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ORIGINAL ARTICLE

Immunohistochemical Evaluation of Extravillous Cytotrophoblasts in the Uteroplacental Bed in Iron-deficiency Anemia of Pregnancy

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Objective: Iron deficiency anemia (IDA) in pregnancy is challenging, inducing a systemic pathological process in women's bodies. In this context, impaired protein metabolism, decreased respiratory enzyme activity, limitation of cells' proliferative potential, oxidative stress, hypoxia, and endothelial dysfunction affect the "mother-placenta-fetal" system. However, several features of this pathogenetic cascade, including the spread of extravillous cytotrophoblasts (ECT), are yet to be investigated. This research aimed to study the immunohistochemical features and quantitative indicators of ECT spread throughout the uteroplacental bed (UPB) in IDA at 37–40-week gestation. **Materials and Methods:** Eighty biopsies of UPB and myometrium were studied postcesarean section with the previous observation of physiological pregnancy and gestation in IDA conditions without clinical signs of placental insufficiency. Histological sections were performed on immunohistochemical techniques with primary antibodies against metalloproteinases 2 and 9, placental lactogen, and antiapoptotic protein BCL-2. Their quantitative parameters in the cytoplasm of endovascular CT cells were determined using computer microdensitometry by calculating optical color density. The arithmetic mean and its statistical error were calculated. The Shapiro–Wilk test of normality was performed. The comparisons between the groups were provided on the unpaired two-tailed Student's test. **Results:** The analysis of the depth of CT invasion on the material of the UPB and the myometrium in conditions of IDA showed the spread of the CT invasion not only in the area of the spiral arteries of the endometrium but also the incorporation of endovascular CT into the myometrial arteries walls. Immunohistochemical study of CT invasion showed the following results: during physiological pregnancy, the concentration of metalloproteinase 2 was 0.232 ± 0.0012 optical density units (ODU), metalloproteinase 9 - 0.219 ± 0.0014 ODU. Immunovisualization of placental lactogen during physiological pregnancy was 0.314 ± 0.0022 ODU in interstitial CT and 0.109 ± 0.0022 ODU in endovascular CT; in the conditions of IDA: 0.337 ± 0.0022 ODU in interstitial CT and 0.110 ± 0.0022 ODU in endovascular CT. **Conclusions:** It is established that, during gestation with IDA, a deepening of CT invasion in the structure of the UPB, an increase in the lining distance of the endothelium-replacing CT in the artery walls, and consequently, a dilatation of spiral and radial arteries takes place in the uterus of pregnant women. However, the invasive ability and synthetic activity of the CT and as a result, the adequacy of the gestational transformation of UPB structures are affected by the hypoxia and depend on the degree of IDA of pregnant women.

Key words: Uteroplacental bed, cytotrophoblast invasion, extravillous cytotrophoblasts, metalloproteinases, iron-deficiency anemia in pregnancy, gynecology

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INTRODUCTION

The uteroplacental bed (UPB) is a place of fertile eggs attachment to the uterus wall. It is the epicenter of gestational alterations of the endometrium, followed by a significant increase of the myometrium of the entire uterus and changes in its configuration. Pathological alterations of the UPB can lead to the development of the uterine-placental form of placental insufficiency, characterized by the violation of the formation of particular placental bed structures such as the spiral arteries and veins and the chorionic tree. [1,2]

The key mechanism in the morphogenesis of the UPB is invasion (CTI) – the process of migration of CTs from the placenta to the endometrium and myometrium with subsequent selective penetration into the uterine arteries and modification of them into the enlarged vascular canals, providing adequate blood supply to the fetus. This process establishes the uterine-placental circulation, which affects the growth and development of the fetus, in particular the successful completion of pregnancy or its loss at different stages. [3,4]

Invasive CTs are classified as interstitial CTs, multinucleated giant cells (MGC), and endovascular or endothelial CT. Invasive CTs synthesize specific metalloproteinases that provide lysis of the extracellular matrix, stroma, and vascular endothelium of the UPB. Endovascular CTs are required for the remodeling of the large radial artery by replacing intimal endothelial cells. MGCs are localized on the edge of the invasive process in the myometrium and are considered a reserve of the CT invasion, which in general determines the possibility of realization of the compensatory-adaptive reaction in the uterine-placental complex and favorable pregnancy outcome. [2-4]

Iron deficiency anemia (IDA) in pregnancy is one of the most challenging conditions of modern obstetrics. Its frequency in different regions varies between 28% and 85%. Iron deficiency leads to a systemic pathological process in women's bodies, caused by impaired protein metabolism, decreased respiratory enzymes activity, limitation of the proliferative potential of cells, oxidative stress, hypoxia, and endothelial dysfunction, which affects the "mother-placenta-fetal" system. [5,6]

However, a vital mechanism of the placentation – the CT's invasion into the uterus wall – has not been well studied. Most of the research in this area focuses on early pregnancy, but CT invasion is not completed at this stage. On the contrary, it continues during the second and third trimester of pregnancy. It can play a significant role in the delivery of a healthy fetus during the last weeks of pregnancy. This is because the reconstruction of the uterus radial arteries is necessary to increase maternal blood flow to the intervillous space of the placenta for the successful completion of pregnancy. [7]

Our research aimed to study the immunohistochemical features and quantitative parameters of the distribution of extravillous CT (ECT) in the uterine-placental bed (UPB) in conditions of IDA in pregnancy.

MATERIALS AND METHODS

Morphological studies were conducted between 2015 and 2019 at the Department of Pathological Anatomy of the Higher State Educational Establishment of Ukraine "Bukovinian State Medical University" (Chernivtsi, Ukraine, IRB protocol number: 9/19062019). The sampling of biomaterials for the study was performed during cesarean sections performed in university hospitals affiliated with the institution above. Pregnant women were informed and provided informed consent for collecting and using biopsies of the placenta and UPB for scientific purposes. IDA in pregnancy is a chronic pathology, so for the validity of the findings, biopsies were obtained only in case of complete clinical development of IDA, namely, when the iron deficiency was recorded from serum iron in pregnant women at least 2 weeks before the development of the typical hematological changes of pregnancy.

Eighty biopsies of UPB and myometrium were investigated including 38 observations of physiological pregnancy and 42 cases of gestation associated with IDA without clinical signs of iron insufficiency (according to the medical records) at 37–40 weeks of gestation. Women of both groups were similar in social status, occupation, and the distance between residence and the factories with harmful emissions. Cases of gestation with IDA combined with eclampsia, preeclampsia, hypertension, and other somatic pathologies were not included in the study.

For the complex morphological evaluation of the IDA in pregnancy, the researchers used general histological, histochemical, immunohistochemical, and morphometric (digital computer histometry, histostereometry, and microdensitometry) statistical methods of investigation. The material was fixed in 10% buffered neutral formalin for 24 h, dehydrated in a rising alcohol battery, and embedded in paraffin. After deparaffinization of histological material (with standard 5 μm thickness), the immunohistochemical analysis with primary antibodies against metalloproteinases 2 and 9, placental lactogen, and antiapoptotic protein BCL-2 with thermal antigen exposure (DAKO). We also used staining with hematoxylin and eosin.

The digital images were analyzed by a computer-aided program – ImageJ (1.48 v, free license, W. Rasband, National Institute of Health, USA, 2015). The color optical density of immunohistochemical concentrations of metalloproteinases 2 and 9 and placental lactogen was classified with "0" or "1." The value of density was graduated from "0" to "255" based on logarithmic transformations. The arithmetic mean and its statistical error also were calculated. The Shapiro–Wilk test of normality was performed. The group comparisons were provided

on the unpaired two-tailed student's test (PAST 3.0 software, free license, O. Hammer, 2015).

RESULTS

Invasive CT in histological sections was found in the structures of the UPB in different positions, all of them difficult the identification of trophoblast. The most problematic task was the visualization of CTs, which replace the endothelium of the spiral arteries. The ability to simulate the phenotype of endothelial cells, and through their phagocytosis, to be internally inserted into the walls of radial arteries and promote their gestational transformation are unique properties of endovascular trophoblastic cells. The main difficulty is that ECT with time flattens, and without the use of special methods, it is impossible to differentiate it morphologically from the endothelium.

Given that trophoblasts consist of epithelial cells, the authors attempted to use polyclonal antibodies to cytokine antibodies. They gave a positive effect for the endothelium-replacing CT, which had a cubic shape. On the contrary, the immunohistochemical method of cytokeratins did not positively affect flat (endothelium like) forms. Methods for determining the specific pregnancy proteins produced by trophoblasts have also been used.

The best results were obtained for placental lactogen. The invasive CT by placental lactogen was verified in both cubic and flatforms. In our opinion, placental lactogen can be recommended as the so-called "gold standard" to identify endothelium-replacing CT [Figure 1].

However, we have obtained reliable results using a method that was not intended to verify endothelial replacement CT, particularly in serial sections of the UPB. It was observed that the antiapoptotic protein bcl-2 identifies the endothelium-replacing CT with an accuracy that placental lactogen did.

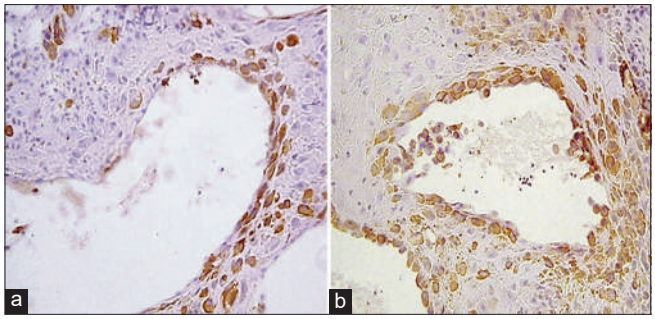

Figure 1: The uteroplacental bed at 39–40 weeks of gestation-endothelium-replacing invasive cytotrophoblast: Observation of physiological pregnancy (a, ×400) and pregnancy with iron deficiency anemia (b, ×400). Immunohistochemical technique with primary antibodies against placental lactogen using diaminobenzidine and nuclei staining with Mayer hematoxylin

In this case, when invasive CT, which did not reach the endothelium of the blood vessels of the UPB, was either negative for bcl-2 or slightly positive [Figure 2]. In our opinion, the most reliable and specific method for identifying endothelium-replacing invasive CT in UPB is the immunohistochemical determination of placental lactogen and protein bcl-2.

Analysis of the depth of invasion of the CT and the distribution of its fractions showed the spread of CTI deep into the area of the spiral arteries of the endometrium and at in walls of myometrial segments of arteries of UPB [Figure 3].

However, the total number of spiral arteries with signs of CTI was significantly lower in the observations with UPB compared with physiological pregnancy. The gestational transformation of the walls of the arteries was not complete. Sometimes, there was a muscular component, a thin layer of fibrinoid without thick walls and dilatation. The criteria of gestational maturity were taken as a percentage of spiral arteries with signs of CTI and complete gestational adjustment: At physiological pregnancies at 37–40 weeks, this value was 99% ± 0.1%, which is significantly higher than in the group with IDA ($P < 0.05$). Accordingly, the specific volume of nonvascular invasive CT was significantly lower in the control group ($P < 0.05$) as indicated in Table 1.

The evaluation of "invasive capacity" and synthetic activity of extravillous CT was performed by immunoimaging metalloproteinases 2 and 9 and placental lactogen differentiated in intravascular cells (endothelium replacing) and extravascular CT.

The averaged indices of ECT metalloproteinase activity overpass the values in physiological pregnancy [Table 2].

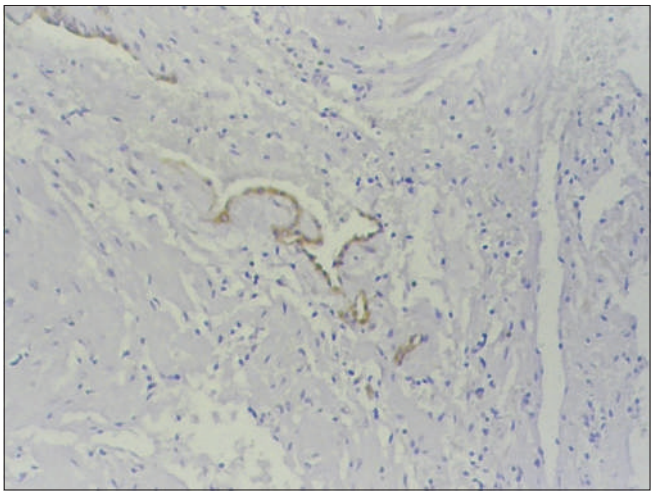

Figure 2: Uteroplacental bed at 39–40 weeks of gestation associated with iron deficiency anemia: The myometrial segment of the artery with the lining of the wall endothelium-replacing cytotrophoblast. The immunohistochemical technique for bcl-2 protein with diaminobenzidine. Nuclei were stained with Mayer hematoxylin (×200)

This obtained difference is statistically significant. Immunoexpression of metalloproteinase 9 in the cytoplasm of the CT of both study groups is illustrated in Figure 3.

According to Table 3, the immunohistochemical concentration of placental lactogen in cells of extravascular trophoblasts in the biopsies of the UPB and myometrium of observations with IDA exceeded those of physiological gestation. In cases with anemia, immunoexpression of placental lactogen was significantly increased in the cells of the extravascular trophoblast.

DISCUSSION

It is known that any hypoxic conditions of the maternal organism, in particular IDA in pregnancy, increase CT invasion, dilatation of the lumens of the spiral arteries of the uterus, and proliferation of cells in the placental villi, to improve gas exchange in the uterine placenta.[8] Such processes are regarded as an adaptive reaction to enhance oxygen exchange between the placenta and the fetus.[9] Remodeling of the UPB and myometrium's major arteries is provided mainly by the intravascular CT, the cells of which, penetrating the walls of the radial arteries, provide their further transformation with lysis of the elastic and muscle components and replacing it with fibrinoid.[10] Therefore, oxygen deficiency reinforces cell proliferation and intravascular invasion of ECT, which provide adequate gestational transformation of the uterine-placental vessels and improve blood perfusion in the placental interventional space.

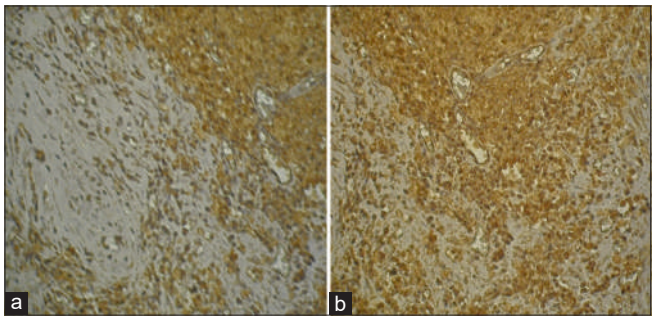

Figure 3: The uteroplacental bed at 39–40 weeks of pregnancy-invasive cytotrophoblast: Observation of physiological pregnancy (a, ×400) and pregnancy with Iron deficiency anemia (b, ×400). Immunohistochemical technique with primary antibodies against metalloproteinase 9 using diaminobenzidine and nuclei staining with Mayer hematoxylin

In our pregnancy observations regarding the etiopathology of IDA, the insufficient gestational transformation of the spiral arteries of the UPB was associated with (1) the preservation of the narrow arteries of the inner elastic membrane or their fragments, (2) with the presence of a broad, muscular membrane around the spiral arteries, and (3) with hyperplastic arteriosclerosis ranging from typical growth up to the complete obliteration of the spiral arteries. All the described changes were confirmed by the specific findings in the histological sections of UPB and the significant difference between the results of the control group and the group with IDA ($P < 0.05$).

The functional–physiological interpretation of the identified morphological traits of uteroplacental and fetal–placental (umbilical-placental) circulation disorders was confirmed by Doppler studies' results based on medical documentation.

Table 1: Morphometric parameters of cytotrophoblastic invasion in the uteroplacental bed ($X \pm S_x$)

Percentage	Physiological pregnancy (n=38)	Pregnancy with IDA (n=42)
Specific volume of nonvascular invasive CT (%)	21±0.12*	29±0.12*
The number of spiral arteries with CT invasion (%)	99±0.13*	93±0.17*

*P<0.05. CT=Computed tomography; IDA=Iron deficiency anemia

Table 2: The optical density of immunohistochemical staining of the cytoplasm of the cytotrophoblast of the uteroplacental bed in determining the activity of metalloproteinases ($X \pm S_x$)

Color optical density (ODU)	Physiological pregnancy (n=38)	Pregnancy with IDA (n=42)
Metalloproteinase 2	0.232±0.0012*	0.251±0.0012*
Metalloproteinase 9	0.219±0.0014*	0.237±0.0016*

*P<0.05. IDA=Iron deficiency anemia; ODU=Optical density units

Table 3: The optical density of immunohistochemical staining of placental lactogen in the extravillous cytotrophoblasts of the uteroplacental bed ($X \pm S_x$)

Color optical density (ODU)	Physiological pregnancy (n=38)	Pregnancy with IDA (n=42)
Extravascular trophoblast	0.314±0.0022*	0.337±0.0022*
Intravascular trophoblast	0.109±0.0022	0.110±0.0022

*P<0.05. IDA=Iron deficiency anemia; ODU=Optical density units

During 29–37 weeks of gestation with IDA, the expected increase in the acceleration of blood circulation in the UPB was not observed. Moreover, peripheral resistance in the system of spiral arteries of the uterus increased. In terms of clinical practice, standardizing the Doppler study of either the acceleration of blood circulation in the UPB or the resistance in the uterine spiral arteries can help quantify the impact of IDA in the last stage of pregnancy. Such imaging biomarkers would require validation with extensive clinical studies. This could enhance the paradigm of translational research in the field, paving the way from the bench to the bedside.

All the described processes affect the uteroplacental hemodynamics and lead to different pregnancy complications, particularly preeclampsia. According to Ali *et al*.,[14] women with severe anemia had a 3.6 times higher risk of preeclampsia than women with no anemia. In our study, we did not observe pregnancies with eclampsia, preeclampsia, or arterial hypertension. Still, our pathomorphological and immunohistological findings in UPB of pregnant women with IDA revealed the signs of placental dysfunction. Thus, even mild, asymptomatic anemia during pregnancy may increase uteroplacental insufficiency, microcirculatory disorders, and dysregulation of maternal and fetal homeostasis. This underlines the importance of early detection of IDA in pregnant women to predict and prevent such pregnancy complications such as preeclampsia. Therefore, in our pathomorphological study, we highlight that the clinicians should pay more attention to commonly available complete blood count and serum iron test and duly detect and offset the iron deficiency. In that case, the pathological changes in the structures of UPB may be interrupted and brought under control before the development of pregnancy complications.

An intensification of trophoblastic invasion and the increase in the distance of the lining of the endothelium-replacing CT of the arterial walls of the UPB and the myometrium was observed in the biopsies of pregnant women with IDA. However, it turned out to be ineffective with regard to adequate gestational transformation of vessels.

It appears that the regulation of the CT cells population in proportion to the severity of IDA can determine the nature and extent of the compensatory gestational transformation of the UPB. This assumption is based on both the quantitative evaluation of placental lactogen's immunovisualization and metalloproteinases' activity in intra- and extravascular CT fractions and on the analysis of morphometric parameters of CT invasion in UPB in IDA during pregnancy.

Mendes *et al*. (2020)[11] suggested that the disruption of redox homeostasis interferes with extravillous trophoblasts' function during placentation. Their *in vitro* study focused on the role of specific oxidative modifications of proteins in vascular endothelial cells and the stromal component of placental stromal cells. Their results elucidated a decrease in motility and invasive capacity of extravillous trophoblast cells in placental morphogenesis in women with IDA.

It can be assumed that oxidative modification of proteins due to iron deficiency and hypoxia contributes to the disruption of CT invasion in the UPB. This assumption needs further investigation. In particular, serum levels of oxidative stress biomarkers could be incorporated in women's regular diagnostic workup with IDA in pregnancy. Such markers include routine tests such as albumin, platelets, or leukocyte count. Still, they may also include more specific markers such as the measurement of S-glutathionylation of proteins involved in placental morphogenesis such as leptin, chorionic somatomammotropin hormone-like 1 (CSHL1), elabela, activin A, and placental growth factor.[12,13]

CONCLUSIONS

Overall, gestation with IDA is associated with deeper CT invasion of the structures of the UPB and an increase in the distance of the lining of the endothelium-replacing CT in the arteries. These result in more significant dilatation of the lumen spiral and radial uterine arteries to improve gas exchange in the "mother-placenta-fetal" system. However, the CT's invasive ability and synthetic activity and as a result, the adequacy of the gestational transformation of UPB structures are determined by the level of hypoxia and depend on the degree of IDA in pregnancy. All the described findings underline the importance of early detection of IDA during pregnancy to avoid pregnancy complications.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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