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Structural, immunohistochemical and molecular features of placentas and placental sites after *in vitro* fertilization with donor eggs (surrogate motherhood)



Evgeniya A. Kogan, Ekaterina E. Rudenko*, Tatiana A. Demura, Nikolay V. Zharkov, Natalia S. Trifonova, Elvira V. Zhukova, Leonid S. Aleksandrov, Sofia N. Bayanova

Sechenov First Moscow State Medical University (Sechenov University), Trubetskaya str. 8, bld.2, 119991, Moscow, Russian Federation

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ABSTRACT

Objective: to identify structural, immunohistochemical and molecular features of placentas and placental sites after *in vitro* fertilization (IVF) with donor eggs (surrogate motherhood).

Study design: morphological and immunohistochemical studies were performed on placental material obtained after delivery by caesarean section. The study included 26 women patients whose pregnancy resulted from IVF with a donor egg (IVF-SM group). The comparison group included 13 women patients whose pregnancy occurred after IVF with their own eggs (IVF-OE). Immunohistochemistry of biopsy material was performed using mouse antibodies to total cytokeratin (clone AE1/AE3) and murine antibodies to HLA-DR (clone TAL1B5). Molecular studies were performed on DNA samples isolated from venous blood. HLA-DNA-TEH reagent kits and polymerase chain reaction were used for genotyping the main human histocompatibility complex class II (DQA1, DQB1 and DRB1).

Results: Histological examination of placenta in IVF-SM group showed a high incidence of central ischemic infarctions (69% of cases), dissociated cotyledon development (61%), pathological villus immaturity (46%) and massive perivillous fibrin deposition (73%). This group also had a pronounced lymphoplasmacytic deciduitis, which was 2 times higher than in the control group, and an expressed inflammatory process in the placental sites. Remodeling of the spiral arteries was incomplete in more than 40% of cases, and 30% of spiral arteries had no gestational changes. In comparison group, a complete gestational adjustment was found in more than 90% of spiral arteries. A focal lymphohistiocytic infiltration in perivascular regions, and a decrease in the number of multinucleated cells as compared with the control were also observed. For seven female surrogate mothers and their children, allelic polymorphisms of genes of HLA II class were studied.

Conclusion: Placental material of women from IVF-SM group is characterized by complex immune response in sites of tight contact between maternal and fetal tissues. The immune pathogenesis is associated with an increase in the number of HLA-DR positive cells, defects in remodeling of the spiral arteries, development of areas of chronic inflammation in perivascular regions, and a decrease in the number of multinucleated cells. Genetic incompatibility between alleles of HLA II genes can be a molecular predictor of impaired immune tolerance.

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Introduction

Surrogate motherhood is a modern assisted reproductive technology (ART), in which donor oocytes (DO) are used in the *in vitro* fertilization procedure. A feature of such pregnancies is gestation of the fetus which does not carry maternal genes and is allogenic to the mother [1]. This results in immunological tolerance

in the "mother-placenta-fetus" system and increases the risk of reactions like "host versus transplant", as well as "transplant versus host" [1,2]. Despite the absence of somatic health problems in surrogate mothers and a positive obstetric history, IVF-SM pregnancy typically occurs with a significant number of complications, such as pre-eclampsia, premature birth, gestational hypertension, etc. [3]. It can be assumed that the predictor of the development of pregnancy complications after oocyte donation in the surrogate motherhood program is a violation of the immunological tolerance in the mother-placenta-fetus system, which is manifested as complex immune response in areas of tight

* Corresponding author.

E-mail address: redikor2@yandex.ru (E.E. Rudenko).

contact between maternal and fetal tissues. Such areas are decidual tissue, placental bed tissues and villous chorion [4]. The purpose of this study is to identify the structural and immunohistochemical features of the placental (afterbirth) and uteroplacental region after IVF-SM.

Study design

The study involved 26 women patients whose pregnancy resulted from IVF with donor eggs (IVF-SM) under the surrogate motherhood program. The comparison group included 13 women patients whose pregnancy resulted from IVF with their own eggs (IVF-OE). The criteria for inclusion in the study were singleton pregnancies, the length of gestation more than 30 weeks, the absence of severe extragenital pathologies and pregnancy complications with well-established pathogenesis (preeclampsia, antiphospholipid syndrome, hereditary forms of thrombophilia, etc.). A morphological study was performed on placental material (placental disk and placental bed), obtained after delivery by caesarean section. The weight and height parameters of newborns measured in the first 2 h of their life were also analyzed.

A macroscopic study of non-fixed placental afterbirth samples was carried out according to a standard protocol [5]. All parts of the afterbirth samples, including the umbilical cord, fetal membrane, and the placental disk, were studied. For each afterbirth, the fetal-placental ratio was calculated as the ratio of the weight of the placental disk to the weight of the fetus (the average value of this ratio for full-term pregnancy is 0.12–0.17 [6]). Histological examination of paraffin sections of placentas (paracentral and peripheral areas) and placental beds stained with hematoxylin and eosin was performed. Pathological changes in the afterbirth tissue, characteristic of maternal malperfusion (central ischemic infarction, decidual vasculopathy, hypoplasia of the distal villi), fetal stromal vascular lesions (vasculopathy of stem villus vessels, pathological villus immaturity, dissociated cotyledon development), compensatory and adaptive (Tenney-Parker changes), and immunopathological processes (massive fibrin deposition, lymphoplasmacytic deciduitis) were studied. The change was considered significant if it was found in more than 5 out of 10 fields of view ($\times 20$) in the characteristic part of the placental disk. A semi-quantitative assessment of the area of chronic inflammation was carried out in the decidual afterbirth and the placental bed tissues according to the Amsterdam Placenta Working Group [7]. Deciduitis progression was determined by the number of lymphohistiocytic cells in the field of view (0 points no/single cells of the lymphohistiocytic series, 1 point up to 30 cells, 2 points up to 50 cells, 3 points more than 50 cells in one field of view).

Immunohistochemical analysis was performed on paraffin sections prepared from biopsy material. Immunohistochemical reactions were performed on dewaxed sections (thickness 4–5 μm), located on glasses coated with a polylysine layer (Menzel Glaser Polylysine, Germany). Polylysine glasses were deparaffinized according to standard procedure and, after rinsing in distilled water, immersed in citrate buffer (pH 6.0) and heated on a water bath at 95 °C for 20 min. The glasses were cooled to room temperature for 20 min and then incubated for 10 min with a peroxidase inhibitor in a humidity chamber. Then they were rinsed with phosphate buffer (pH 7.0–7.6) and incubated with Ultra-V-Block (LabVision, USA) for 30 min. Excess reagent was carefully removed from the glasses.

Mouse antibodies to total cytokeratin (clone AE1/AE3) and mouse antibodies to HLA-DR (clone TAL.1B5) (Dako, 1:50 dilution) were used as primary antibodies. The sections were incubated with antibodies for 30 min according to specification from the antibody manufacturer. Then the sections were thoroughly washed in phosphate buffer (pH 7.0–7.6). For the detection of primary

antibodies bound to the corresponding antigens, the universal polymeric system Histofine® Simple Stain MAX PO (MULTI) (Nichirei, Japan) was used. This system contains a dextran framework with multiple attached horseradish peroxidase molecules and secondary antibodies to anti-mouse and anti-rabbit immunoglobulins (Ig). The sections were incubated with the detection system in a humidity chamber for 30 min and then rinsed in phosphate buffer (pH 7.0–7.6). To visualize the site of binding of antibody to antigen, the chromogenic substrate 3,3-diaminobenzidine (DAB) was oxidized with horseradish peroxidase in the presence of hydrogen peroxide. The final reaction product was formed which was visible as brown staining of specific cell structures (N-Histofine® DAB-2V, Nichirei, Japan). The sections were incubated with DAB for 5–10 minutes. Then the glasses were washed in distilled water, and the nuclei were stained with hematoxylin for 2–3 minutes. The glasses were dehydrated in a battery consisting of distilled water, alcohol (70%, 80%, 95%, and 100% aqueous solutions) and xylene (three portions). Then the sections were covered with cover glasses using BioMount synthetic medium.

When conducting immunohistochemical reactions, positive and negative controls were used. As negative controls, samples of the studied sections processed without incubation with primary antibodies were used. The positive control for each antibody was selected in accordance with the recommendations of the antibody manufacturer.

Molecular studies were performed on DNA samples isolated from EDTA-stabilized venous blood using QIAamp DNA Blood Mini Kit (QIAGEN, Germany). HLA-DNA-TEC reagent kits (DNA technology, Russia) were used for genotyping of the main human histocompatibility complex (HLA) class II (DQA1, DQB1 and DRB1). The polymerase chain reaction was carried out using DT96 detecting amplifier (DNA technology, Russia) according to the manufacturer's protocol. Alleles of HLA II gene (DQA1, DQB1, DRB1) in seven surrogate mother-child pairs were identified.

Statistical data processing was carried out using IBM SPSS Statistics 23 statistical software package (IBM, USA). For comparison of two or more samples, non-parametric statistical significance assessment methods, such as χ^2 criterion, Fisher exact method and Mann-Whitney criterion, were used. To determine the statistical significance of differences in averages in independent samples, Student's *t*-test was used. Differences were considered significant at $p < 0.05$ (**) or $p < 0.01$ (*).

Results and discussion

The macroscopic study of the placental samples taken from IVF-SM group showed an increase in the average weight of placenta, as well as an increase in the average placental-fetal ratio, compared with IVF-OE group. False nodes of the umbilical cord (in 2 cases), central and peripheral infarctions (in 5 and 8 cases, respectively) were also found in IVF-SM group. Intervillous blood clots were detected in 2 cases. The observed differences were not statistically significant, and the described macroscopic indicators corresponded to those reported in literature (Table 1).

Table 1
Macroscopic indicators of placentas in women from IVF-SM and IVF-OE groups.

indicator	IVF-SM (n=26)	IVF-OE (n=13)	p(F)
average weight of placenta, g	688 ± 81	490 ± 25	–
average placental-fetal ratio	0.21 ± 0.15	0.18 ± 0.02	–
umbilical cord separation, n	0	1	p>0.05
false nodes of the umbilical cord, n	2	3	p>0.05
central infarctions, n	18	8	p>0.05
peripheral infarctions, n	14	10	p>0.05
intervillous blood clots, n	2	1	p>0.05

Histological examination of placentas in IVF-SM group (Table 2) demonstrated a high incidence of central ischemic infarctions (69% of placentas), development of impaired (dissociated) cotyledons (61%), pathological immaturity of villi, mainly with the predominance of intermediate differentiated villi (46%), and massive perivillous fibrin deposition (73%). The differences with the comparison group were significant ($p < 0.05$). At the same time, no significant differences between the studied groups were observed in such indicators as Tenney-Parker changes, distal hypoplasia, decidual vasculopathy and vasculopathy of stem villus vessels.

Lymphoplasmacytic deciduitis was observed in more than 80% of placentas of women from IVF-SM group. This pathology is typically characterized by the presence of an increased number of lymphocytes and macrophages in the basal or parietal decidual tissues, which can be associated with a chronic infectious process. While the presence of macrophages and lymphocytes is a variant of the norm, the presence of plasma cells in decidual infiltrates is regarded as a pathology [8]. The IVF-SM group had a pronounced lymphoplasmacytic deciduitis, which was 2 times higher than in the control group (1.23 ± 0.4 vs. 0.5 ± 0.3), and the expressed inflammatory process in the placental sites (2.15 ± 0.3).

An increased formation of lymphoplasmacytic infiltrate around the vessels of the placental site is associated with maternal malperfusion due to incomplete gestational remodeling of the spiral arteries. The term "placental site" is commonly understood as a part of the uterus (decidual plate and myometrium), located directly below the place of implantation of the ovum and formation of the placenta [9]. The spiral arteries of the placental site (a total of 100–150 pieces) are involved in the process of gestational remodeling, which leads to complete transformation of up to 90% of the myometrial segments of these arteries. Under the influence of proteases secreted by the non-villous trophoblast, lysis of elasto-muscular elements of the spiral and radial arteries of the uterus occurs with the full replacement of all the wall elements of these vessels with fibrinoid, which contributes to a constant increase of maternal arterial blood flow to the villi of the placenta [10].

Morphological study of the placental sites in IVF-SM group demonstrated that the remodeling of the spiral arteries was incomplete in more than 40% of cases, and that 30% of spiral arteries had no gestational changes. In the comparison group, a complete gestational adjustment was found in more than 90% of spiral arteries. A focal lymphohistiocytic infiltration of the perivascular regions was also observed, as well as a decrease in the number of multinucleated cells as compared with the control. Thus, pregnancy with an allogeneic fetus leads to the development of pathology of the placental bed, consisting in defects in remodeling of the spiral arteries, areas of chronic inflammation in the perivascular areas, and a decrease in the number of giant multinucleated cells (Fig. 1).

To assess the degree of development of the immunological response, an immunohistochemical study of placentas and

placental beds with antibodies to HLA-DR was conducted. HLA-DR is one of the MHC class II antigens which can be used as a marker of activated cells and an indicator of transplant rejection reactions. A significant ($p < 0.05$) increase in the number of HLA-DR-positive cells in the placenta and placental bed (dendritic cells and macrophages of maternal origin) was detected in IVF-SM group compared to control, which confirms the long-term immunization of the mother's tissues with allogeneic fetal antigen.

An immunohistochemical study with antibodies to pancytokeratins (Pan-CK) revealed multinucleated giant cells (MGC) of the trophoblast. We also observed a decrease in the number of MGC and in the depth of invasion of the non-villous trophoblast into myometrium (Table 3).

The sites of tight contact between maternal and fetal tissues are the basal decidual tissue, the syncytiocapillary membrane in villi, and the invasive trophoblast of the placental bed. The lymphohistiocytic infiltration at these points suggests the development of immune processes [11] (Fig. 1).

Our study revealed a significant progression of basal deciduitis and immuno-inflammatory processes in the placentas and placental sites in IVF-SM group without pregnancy complications, compared with IVF-OE group, which is consistent with the literature data. The immune interactions initiated from the stage of blastocyst implantation form an immune tolerance in the mother-fetus system. The proliferating trophoblast invades the tissues of the uterus and spiral arteries. As a result, the maternal organism produces anti-father antibodies which are fixed on placenta. They have an immunotropic effect, blocking the efferent link of the immune response at the local level, which makes placenta an immunologically privileged tissue [12]. The second wave of allogeneic trophoblast invasion into the uteroplacental segment of the spiral arteries can also be disrupted due to pronounced immune response of the maternal organism. However, despite the similarity of these processes with transplant rejection reactions, the pregnancy with a donor egg is not interrupted and continues until delivery. The trophoblast carrying only alien antigens probably activates other mechanisms of tolerance, which are clinically manifested as an increased risk of developing pregnancy complications. The lymphohistiocytic infiltration at the sites of tight contact between maternal and fetal tissues suggests the development of immune processes [11]. The activity and duration of these immune reactions can be confirmed by an increase in the number of HLA-DR positive cells, which is described in literature as the transplant rejection reactions.

The study of molecular mechanisms of tolerance violation in the mother-fetus system is a highly important task, since oocyte donation technology is the only ART that allows selection of mother-child pairs based on their genetic compatibility. Such a selection minimizes the risks of development of obstetric pathologies. In this work, for 7 female surrogate mothers and their children, a molecular analysis was performed to study the allelic polymorphism of three HLAII class genes (DQA1, DQB1, DRB1). The types of alleles and the frequency of their occurrence in

Table 2
Histological characteristics of the placental samples.

Indicator (n)	IVF-SM (n = 26)	IVF-OE (n = 13)	p value
central ischemic infarctions	18	6	$p < 0.05$
Scintial nodes (Tenney-Parker changes)	7	5	$p > 0.05$
distal hypoplasia	10	4	$p > 0.05$
decidual vasculopathy	9	6	$p > 0.05$
dissociated cotyledons	16	6	$p < 0.05$
vasculopathy of stem villus vessels (segmentary)	9	6	$p > 0.05$
pathological immaturity of villi	12	2	$p < 0.05$
massive perivillous fibrin deposition (pseudoinfarctions)	19	3	$p < 0.05$
lymphoplasmacytic deciduitis	24	4	$p < 0.05$

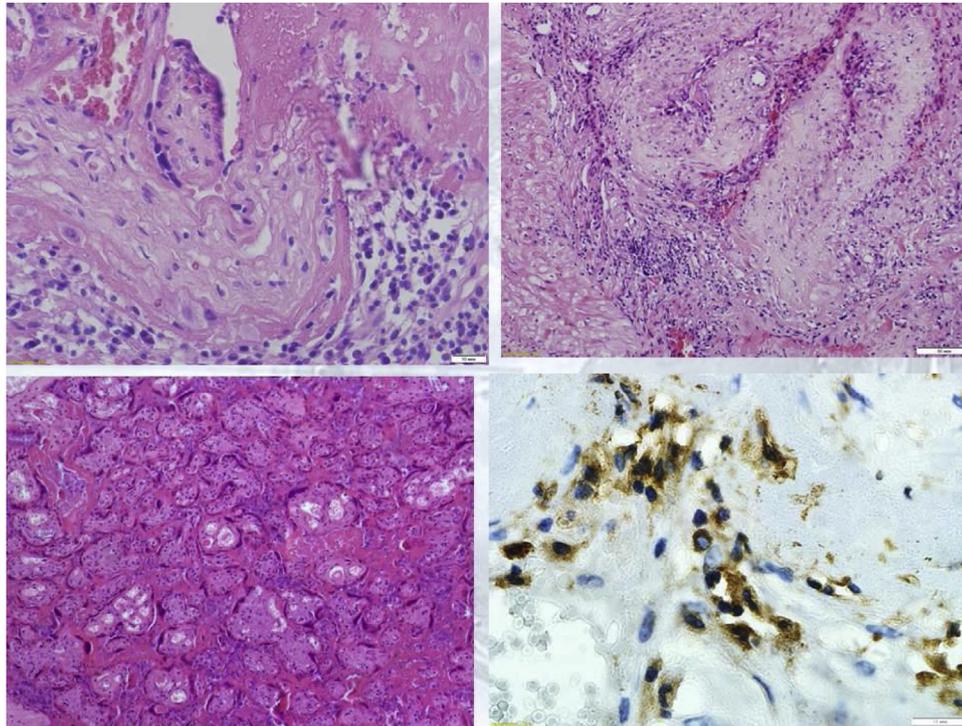


Fig. 1. Histological changes in placental samples of women from IVF-SM group. **A.** Lymphoplasmacytic deciduitis. **B.** Perivascular area of chronic inflammation in the tissues of the placental bed without remodeling of the spiral artery, and without clinical manifestations of preeclampsia. **C.** Ischemic infarction up to 7 days old. **D.** Immunohistochemical picture of HLA-DR expression by dendritic cells in decidual tissue.

Table 3
Histological and immunohistochemical characteristics of placental sites.

	IVF-SM (n = 26)	IVF-OE (n = 13)	Statistical method	p value
Immunoinflammatory processes in placental bed	2.15 ± 0.69	0.50 ± 0.47	Mann-Whitney U-criterion	p < 0.05
HLA-DR cells (cells/10 fields of view)	41.9 ± 5.3	29.0 ± 1.9	Student's t-criterion	p = 0.0388
MGC cells (cells/5 fields of view)	127.4 ± 5.59	65.7 ± 14.5	Student's t-criterion	p = 0.001561
complete remodeling of the spiral arteries (%)	23	96	–	–
partial remodeling of the spiral arteries (%)	46	4	–	–
absence of remodeling (%)	31	0	–	–

Table 4
Polymorphism of HLA II genes (DQA1, DQB1, DRB1) in a group of surrogate mothers.

patient	DQA1		DQB1		DRB1	
	mother	child	mother	child	mother	child
1	*0102, *0301	*0201, *0501	*0302, *0602-8	*02, *0303	*04, *13	*03, *07
2	*0102, *0103	*0101, *0201	*0502/*0504, *0602-8	*02, *0503	*13, *16	*07, *14
3	*0102, *0501	*0301, *0301	*0301, *0502/*0504	*0302, *0401/*0402	*11, *16	*04, *04
4	*0201, *0501	*0101, *0102	*02, *0301	*0501, *0602-8	*07, *13	*01, *15
5	*0101, *0102	*0301, *0501	*0501, *0602-8	*02, *0301	*01, *15	*02, *04
6	*0401, *0501	*0102, *0501	*02, *0301	*0301, *0602-8	*03, *02	*11, *13
7	*0101, *0102	*0401, *0501	*0501, *0502/*0504	*02, *0301	*01, *16	*03, *11

surrogate mother-child pairs were identified for each of these genes (Table 4).

For DQA1 gene, the most common alleles were *0102 for mothers and *0501 for children (frequency of occurrence 36.7% and 28.5%, respectively). For DQB1 gene, the most common alleles were *0502/*0504 and *0602-8 (21.4% in both cases) for women, and *02 (36.7%) for children. For DRB1 gene, the most common alleles were *13 and *16 (21.4% in both cases) for women, and *04 (21.4%) for children. For a mother-child pair 6, coincidences were found for DQA1*0501 and DQB1*0301 alleles. It is noteworthy that a severe

form of preeclampsia was clinically diagnosed in this woman, which was histologically manifested as a characteristic placental alteration.

Conclusions

Structural features of placentas and placental sites of women whose pregnancy resulted from IVF with a donor egg, are characterized by pronounced immune alteration of the afterbirth, manifested in a high frequency of development of immune

reactions at the sites of tight contact of maternal and fetal tissues. These reactions include massive perivillous fibrin deposition, the presence of basal deciduitis and immuno-inflammatory infiltrates in placental sites. Immune pathogenesis of these processes is confirmed by immunohistochemical studies demonstrating an increase in the number of HLA-DR positive cells. The allogenic trophoblast invasion leads to significant pathology of the placental bed which involves defects in remodeling of the spiral arteries, development of areas of chronic inflammation in the perivascular regions, and decrease in the number of multinucleated giant cells. As a result, maternal malperfusion is formed. It can be assumed that the immune response in the placenta of surrogate mothers is developed due to violation of immune tolerance in the mother-fetus system, caused by the allogeny of the donor egg. Genetic incompatibility between alleles of HLA II genes can be a molecular predictor of impaired immune tolerance.

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