues from miscarriage group and controls.

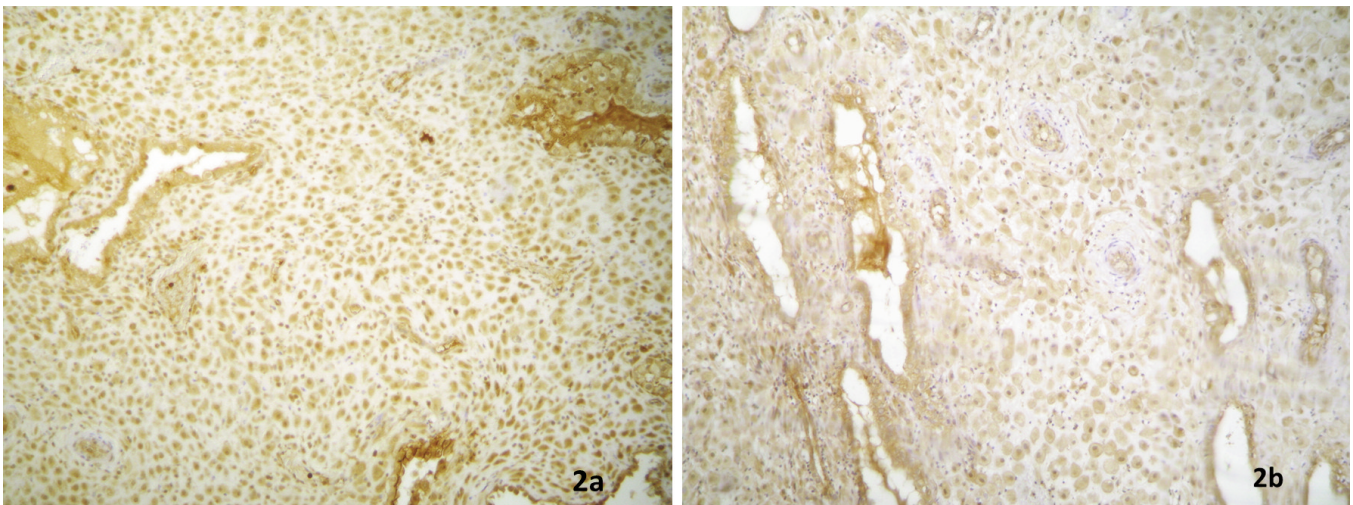|	Miscarriage Group Decidua Basalis	Control Group Decidua Basalis	P	Miscarriage Group Decidua Parietalis	Control Group Decidua Parietalis	P
PPAR-δ						
(-)	0 (0%)	2 (16.7%)	>0.05	1 (8.3%)	5 (41.7%)	>0.05
(+)	2 (16.7%)	3 (25%)		3 (25%)	4 (33.3%)	
(++)	8 (66.7%)	3 (25%)		7 (58.3%)	1 (8.3%)	
(+++)	2 (16.7%)	4 (33.3%)		1 (8.3%)	2 (16.7%)	
PPAR-γ						
(-)	10 (83.3%)	7 (58.3%)		12 (100%)	9 (75%)	
(+)	2 (16.7%)	4 (33.3%)		0 (0%)	2 (16.7%)	
(++)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
(+++)	0 (0%)	1 (8.3%)		0 (0%)	1 (8.3%)	
TNFα						
(-)	0 (0%)	6 (50%)	<0.05	0 (0%)	5 (41.7%)	<0.05
(+)	0 (0%)	4 (33.3%)		0 (0%)	5 (41.7%)	
(++)	3 (25%)	2 (16.7%)		3 (25%)	2 (16.7%)	
(+++)	9 (75%)	0 (0%)		9 (75%)	0 (0%)	

Fig. 2. Staining for PPAR- δ in Decidua Parietalis. **a.** Control Group. Moderate (++) intensity. **b.** Miscarriage Group. Moderate (++) intensity. x 40

Immunohistochemical staining for TNFα in decidua basalis of control group was found negative (-) in 6 sections (50%) and positive for the rest. Analytically, 4 sections (33.3%) were weakly (+) stained for TNFα and 2 (25%) presented moderate (++) staining (Fig. 5a). On the contrary, in decidua basalis of miscarriage group, all sections (n=12) were positive. Moderate (++) staining was observed in 3 of them (25%) and strong (+++) in the other 9 (75%) (Fig. 5b). Regarding decidua parietalis of miscarriage group, all sections (n=12) were positive. TNFα expression was found moderate (++) in three of them (25%) and strong (+++) for the rest (75%) (Fig. 6b). As for the sections of decidua parietalis from control group, 5 sections (41.7%) were found negative (-) and 7 were positive, with 5 (41.7%) presenting weak

(+) staining and 2 (16.7%) strong (+++) staining (Fig. 6a). A statistically significant difference in TNFα expression of both deciduas between control and miscarriage group was reported (p<0.05) (Table 1).

Discussion

This study detected a possible involvement of PPARs and TNFα in the suspected immunological mechanism of unexplained recurrent spontaneous abortion of first trimester. Although much research has already been conducted concerning the immunological changes in feto-maternal interface of women dealing with recurrent miscarriage, only a few cohorts tried to enlighten the possible involvement of PPARs in multiple
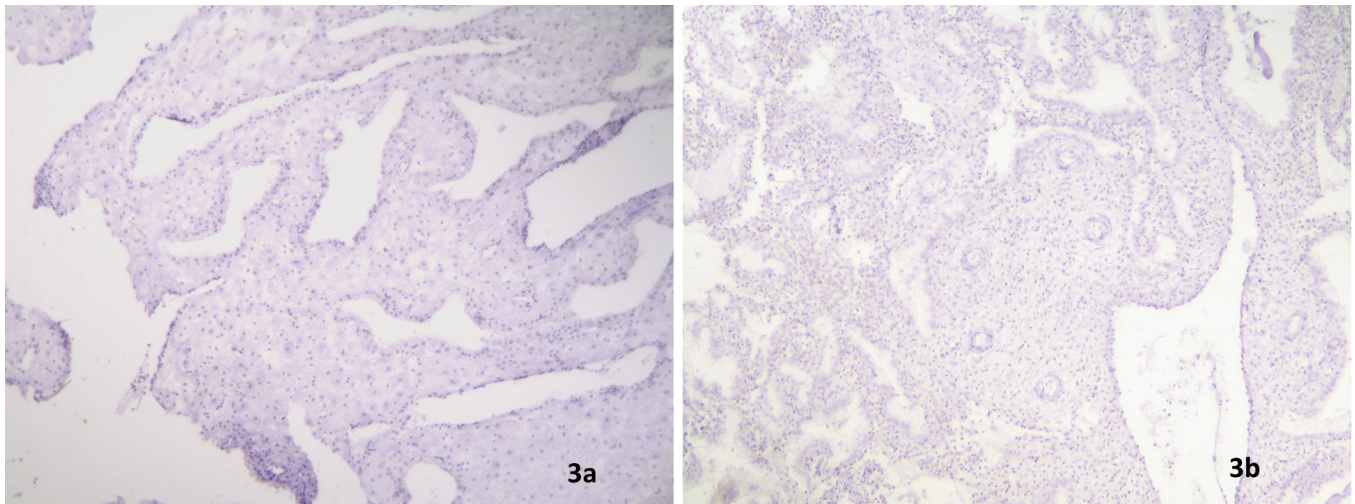

Fig. 3. Staining for PPAR-γ in Decidua Basalis. **a.** Control Group. Negative (-) staining. **b.** Miscarriage Group. Negative (-) staining. a, x 40; b, x 32
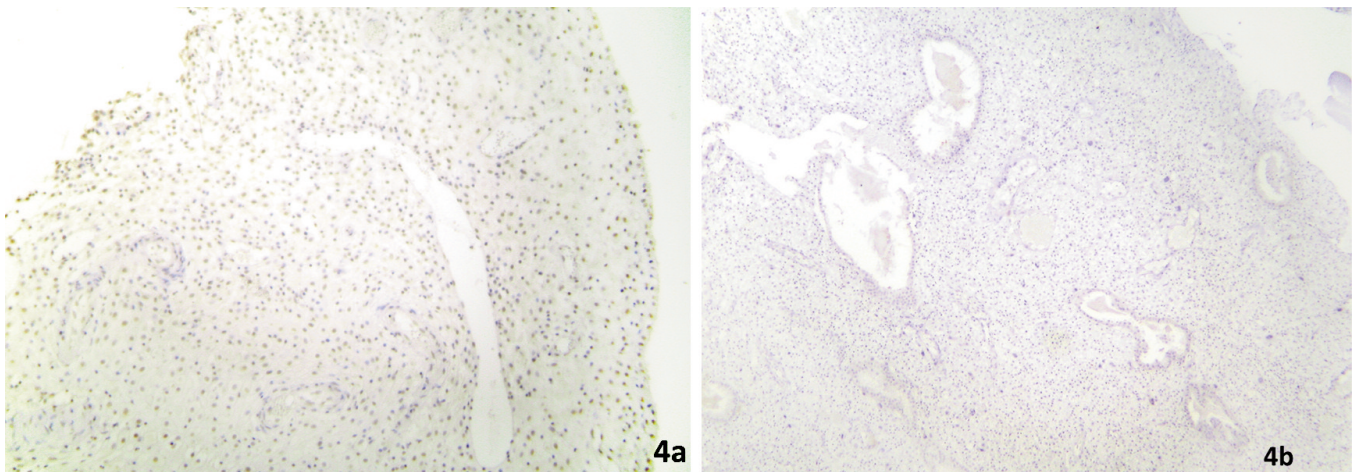

Fig. 4. Staining for PPAR-γ in Decidua Parietalis. **a.** Control Group. Weak(+) intensity. **b.** Miscarriage Group. Negative (-) staining. a, x 40; b, x 24

abortions. On the contrary, previous reports studying TNFα expression in recurrent miscarriage support our hypothesis, as they also detected alterations of TNFα expression in serum and deciduas of women with recurrent pregnancy loss in contrast with controls (Walia et al., 2008; Banerjee et al., 2013).

According to previous research, PPARs may be involved in the pathophysiology of gestational abortogenic complications like diabetes mellitus, intrauterine growth restriction and preeclampsia (Holdsworth-Carson et al., 2010). Specifically, PPAR-γ regulates inflammatory response, proinflammatory cytokine expression and prostaglandin production in human gestational tissues and cells (Kniss, 1999; Lappas et al., 2002; Ackerman et al., 2005; Berry et al., 2005; Lappas et al., 2006) in order to promote the quiescence of the uterus (Borel et al., 2008). In addition, PPAR-δ plays a central role at various stages of pregnancy, like implantation, decidualization, and placentation (Wang et al., 2007). In our study, we identified PPAR-γ expression in both decidua basalis and decidua parietalis from control group and decidua basalis from miscarriage group. No expression of PPAR-γ was observed in decidua parietalis from miscarriage group (Table 1). Furthermore, PPAR-δ expression was detected in decidua basalis and parietalis from both groups (Table
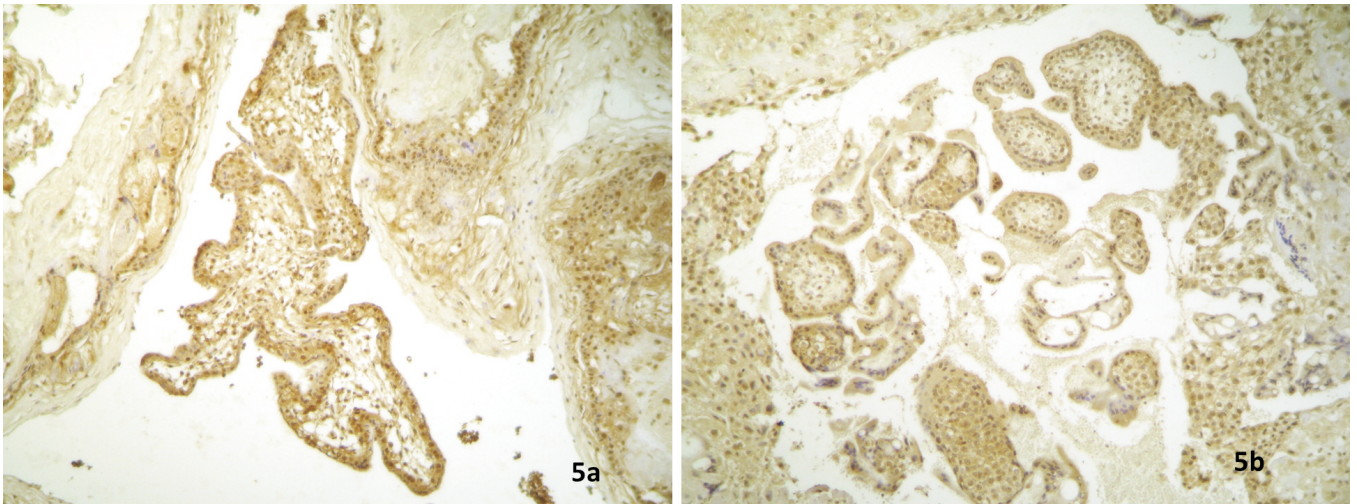

Fig. 5. Staining for TNF-α in Decidua Basalis. **a.** Control Group. Moderate (++) intensity. **b.** Miscarriage Group. Moderate(++) intensity. x 40
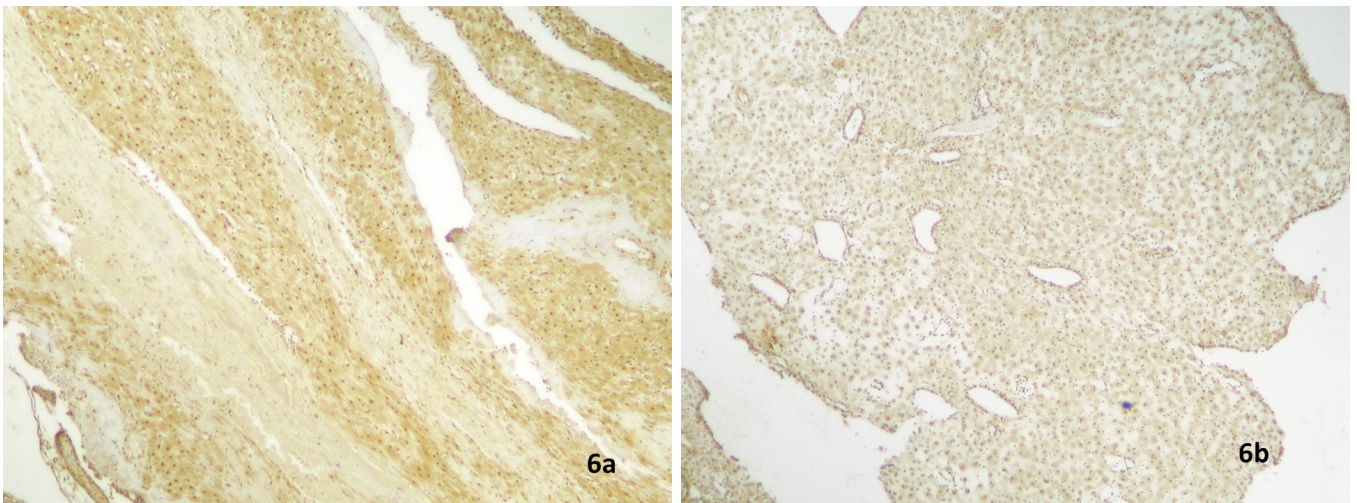

Fig. 6. Staining for TNF-α in Decidua Parietalis. **a.** Control Group. Moderate(++) intensity. **b.** Miscarriage Group. Moderate(++) intensity. x 24

1). Our results are in accordance with previous research (Toth et al., 2008; Knabl et al., 2013). Additionally, in our report, we discovered a trend for a downregulation of PPAR-γ and an upregulation of PPAR-δ in deciduas derived from the miscarriage group (Table 1), without statistical significance, though (p>0.05). Our results disagree with previous studies which mentioned that in miscarriage patients, PPAR-γ and PPAR-δ expression in the extravillous trophoblast was significantly elevated compared to normal pregnancy between 6-12th week of gestation (Toth et al., 2008, 2009; Alexandrova et al., 2012). Knabl et al also observed that PPAR-γ expression was upregulated in deciduas from women with spontaneous abortions (Knabl et al., 2013). However, none of these reports had studied women with recurrent miscarriage. To our knowledge, there are only preliminary data about the expression of PPARs in deciduas from women with recurrent miscarriage and their role in unexplained recurrent pregnancy loss (Toth et al., 2010). Based on our results we can not suggest a possible implication of PPARs to recurrent abortions. Nevertheless, our data should be interpreted with caution, due to the small sample size we used for our research and the insufficient bibliographic reports.

An enhanced Th1 immunological profile has already been associated with recurrent miscarriage (Lim et al., 2000; Makhseed et al., 2000; Clark et al., 2001; Bates et al., 2002; Walia et al., 2008). Tumor necrosis factor alpha as a Th1 cytokine and its levels during early implantation plays a major role in normal pregnancy, and its alterations in serum (Makhseed et al., 2000; Dabbagh, 2000; Laird et al., 2003; Vitoratos et al., 2006) or uterus (Lea et al., 1997; Walia et al., 2008) are implicated with early pregnancy failure. In our study, we identified TNFα expression in both decidual basalis and parietalis of control and miscarriage group (Table 1). Previous research has also reported expression of TNFα in deciduas with a similar pattern (Chen et al., 1991; Lea et al., 1997). In our cohort, we also reported a statistically significant increase in TNFα expression (p<0.05) in both decidua basalis and parietalis from women with unexplained recurrent miscarriage (Table 1). Previous reports, in their majority, support our data (Walia et al., 2008; Banerjee et al., 2013). On the contrary, Lea et al supported that there was no statistically significant difference in TNFα expression between recurrent miscarriage group and controls (Lea et al., 1997). However, in Lea's research there was no karyotype data about the women studied (Lea et al., 1997). According to our results, a possible involvement of TNFα in the immunological mechanism of recurrent abortions could be hypothesized.

To conclude, our research data confirmed the expression of PPAR-γ in decidua basalis and decidua parietalis of the control group and decidua basalis of miscarriage group. They also confirmed the expression of PPAR-δ in deciduas from both groups. However, our evidence does not support a possible connection between the expression of these receptors and recurrent pregnancy loss. Further research should be performed due to insufficient data from previous cohorts. Regarding TNFα, our results confirmed its expression in deciduas and its possible role in the pathophysiology of recurrent abortions in accordance with previous reports.

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