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Immunohistochemical study of immunological markers: *HLAG*, CD16, CD25, CD56 and CD68 in placenta tissues in recurrent pregnancy loss

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Summary. Introduction: Recurrent pregnancy loss (RPL) of unknown etiology is correlated with immunological alterations during pregnancy. Normally, changes in leukocyte subpopulations and *HLA* expression take place in pregnant uterus in order to tolerate the semi-allogenic embryo. Objective: Our research tries to enlighten the immunological changes that take place in the uterus of women with recurrent abortions of unknown etiology during first trimester of pregnancy. Materials and methods: The miscarriage group was obtained from 25 women who miscarried between the ages of 35 to 42 years and controls consisted of 25 healthy women between the ages of 27 to 39 years, who had electively terminated their pregnancies during the first trimester of pregnancy. The abortion was processed and specimens taken were studied using immunohistochemical methods. Specimens were taken from decidua basalis and decidua parietalis. Monoclonal antibodies were used against *HLAG* (*Human Leukocyte Antigen G*), CD68(Cluster of Differentiation 68), CD56, CD16 and CD25. The results were statistically analysed with Mann-Whitney test. Results: *HLA-G* expression in decidua basalis from miscarriage group was found to be decreased. CD25+ cell expression was found to be invariable in deciduas from both groups. CD16+ cell and CD68 + cell expression was found to be increased in deciduas from the miscarriage group. CD56+ cell expression was found to be increased in decidua parietalis from miscarriage group. Conclusion : Several differences in the immunological profile of deciduas from RPL group were observed. Changes in feto-protective HLA-G expression and a possible implication of macrophages and NK cells were found.

Key Words: Recurrent miscarriage, NK cells, CD68, CD25, HLA-G

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Introduction

Recurrent pregnancy loss (RPL) constitutes a challenge in the field of reproductive medicine, as the etiology in approximately 50% of cases of RPL, remains unknown. It has been assumed that immunological alterations are associated with RPL, with few available evidence-based diagnostic and treatment guidelines. The etiology of RPL is considered complicated, subdivided into embryological (mainly due to an abnormal embryonic karyotype) and maternal causes, affecting the endometrium and/or placental development (coagulation disorders, autoimmune defects, endocrine disorders and endometrial defects) (Ford and Schust, 2009).

Various alterations take place in the maternal immunity system during gestation, ensuring the protection of the semi-allogenic fetus and the maintenance of pregnancy. More specifically, Human Leukocyte Antigen (*HLA*) gene expression status, lymphocyte populations and complement proteins are all

submitted to specific changes in order a possible abortion of the fetus to be avoided. It seems that trophoblast cells control monocyte migration and differentiation, producing a pro-inflammatory cytokine and chemokine profile, essential for their survival and growth (Fest et al., 2007).

The attack of maternal immune cells against paternal *HLA* class 1 antigens can be prevented by regulating firmly the expression of specific HLA class 1 molecules in subpopulations of trophoblasts (Hunt and Orr, 1992). The extravillous trophoblasts migrating into the deciduas display a unique pattern of *HLA* class 1b molecules, with *HLA-E*, *F* and *G* predominating. *HLA-G* activates different pathways in uterine NK cells and macrophages and alters their killer function by interacting with leukocyte inhibitory receptors (LIR's) on uterine natural killer cells (uNK) and macrophages, and with the T cell receptor on CD8+ (Cluster of Differentiation) cells (Long, 1999; Shakhawat et al., 2010). *HLA-G* expression in antigen-presenting cells suppresses T-cell proliferation, via apoptosis (Naji et al., 2007). Polymorphism and methyliosis of *HLA-G* antigen are associated with recurrent pregnancy loss (Ober et al., 2003; Hviid et al., 2004). Moreover, HLA-G is not involved in T-cell suppression exclusively with its membrane-bound form, but also with a soluble isoform in maternal serum (Fournel et al., 2000; Hunt et al., 2000; Pfeiffer et al., 2000; Morales et al., 2007).

One of the first histological variations in the maternal immune system is a dramatic change in the relative proportions of leukocyte subpopulations in the uterus. T and B cells exhibit a significant decrease in their levels and the uNK cell population shifts from endometrial NK cells to decidual NK cells. Collaboration between extravillous trophoblastic cells, endothelial cells, smooth muscle cells and decidual leukocytes (macrophages and uterine NK cells) render the trophoblast receptive to invasion and expedite vascular cell loss and extracellular matrix remodeling (Harris, 2010). Moreover, some studies suggest that trophoblast-macrophage collaboration promotes the normal forming of placenta by enabling vascular remodeling and tissue homeostasis (Nagamatsu and Schust, 2010).

Uterine NK cells, the largest cell population at the implantation site, are supposed to be part of the "decidualization" process (De Carolis et al., 2010) and play a major and unique role in pregnancy course, as they have low cytotoxicity and participate in trophoblast attraction and invasion, decidual and placental angiogenesis, fetal vasculogenesis, and vascular modifications in the uterus (Hanna et al., 2006; Tabiasco et al., 2006; Yagel, 2009; Lash et al., 2010; Harris, 2010). The majority (90%) of NK cells found in the endometrium (uNK cells) express high levels of CD56, but are CD16 and CD3 negative. The remaining 10% of uNK cells resemble peripheral blood NK cells and are CD16+ (Laird et al., 2011). Variations of decidual NK cell quantitative status and activation level may induce pregnancy complications, such as immunologic infertility and recurrent pregnancy loss (Hiby et al., 2008). Decidual CD68+ cells (macrophages) excrete immunosuppressive agents (Prostaglandin E2), and downregulate inflammatory responses at the fetomaternal interface that help to prevent some uterine infections in pregnant women. The quick and effective removal of apoptotic cells by tissue macrophages prevents the release of self-antigens, and in the case of pregnancy, paternal alloantigens (Abrahams et al., 2004). Moreover, during pregnancy, there is a T regulatory CD25+ cell accumulation in deciduas and an elevation in maternal serum early in the first trimester. A decline in quantity or functionality is associated with adverse consequences, such as infertility, miscarriage and pre-eclampsia (Guerin et al., 2009).

Our aim was to examine the immune profile of women with recurrent pregnancy loss of unknown etiology, during the first trimester of their pregnancy, using four immunological markers (CD16, CD56, CD25 and CD68) as well as *HLAG* expression in two groups of decidual tissues.

Materials and methods

The miscarriage group was obtained from 25 women, between the ages of 35 to 42 years, who miscarried during the 1st trimester of gestation and controls consisted of 25 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 consent from patients. All 25 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 decidua, villus chorion 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 the distinguished decidua, villus chorion and the embryo parts. Specimens were stabilized in aqueous solution that consisted of neutral formalin 10% v/v for 12-24 hours. Following this, specimens were placed in an automatic machine for further processing, including fixation,

dehydration, xylene clarification and paraffin embedding. Then, 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. After this step, 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 immunohistochemical study.

Immunohistochemistry

In all specimens, decidua basalis was identified using the antibody cytokeratin (CK7), which is positive in trophoblastic cells (Fig. 1). Furthermore, 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 (Fig. 2).

The unstained specimens were further processed using an automatic machine (Bond Max) that carried out the following procedures. First, deparaffinization was performed in xylene. Afterwards, specimens were immersed in absolute alcohol, in degressive densities 100%, 96% and 70% v/v consecutively and were rinsed with distilled water. Antigen retrieval was performed by incubation at various temperatures, depending on the antibody that was examined each time.

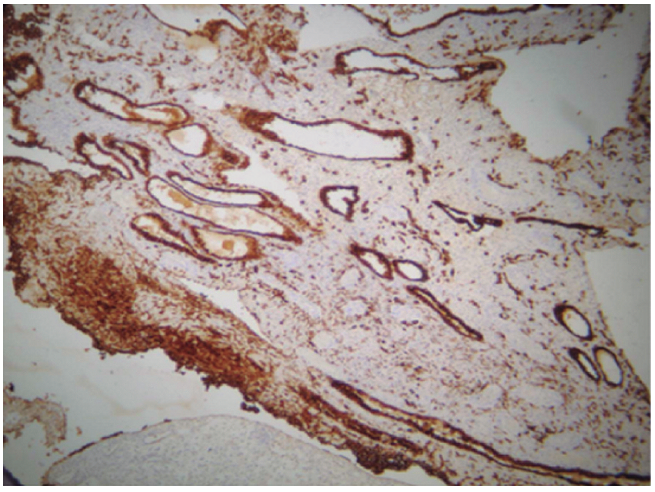

Fig. 1. Control group. Decidua basalis. CK7. Detection of trophoblastic cells. x 16

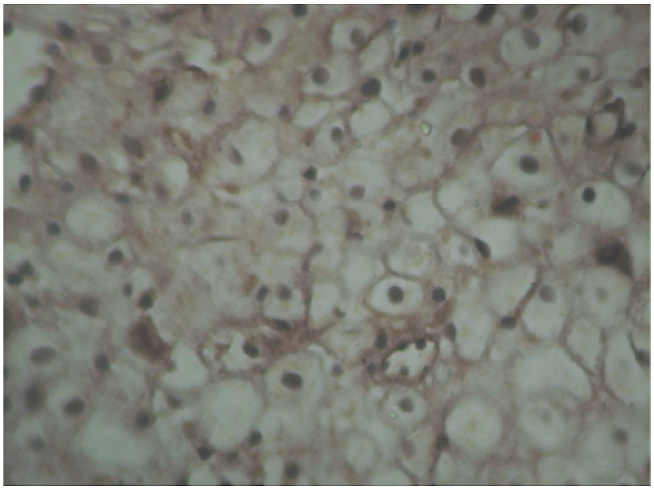

Fig. 2. Decidua basalis. Prolactin. Detection of decidual cells. x 160

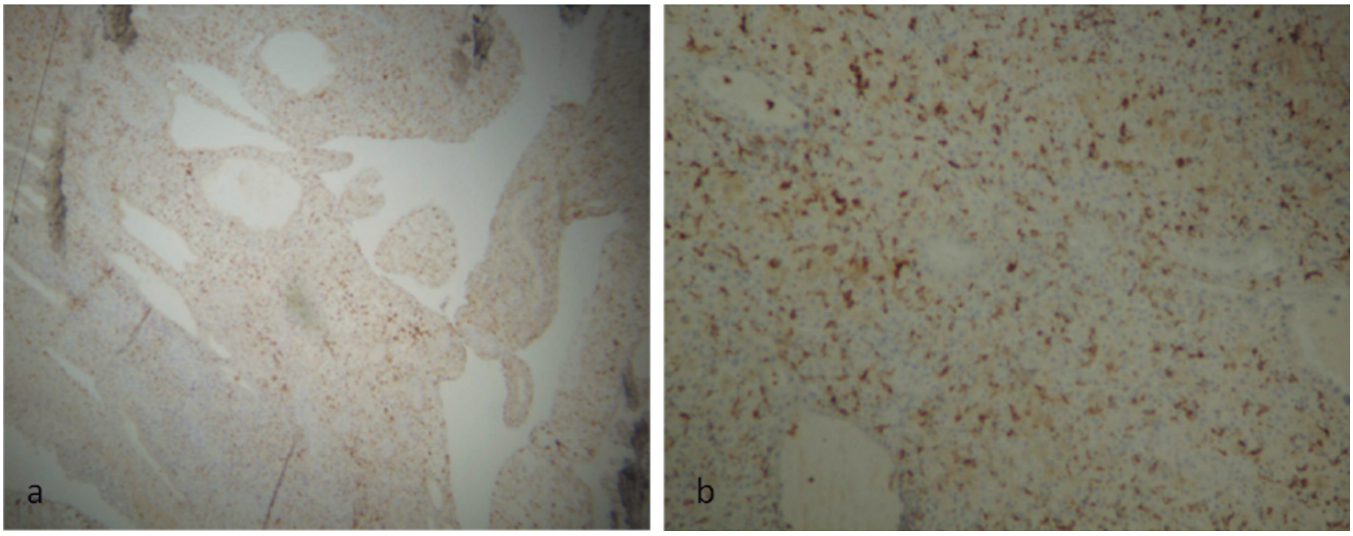

Fig. 3. Staining for CD68+ cells in decidua basalis. **a.** Control group. Negative (-) .**b.** Miscarriage group. Strong (+++) intensity. x 16

Following this procedure, 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 five used in our study. These antibodies as mentioned above are: HLAG (clone MEM-G/2: sc-51676, Santa Cruz Biotechnology, Inc USA), CD68 (clone 514H12, Catalog No: PA0273, Leica Microsystems UK), CD56 (clone CD564, Catalog No: PA0191, Leica Microsystems UK), CD16 (Novocastra Lyophilized Mouse Monoclonal antibody, clone 2H7, Product Code: NCL- CD16, Leica Microsystems UK) and CD25 (clone 4C9, Catalog No: PA030 Vision BioSystems Europe Ltd UK). Eventually, specimens were washed using WAS solution.

For the detection of immunohistochemical staining, specimens were firstly immersed in Post-Primary solution. After being washed , specimens were immersed in Polymere solution and then in chromogen diaminobenzidine (DAB) solution. Finally, specimens were stained with Haematoxylin- eosin. Following the previous stages that were performed by the automatic processor, specimens were rinsed in tap water and dehydrated with escalating densities of ethanol solution (70, 96 and 100% v/v consecutively) and xylene. Then, they were covered with tape, placed in glass plates and immersed in Canada balsam.

The previously reported immunohistochemical staining procedure was repeated for each of the 5 antibodies that were examined in this study. The monoclonal antibodies CD56, CD25 and CD68 were already set and ready to be used, while the monoclonal antibodies CD 16 and HLAG needed to be in dilutions 1:20 and 1:50 respectively.

Microscopic evaluation was performed on the cells of the intermediate trophoblast on decidua basalis and decidua parietalis of recurrent miscarriage and elective abortion material. Specimens were examined using an optical Zeiss™ microscope and photographs were taken using a Contax™ camera, attached to the microscope. In total, 100 specimens (50 from decidua basalis and 50 from decidua parietalis) were examined. Intensity of staining was evaluated as negative (-), weak (+), moderate (++) and strong (+++). Finally, the results were statistically analysed and checked for their significance using the Mann-Whitney test.

Results

Regarding the immunohistochemical staining for CD-68 cells, all sections (n=25) of decidua basalis in control group were found to be negative (-) (Fig. 3a). In contrast, the staining for CD-68 cells was detected as positive in all sections (100%) of decidua basalis in the miscarriage group (Fig. 3b). Specifically, 3 out of 25 (12%) sections of miscarriage group presented moderate (++) staining, with the rest (88%) strong positive (+++) (Fig. 3b). Observing the sections of decidua parietalis of control group, CD68 cells were not identified in any of them (0%). On the contrary, intensity of immunohistochemical staining for CD-68 cells on decidua parietalis of women from miscarriage group was detected as moderate (++) in all sections (n=25). It is obvious that there is a statistically significant difference between miscarriage and control group, in regards to infiltration of CD-68 cells in both decidua basalis and parietalis ($p<0.05$) (Table 1).

Immunohistochemical staining for CD 25+ cells was found to be positive (+) in all sections (100%) from decidua basalis in control group, presenting a weak (+) staining. All sections (100%) from decidua basalis in the

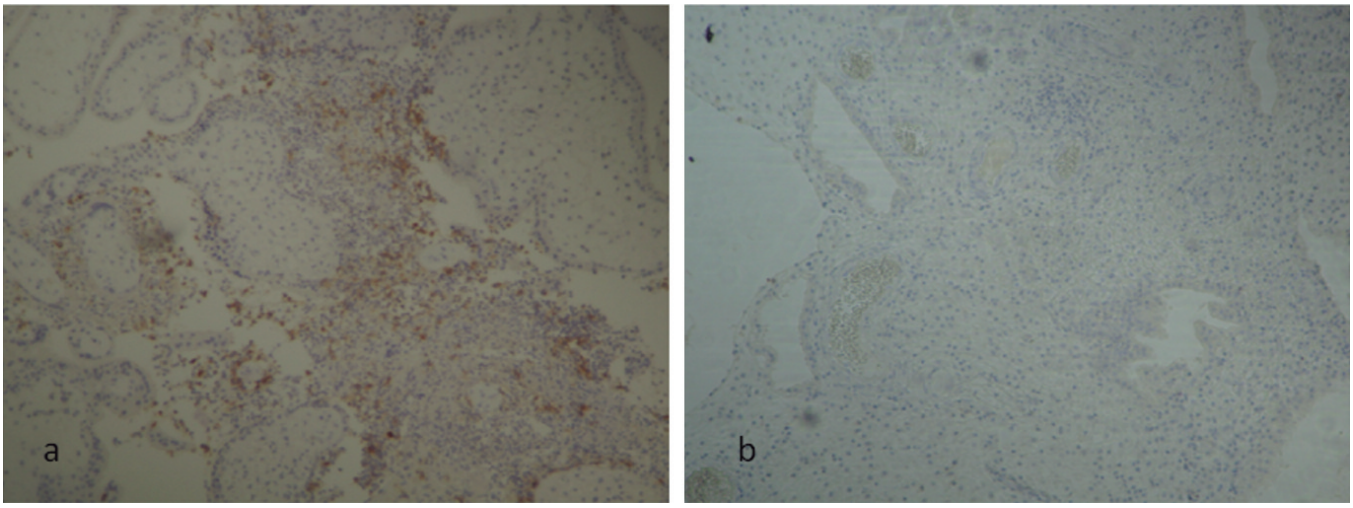

Fig. 4. Staining for CD25 cells. **a.** Decidua basalis. Miscarriage group. Weak (+) intensity. **b.** Decidua parietalis. Control group. Negative (-) staining. x 40

miscarriage group were also detected with weak positive (+) staining (Fig. 4a). On the contrary, immunohistochemical staining for CD 25+ in all sections (100%) from decidua parietalis, was detected negative (-) in both control and miscarriage group (Fig. 4b). Consequently, it is apparent that there was not a statistically significant difference in CD 25+ cell expression between women of miscarriage and control group, in both decidua basalis and parietalis sections ($p<0.05$) (table 1).

Considering immunohistochemical staining for CD16+ cells, all sections (n=25) from decidua basalis in the control group, were found to be positive (+) (Fig. 5a). Analytically, 5 sections out of 25 (20%) presented weak (+) staining, while the rest of them (80%) showed a moderate positive (++) staining. Furthermore, all sections (n=25) from decidua basalis in miscarriage

Table 1. Intensity of staining for immunological cell markers in decidual tissues from miscarriage group and control group

	Miscarriage Group	Control Group		Miscarriage Group	Control Group	
CD68	Decidua Basalis	Decidua Basalis	p	Decidua Parietalis	Decidua Parietalis	p
(-)	0 (0%)	25 (100%)	<0.05	0 (0%)	25 (100%)	<0.05
(+)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
(++)	3 (12%)	0 (0%)	<0.05	25 (100%)	0 (0%)	<0.05
(+++)	22 (88%)	0 (0%)		0 (0%)	0 (0%)	
CD56						
(-)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
(+)	25 (100%)	25 (100%)		0 (0%)	2 (8%)	
(++)	0 (0%)	0 (0%)		3 (12%)	23 (92%)	<0.05
(+++)	0 (0%)	0 (0%)		22 (88%)	0 (0%)	<0.05
CD16						
(-)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
(+)	0 (0%)	5 (20%)		0 (0%)	25 (100%)	<0.05
(++)	3 (12%)	20 (80%)	<0.05	2 (8%)	0 (0%)	
(+++)	22 (88%)	0 (0%)	<0.05	23 (92%)	0 (0%)	<0.05
CD25						
(-)	0 (0%)	0 (0%)		25 (100%)	25 (100%)	
(+)	25 (100%)	25 (100%)		0 (0%)	0 (0%)	
(++)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
(+++)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
HLA-G						
(-)	25 (100%)	0 (0%)	<0.05	25 (100%)	25 (100%)	
(+)	0 (0%)	25 (100%)	<0.05	0 (0%)	0 (0%)	
(++)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	
(+++)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	

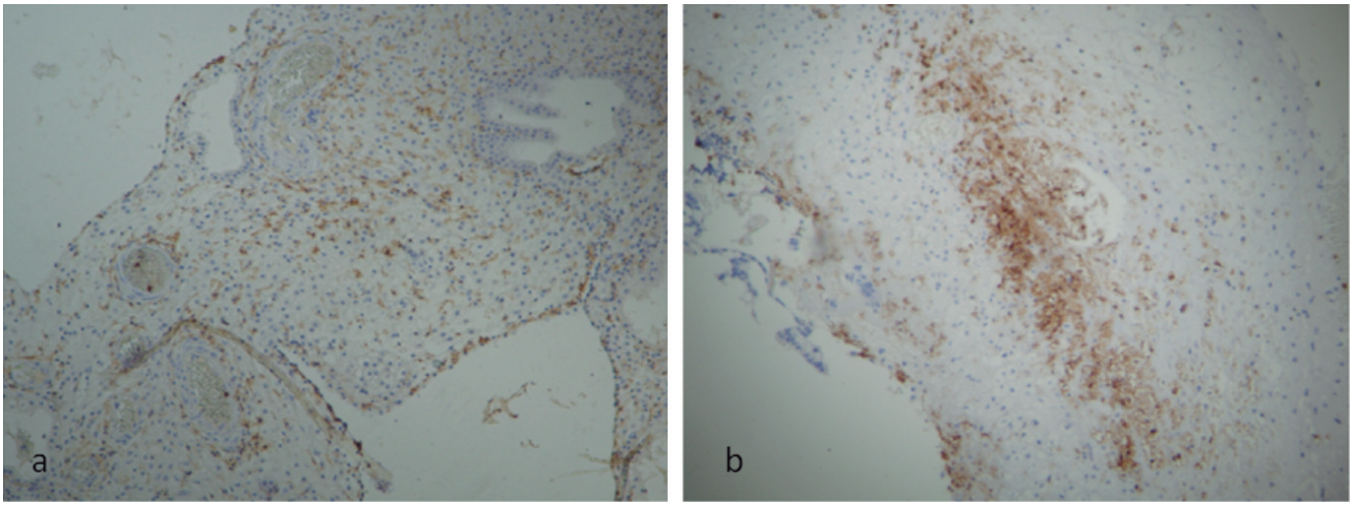

Fig. 5. Staining for CD16+ cells in decidua basalis. **a.** Control group. Weak (+) intensity. **b.** Miscarriage group. Strong (+++) intensity. x 40

group were also detected positive (+), with the majority of them (88%) being strongly (+++) stained (Fig. 5b). A statistically important difference in CD16+ cells expression was observed between women of miscarriage group and those from control group, in sections from decidua basalis ($p<0.05$) (Table 1). Observing sections from decidua parietalis in the control group, CD 16+ cells were identified in all of them (n=25), presenting a weak intensity staining (Fig. 6a). Regarding sections from decidua parietalis in miscarriage group, all sections (n=25) were found to be positive (+), with 2 of them showing moderate staining and the other 23 sections being strongly stained (Fig. 6b). A statistically important difference in CD16+ cell expression was detected between miscarriage group and control group , in sections from decidua parietalis. ($p<0.05$) (Table 1).

In both control and miscarriage group, all sections from decidua basalis presented weak positive immunohistochemical staining for CD56+ cells (Fig. 7a). Regarding the immunohistochemical staining for CD56+ cells of decidua parietalis, all sections in the control group were positive, with the majority of them expressing a moderate intensity staining (Fig. 7b). Similarly, in the miscarriage group, all sections were positive, with 88% of them showing a strong staining of CD56+ cells. It is obvious that a statistically important difference in CD56+ cell expression was detected in decidua parietalis between the miscarriage group and control group ($p<0.05$) (Table 1).

Regarding immunohistochemical staining for HLA-G antibody in sections of decidual basalis, there was statistically significant difference between control and miscarriage group. All sections (n=25) of decidua basalis in control group were found to be weak positive (+) (Fig. 8a), while all sections in the miscarriage group were negative (-) (Fig. 8b).As for the sections from decidua parietalis, in both control and miscarriage group HLA-G antibodies were not found (Table 1).

Discussion

Based on the results of our study, the immunological profile of women with recurrent pregnancy loss is quite different compared with that of controls. It seems that recurrent pregnancy loss syndrome is provoked by a 'malfunction' of the immunity system, which fails to protect the fetus. Previous reports concerning this syndrome, seem to support such a hypothesis, as they also detect alterations of the relative populations of the immunity cells in deciduas in contrast with controls.

The role of macrophages (CD 68+ cells) in formation of feto-maternal interface is well known. Previous studies highlight that macrophages, in human uterus, participate in apoptotic cell phagocytosis (Abrahams et al., 2004), interact with the trophoblast to perform vascular remodeling in the uterus (Harris, 2010), increase monocyte migration at decidua basalis and induce a significant increase in the secretion and production of the pro-inflammatory cytokines and chemokines (Fest et al., 2007). In our study, we detected a significant increase of CD68+ cell expression in both decidua basalis and parietalis of women with recurrent miscarriage, in contrast with controls. Based on the available reports, the number of macrophages in decidua of women with recurrent pregnancy loss seems to be similar with the normal population (Laird et al., 2003). An increase in macrophage population was observed only in non pregnant endometrium of women with recurrent miscarriage (Laird et al., 2003). Our research suggests a possible involvement of CD68+ cells in recurrent pregnancy loss pathophysiology.

Previous research claims that CD25+cell (T-regulatory cells) accumulation during the first trimester of pregnancy protects the fetus from possible abortion

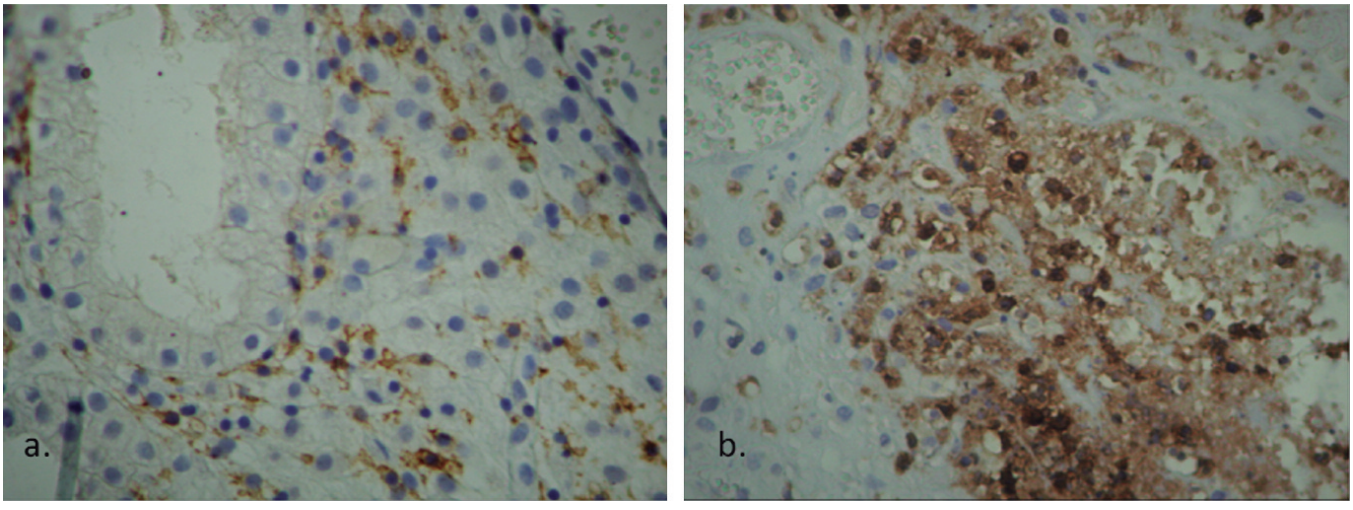

Fig. 6. Staining for CD16+ cells in decidua parietalis. **a.** Control group. Weak (+). **b.** Miscarriage group. Moderate (++). x 160

(Guerin et al., 2009). Bao et al (2011) supported that the percentage of CD25+cells over the total population of T cells in decidua was decreased in miscarriage group in comparison with control group (Bao et al., 2011). Our results indicate no change in CD25+cell expression between miscarriage group and controls in both decidua basalis and parietalis, in contrast with previous research, which demonstrates that the number of CD25+cell in decidual samples of women with recurrent pregnancy loss was higher in comparison with elective abortions (Quack et al., 2001). However it should be noted that a quantitative calculation of the exact number of such cells in human deciduas is more appropriate to detect possible alterations in CD25+cells population.

Uterine NK cells play a major role in pregnancy and variations in their quantity and activation may lead to infertility or recurrent pregnancy loss (Hiby et al., 1998). Contradictory results regarding the alterations in CD56+ cell population in women with RPL are reported (Quack et al., 2001; Laird et al., 2003; Bulmer et al., 2010). Based on our results, CD56+cell expression was increased in decidua parietalis of women from the miscarriage group, compared with tissues from control group. On the contrary, in decidua basalis, intensity of staining for CD56 factor was found to be invariable in both groups of the present study. Furthermore in our study, the number of CD16+ cells was found to be significantly higher in both decidua basalis and decidua parietalis tissues of women from the miscarriage group compared with controls. A possible interpretation of the above mentioned results could be the different subsets of NK cells in human peripheral blood, endometrium and

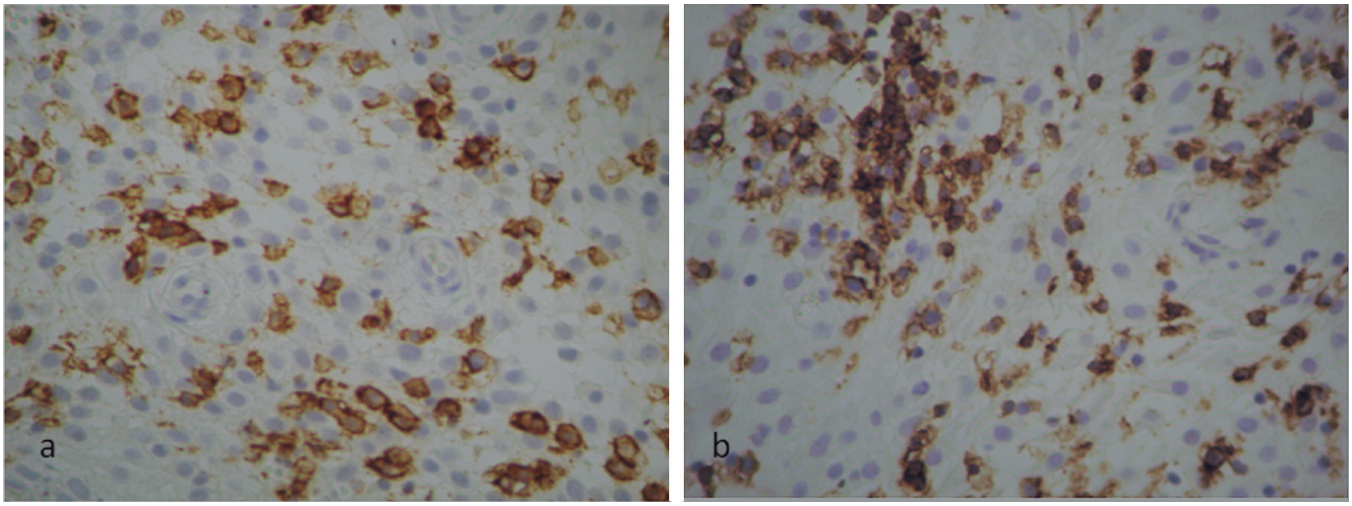

Fig. 7. Staining for CD56+ cells in decidua parietalis. **a.** Control group. Moderate (++) intensity. **b.** Miscarriage group. Strong (+++) intensity. x 160

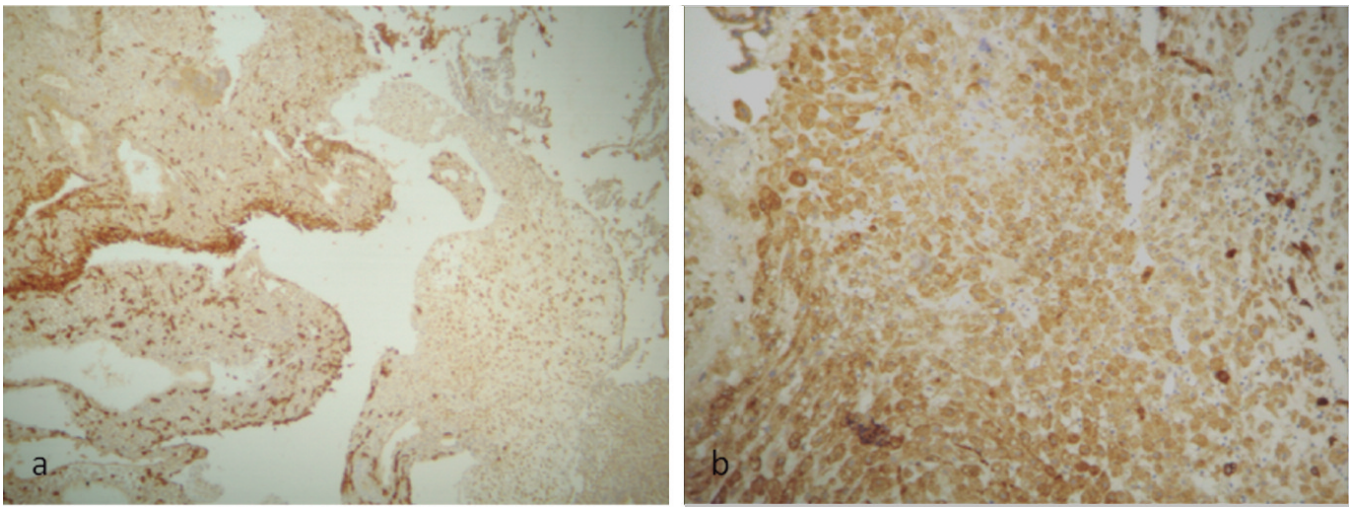

Fig. 8. Staining for HLA-G in decidua basalis. **a.** Control group. Weak (+) intensity. **b.** Miscarriage group. Negative (-) intensity.

decidua (Laird et al., 2003).

HLA-G expression in antigen-presenting cells suppresses T-cell proliferation and alters the killer function of uterine macrophages and NK cells, protecting the semi-allogenic fetus (Long, 1999; Naji et al., 2007; Shakhawat et al., 2010). In our study, HLA-G expression was found to be decreased in decidua parietalis of women with RPL in comparison with women with elective abortions (control group). In decidua basalis, in both groups, *HLA-G* expression was found to be invariable. Our results are in accordance with the relative previous reports, which claim that in decidua from women with recurrent miscarriage an increased NK cell marker expression of both CD56 and CD16 was accompanied by a decreased expression of HLA-G (Emmer et al., 2002). However, our conclusions should be interpreted with caution, as other reports support that *HLA* class 1b antigens, *HLA-E, HLA-F,* and *HLA-G*, are detectable only on some subpopulations of trophoblast (Hunt and Orr, 1992; Hunt, 2006). In the same direction, Bhalla (2006) stated that HLA-G is expressed only in the extravillous trophoblast and there is no significant difference in expression pattern between the recurrent miscarriage and the control group (Bhalla et al., 2006).

Although the cells of the immunity system and their factors seem to be involved actively in feto-maternal interface disorganization and unexplained recurrent pregnancy loss, much research is yet needed in order to decrypt fully the multifactor etiology of this clinical entity.

Conclusions

In the present study, statistically important alterations in the expression of specific subpopulations of leucocytes and HLA-G were observed in the decidual tissues of women with recurrent pregnancy loss of unknown etiology. Feto-protective HLA-G expression was found to be decreased . Expression of macrophages was found to be increased, suggesting a possible involvement of these cells in the pathogenesis of the syndrome. NK cells had also been found to be increased, although they were considered by a lot of studies as feto-protective. A possible implication of this subpopulation in recurrent pregnancy loss can be hypothesized. The bibliography offers contradictory results about the effect of these subpopulations in RPL syndrome. Their study is difficult and further research is needed in order to reveal how exactly these cells act in human pregnant uterus.

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