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MACROPHAGES AND PLACENTAL/DECIDUAL APOPTOSIS IN RECURRENT SPONTANEOUS ABORTION: IMMUNOHISTOCHEMICAL STUDY

TAREK A ATIA[1-2] AND MOHAMED ALI [3*]

1: College of Applied Medical Sciences, Prince Sattam Bin Abdul Aziz University, KSA

2: Histology Department, Faculty of Medicine, Al-Azhar University, Cairo, Egypt

3: Obst & Gyn Department, Faculty of Medicine, Al-Azhar University, Cairo, Egypt

***Corresponding Author: E Mail: drmohamednada@yahoo.com; Tel.: 01008172225**

ABSTRACT

Recurrent spontaneous abortion (RSA) is a common pregnancy complication with variable causes, where up to 15% of pregnancies result in spontaneous loss. Several studies had investigated the role of placental apoptosis and macrophages, separately, in RSA. Objective was to investigate the M1/M2 dynamic activities in cases of RSA and their role in placental apoptosis. Placental/decidual samples were collected from 40 women with RSA (study group) and 30 cases with sporadic abortion (control group). Samples were prepared and stained immunohistochemically with markers for macrophages (CD80, CD163); anti-apoptotic Bcl-2 antibody and apoptotic protein (p53). There was a significant increase of M1/CD80 macrophages in placental tissues of RSA compared to M2/CD163 cells, (P = 0.002). Additionally, there was significant increase of M1/CD80 macrophages in RSA cases compared to that of sporadic abortion cases, (P = 0.001). On the other hand, the number of apoptotic (p53) immunopositive cells in placental tissues of RSA was significantly higher in study group than that in control group, (P = 0.003). In contrast, the mean number of anti-apoptotic (Bcl-2) immunopositive cells was significantly higher in control placental tissues than that in RSA cases, (P = 0.025). Placental/decidual apoptosis and macrophages have a crucial role in pregnancy continuation. However, increased inflammatory M1 macrophages and increased p53 expression in placental tissue during the early stages of pregnancy could lead to RSA.

Keywords: RSA, Placenta/Decidual, Apoptosis, Macrophages, Immunohistochemistry

INTRODUCTION

Recurrent spontaneous abortion (RSA) is a common pregnancy complication, where up to 15% of pregnancies result in spontaneous loss. RSA defined as three or more consecutive pregnancy losses prior to 20 gestational weeks[1]. The etiology of RSA is variable, but unbalanced placental apoptosis and macrophages have a crucial role**.** Macrophages developed very early in maternal/fetal interface, present in all components of conception, and remain active throughout pregnancy. Macrophage populations are derived from both maternal (decidual) and fetal (chorionic villi) macrophages. Additionally, their number is variable during the different stages of pregnancy. These cells have a variety of functions that are important for successful pregnancy[2]. Macrophages help in remodeling the uterine connective tissues, placental vascularization, regulating immune-tolerance toward fetal antigens and initiating parturition[3]. Decidual macrophages are the second most prominent cells in the maternal-fetal interface after the Natural Killer cells. They produce several cytokines as part of their function as antigen presenting cells[4]. Macrophages are involved in modulating the placental response to infection via regulating T-cell activities[5.6].

Macrophages are usually activated in response to the environmental changes and they are subdivided functionally and phenotypically into two types. First, pro-inflammatory M1 macrophages, CD68/CD80 positive cells, which can present antigens, form IL-12 and IL-23, and activate TH1 cells or cell mediated immune response. Second, anti-inflammatory M2 macrophages, CD68/CD163 positive cells, that can mediate tissue remodeling, activate TH2 or antibody mediated immune response, and possess immunosuppressive activity[7-10]. Additionally, macrophages accumulate near the extra-villous trophoblasts, phagocytose the apoptotic decidual cells and enhance the extra-villous trophoblasts invasion. However, increased number of macrophages and the associated aberrant apoptosis could adverse the pregnancy outcomes[11].

Normal placental development undergoes sequences of cell division and differentiation, followed by invading the embryonic trophoblast cells into the decidua with remodeling the vasculature to increase blood flow into the placenta and the fetus. With pregnancy continuation, the placenta develops series of tissue remodeling with regular loss of trophoblast cells by apoptosis[12]. Additionally, many studies have indicated the importance of apoptosis in promoting maternal immune tolerance to the paternal antigens expressed by the trophoblast cells[13]. Several studies reported

apoptotic changes in both the maternal-fetal interfaces of the placenta during the normal as well as in some cases of complicated pregnancies[14].

Apoptosis is an interactive and dynamic biological process involved in degradation of undesirable cells to maintain the normal tissue function. The interaction between pro- and anti- apoptotic pathways can regulate, stimulate or inhibit cellular apoptosis. Apoptosis are initiated by a family of cysteine proteases, caspases, that could be activated through intrinsic (mitochondrial mediated) or extrinsic (death receptor mediated) apoptotic pathways[15,16]. In intrinsic pathway, the apoptotic signals are initiated by the mitochondria in response to cellular stresses such as DNA damage. P53, a tumor suppressor protein, could activate the mitochondrial pathway that in turn can activate pro-apoptotic Bcl-2 family members. In addition, p53, a negative cell cycle regulator, is important for numerous biological processes, such as cell cycle, DNA repair, cell differentiation and apoptosis. However, in extrinsic pathway, apoptosis is initiated by members of the TNF (Tumor Necrosis Factor) death receptor family. Indeed, the extrinsic and intrinsic pathways are not completely independent, as p53 can up-regulate the expression of certain death receptors, and the mitochondrial pathway may act to amplify signals triggered by the death receptor pathway, suggesting that crossover can occur between the two pathways[17,18].

Numerous studies had reported that balance between M1/M2 activities in all stages of pregnancy is essential for successful pregnancy[10]. Additionally, the number of macrophages[19] and apoptotic cells[20] is increased dramatically in placental tissues in cases of recurrent abortion. In the current study, we have used Immunohistochemical method to investigate the M1/M2 dynamic activities and their role in placental/decidual apoptosis in cases of RSA.

PATIENTS AND METHODS

Patients

After obtaining the approval from the Hospital Ethics Committee, this prospective case-control study was carried out between December 2013 and January 2015 at the Obstetrics and Gynecology department, Al-Azhar University Hospitals, Cairo, Egypt. It included 40 women with RSA (study group) and 30 women with sporadic abortion (abortion for the first time, after having at least a normal pregnancy) as a control group and matched for age for those with RSA. Each female agreeing to participate in the study provided a written informed consent prior to enrollment.

Decidual/placental samples have been taken from all cases by dilatation and evacuation

without any prior pharmaceutical induction within the first 24 hours after diagnosis. All women enrolled in the study had a null medical and family history. Then the following data were collected; age, parity, body mass index, number of previous abortion and maternal previous diseases. After that a thorough clinical examination was performed, aiming to exclude apart from common disorders already known as aggravating factors for increased risk for abortion. Following evacuation, specimens from both groups were fixed in 10% neutral-buffered formalin, routinely processed, embedded in paraffin wax, then sectioned and mounted onto APES coated slides.

Immunohistochemical staining

Immunohistochemical assay for macrophage markers for M1 and M2 cell populations, anti-apoptotic antibody Bcl-2 and apoptotic protein p53 expression were performed on formalin fixed, paraffin-embedded tissue sections using the peroxidase labeled avidin-biotin method. Commercially available antibodies for CD80 antibody [B7-1 Antibody (H-208): sc-9091] for M1 macrophages, CD163 antibody [(RM3/1): sc-33715] for M2 macrophages. Additionally, we have used Bcl-2 antibody [100 : SC-509] and p53 antibody [(BP 53.12): sc-81168] for apoptotic cells (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) were used to recognize these antibodies according to the manufacturer's instructions.

Sequential slides with 4μm-thick tissue sections were deparaffinized and hydrated in sequential treatment of xylene, ethanol and water. Heated citrate buffer (0.01 M citric acid, pH 6.0) was used to retrieve antigens. Endogenous peroxidase activity and non-specific bindings were blocked with 3% H2O2. Sections then incubated with primary antibodies (dilution 1:200) overnight at 4°C. The following day, biotinylated secondary antibody and streptavidin-horseradish peroxidase were added. The peroxidase reaction was developed with 3,3'-diaminobenzidine (DAB; Sigma Chemical Co.) to give a brown colored product. Counterstaining was done lightly with hematoxylin, and the sections were dehydrated in alcohol before mounting. Appropriate positive controls were performed in each staining run, and negative controls were performed for each sample by replacing the primary antibody with mouse IgG.

Data registration and Statistical analysis:

The number and the optical density of immunopositive CD80 (M 1), and CD163 (M 2), Bcl-2 and p53 cells were assessed. Digital images of 10 randomly selected high-power fields [hpf] (X400) captured and analyzed using Carl Zeis microscope and Zen Image

software (2012, blue edition). Optical density was used to measure the strength of immunohistochemical reactivity accurately[1], instead signing the reaction as (+, or weak), (++ or moderate), and (+++ or strong reaction). Then, the data are presented as mean ± standard deviation (SD). Continuous variables were compared using paired and independent Student's t-test. Values of $P<0.05$ were considered statistically significant.

RESULT

1- Maternal Demographic data

Seventy women were included in the study; 40 women with RSA (Study group) and 30 women with sporadic miscarriages and matched for age for those with RSA recruited as (control group). The two groups were similar; there were no significant differences with respect to age ($p = 0.7$), parity ($p = 0.9$), body mass index ($p = 0.4$), and gestational age ($p = 0.4$) at time of abortion, but the number of abortions was significantly higher among the study group than among those of control group ($P =0.001$) (**Table 1**).

2- Macrophage population (M1/CD80 and M2/CD163):

The placental M1 cells were immuno-stained against the CD80 and M2 cells against CD163 antibodies. In RSA cases, the mean number of M1/CD80 immuno-positive cells (fig. 1-A) was significantly higher than that of M2/CD163 immuno-positive cells (fig. 1-B); (mean = 81 ± 13.6544661 vs. 49 ±17.93739) respectively, ($P = 0.002$). Additionally, the mean number of M1/CD80 immuno-positive cells in RSA cases (fig. 1-A) was significantly increased compared to that of sporadic abortion cases (fig. 2-A); (mean = 81 ± 13.6544661 *vs.* 48 ±10.93288) respectively, ($P = 0.001$).

On the other hand, the mean number of M1/CD80 immuno-positive cells (fig. 2-A) was non-significantly different from that of M2/CD163 immuno-positive cells (fig. 2-B) in sporadic abortion cases; (mean = 48 ± 10.93288 vs. 45 ±10.64712) respectively, ($P = 0.190$). Additionally, the mean number of M2/CD163 immuno-positive cell population in RSA cases (fig. 1-B) was also non-significantly different from that of sporadic abortion cases (fig. 2-B); (mean = 49 ± 17.93739 vs. 45 ± 10.64712) respectively, ($P = 0.718$). Tables (2 and 3) show the statistical variations and significances between the study and control groups.

3- Apoptosis detection: Anti-apoptotic Bcl-2 and p53 expression:

Apoptotic changes in placental tissues were examined by detecting the expression of p53 protein in comparison to anti-apoptotic expression of Bcl-2 antibody. In placental tissues of RSA (fig. 3-A) the mean number of apoptotoc/p53 immunopositive cells was

significantly higher than that of control group (**Figure 3**-B) (mean= 39 ± 8.831761 vs. 18±6.839428) respectively, (P = 0.003), **Table 2**. In contrast, the mean number of anti-apoptotic/Bcl-2 immunopositive cells was significantly higher in placental tissues of control group (**Figure 4**-B) than that in RSA cases (**Figure 4**-A) (mean= 30±10.95648 vs. 16±7.258788) respectively, (P = 0.025).

Interestingly, the optical densities of all immunopositive cells showed non-significant variation between the investigated groups.

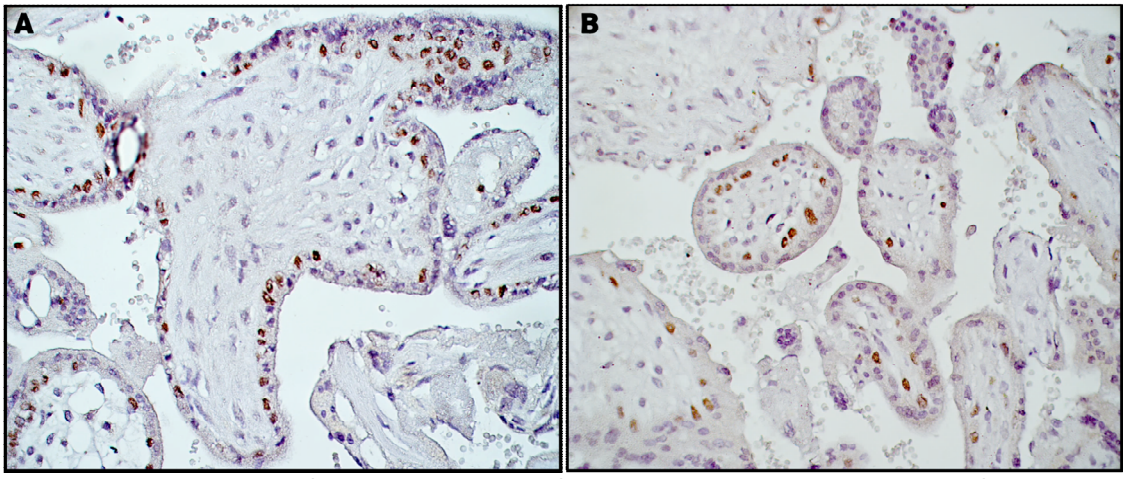

Figure (1): Immuno-histogram of chorionic villi samples from RSA cases showing the expression of CD80 immuno-positive M1 cells (A), which is significantly higher than the expression of CD163 immuno-positive M2 cells (B) [P= 0.002]. Immunopositive cells appeared as brown color in M1 and M2 cells. [X400]

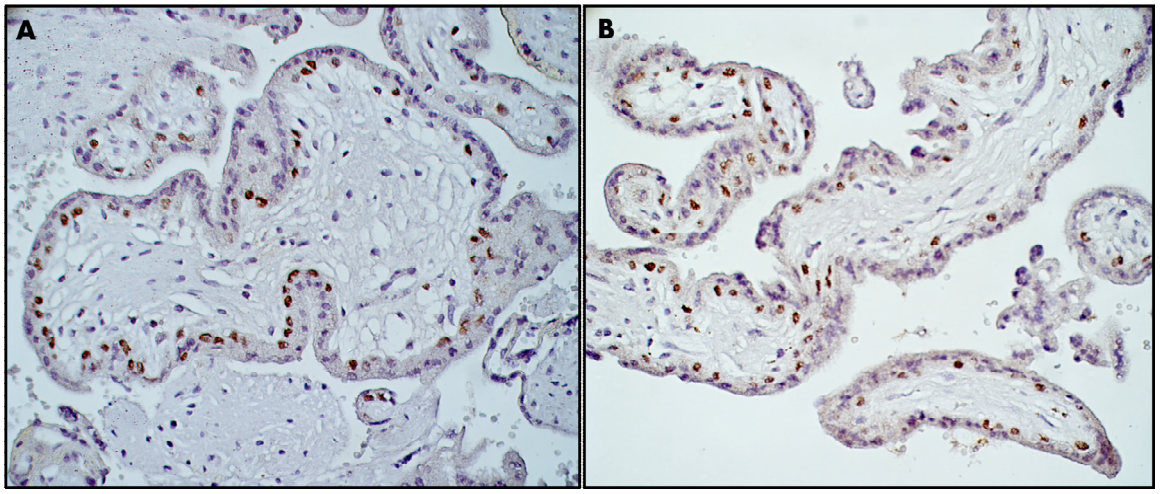

Figure (2): Immuno-histogram of chorionic villi samples from sporadic abortion cases showing the expression of CD80 immuno-positive M1 cells (A), which is non-significantly different from the expression of CD163 immuno-positive M2 cells (B) [P = 0.190]. Immunopositive cells appeared as brown colour in M1 and M2 cells. [X400]

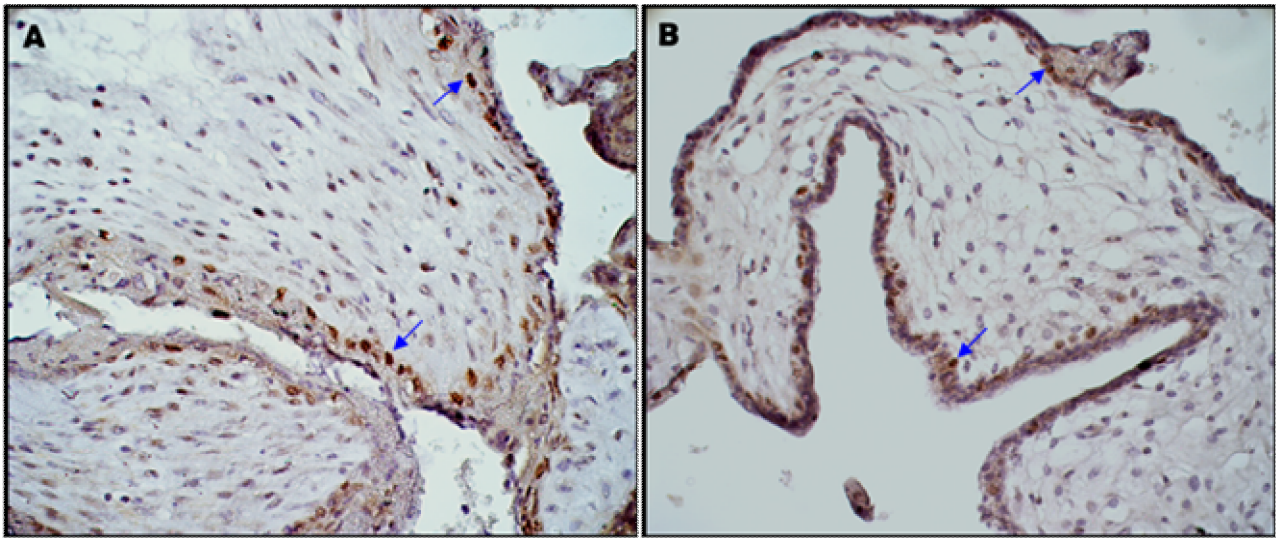

Figure (3): Immuno-histogram of chorionic villi samples from RSA cases showing the expression of p53, apoptotic protein, in the chorionic villi of RSA cases (A), which is significantly higher than that of sporadic abortion cases (B) [P= 0.003]. Expression of p53 appeared as brown color in the nuclei (blue arrow). [X400]

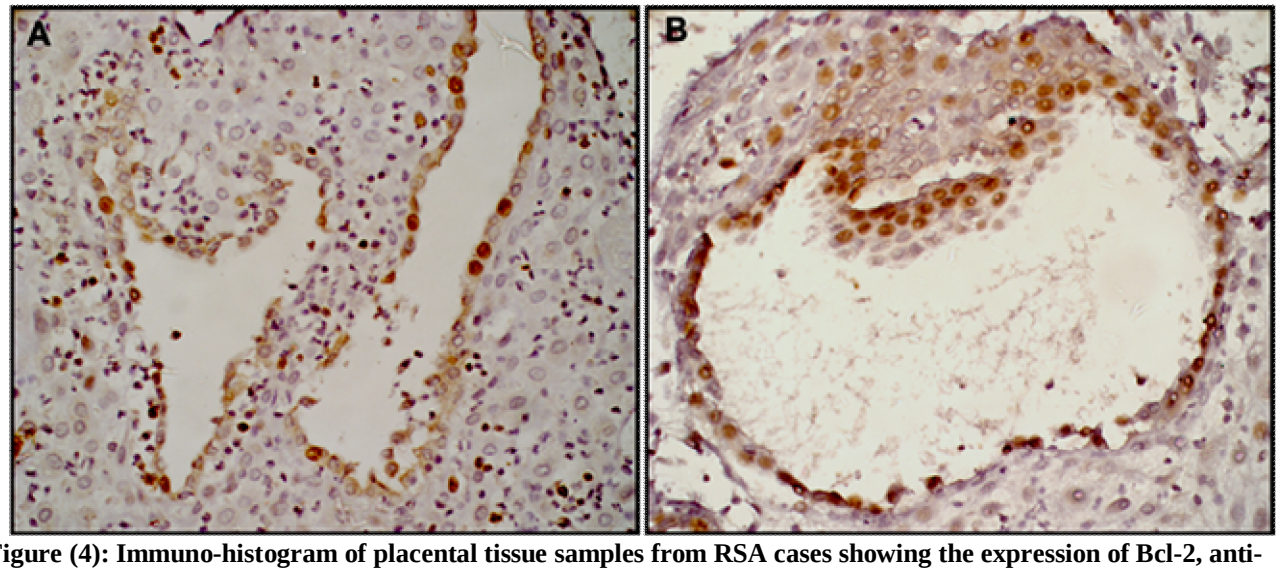

Figure (4): Immuno-histogram of placental tissue samples from RSA cases showing the expression of Bcl-2, anti-apoptotic protein, (A), which is significantly lower than that of sporadic abortion cases (B) [P= 0.025]. Expression of Bcl-2 appeared as brown color in the nuclei. [X400].

Table 1: Maternal Demographic data; where BMI= Body Mass Index

	Study group (n =40)	Control group (n =30)	P value
Maternal age (years ± SD)	22.5± 3.2	23.7 ± 2.1	0.7
Parity	2.7 ± 1.4	2.6 ± 2.4	0.9
BMI (Kg/m^2)	24.1 ± 3.3	23.8 ± 2.6	0.4
Gestational age at time of abortion	12.6 ± 2.1	11.3 ± 3.3	0.4
Number of Abortions	5.1 ±0.7	0.00	0.001

Table 2: Statistical analysis showing the mean, SD and P-values of immunopositive M1/CD80, M2/CD163, Bcl-2 and p53 cells and their optical densities in RSA vs Sporadic Abortion cases.

	Study group (n=40)	Control group (n=30)	P- value
CD80- Cell count	81± 13.6544661	48±10.93288	0.001
CD80- Optical dens	0.29830446± 0.205059244	0.352578684±0.238417836	0.612
CD163 Cell count	49±17.93739	45±10.64712	0.718
CD163- Optical dens	0.425390687±0.122655799	0.373248163±0.160641079	0.450
p53- cell count	39±8.831761	18±6.839428	0.003
P53- Optical dens	0.402600259±0.226405335	0.455682228±0.20412076	0.618
Bcl-2 cells count	16±7.258788	30±10.95648	0.025
Bcl-2 cell Optical dens	0.425854946±0.139872846	0.493779789±0.112514728	0.262

Table 3: Statistical analysis shows the mean, SD and P-values of the immunopositive M1/CD80 and M2/CD163

	CD80- Cell count	CD163- Cell count	P- value
Study group (n=40)	81± 13.6544661	49±17.93739	0.002
Control group (n=30)	48±10.93288	45±10.64712	0.190

DISCUSSION

Our study result indicated that the number of immunopositive M1/CD80 macrophages was significantly increased in RSA cases compared to that in sporadic abortion cases (P=0.001). Additionally, the number of M1/CD80 immunopositive cells was significantly higher than the number of M2/CD163 immune-positive cells in RSA cases (P= 0.002). Our results are in agreement with Nakashima et al[21] and Wang et al[22]. They noticed marked increase in decidual macrophages in women suffered RSA, without determining which subtype; M1 or M2 cells. However, Guenther et al[19] indicated that M1 cells was significantly increased in RSA cases. In contrast, Vassiliadou et al[23] reported that the macrophage cell population was increased not significant in placental tissues of RSA cases, while Quack et al[24] demonstrated that the number of macrophages was not increased.

Several investigators reported that pregnancy is a process accompanied with systemic inflammation. They found significant increase of the plasma levels of some inflammatory and pro-inflammatory cytokines; such as tumour necrosis factor alpha (TNF-α) and interferon gamma (INF-ϒ) in pregnant females compared to non-pregnant females[25,26]. However, these inflammatory and pro-inflammatory cytokines are much higher in complicated pregnancy such as pre-eclampsia and miscarriage[26]. Macrophages subdivided into two phenotypes; M1 and M2 cells. M1 cells are related to inflammation and have the capacity to produce IL-12, and IL-23 and TNF-α. In contrast, M2 cells being anti-inflammatory cells produce IL-

10, Transforming Growth Factor- beta (TGF-β) and macrophage mannose receptors that activate the host defence and help in removing the products of inflammatory processes[19,27]. Thus, we can estimate that M2 macrophages are part of normal pregnancy continuation, while M1 macrophages have diverse functions that could lead to spontaneous abortion.

Balanced cell apoptosis and cell proliferation in maternal-fetal interface are important for normal embryogenesis, while unbalanced apoptosis may affect embryogenesis leading to fetal loss[28]. In the current study, we investigated the apoptotic and anti-apoptotic activities in placental tissues from RSA and sporadic abortion cases using p53 and Bcl-2, respectively. Our result revealed significant increase of apoptotic cells that express p53 antibody [P= 0.003], with significant reduction of Bcl-2 expression [P= 0.025] in placental tissues of RSA compared to that in cases with sporadic abortion. Numerous investigators reported increased number of apoptotic cells with over-expression of p53 in patients with RSA[29]. Additionally, Kelten et al[30] had reported that Bcl-2 expression was down-regulated in RSA, and the number of Bcl-2 immuno-positive cells was highly decreased in RSA in comparison to control group. These findings are in agreement with our result. Therefore, we speculated that upregulated Bcl-2 expression with down-regulated p53 expression is essential for normal placental development and pregnancy continuation. However, increased placental apoptosis with over-expression of p53 leads to pregnancy failure[31]. But other indicated that abnormal expression of some apoptosis related genes, like TNF-α and TGF-β in placental/decidual tissues can cause RSA[32].

In a previous study[1], we noticed a significant increase of macrophages/CD68 cells in placental/decidual tissues of RSA cases compared to that of control group. However, excess macrophages can exaggerate the maternal inflammatory response to the invading trophoblasts. That in turn will help excess M1 polarization, with production of IL-12 and IL-23. These cytokines can promote cell apoptosis through stimulating the intrinsic mitochondrial (p53) pathway[33]. Therefore, there was a strong correlation between increased M1 polarization and increased level of tissue apoptosis; and increased the incidence of pregnancy failure; as a result.

CONCLUSION

Placental/decidual macrophages and apoptosis have a crucial role in pregnancy continuation. However, increased the inflammatory M1 macrophages and increased 53 expression in placental tissue during the early stages of pregnancy could lead to pregnancy failure. Our

results highlight a new aspect on the correlation between placental M1 macrophages and apoptosis in the pathogenesis of RSA. Indeed, further studies are needed to verify the current results.

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