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V.V. Ilika, O.V. Lazaruk, O.V. Garvasyuk, S.V. Namestiuk, N.Ya. Kozariichuk, A.Ya. Velyka, M.O. Andrushchak

Some histochemical features of proteins of deciduocytes of the basal plate of the placenta in chronic basal deciduitis against the background of iron-deficiency anaemia in pregnant women

Bukovinian State Medical University, Chernivtsi, Ukraine

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Free radicals and their impact on the placenta are the subject of active scientific research, as they may have significant implications for the health of pregnant women and fetal development.

Aim: to use histochemical methods to establish the quantitative characteristics of oxidative protein modification and limited proteolysis in deciduocytes of the basal plate of the placenta in cases of chronic basal deciduitis against the background of iron-deficiency anaemia in pregnant women.

Material and methods. A total of 82 placentas were examined using chemiluminescence and histochemical techniques, including methods by A. Yasuma and T. Ichikava, as well as bromophenol blue staining by Mikel Calvo and Bonheg.

Results. In cases of chronic basal deciduitis, the luminescence intensity of nitroperoxides in deciduocytes increased to 170±4.8 arbitrary units (arb. units). The quantitative analysis revealed an R/B (Red/Blue) coefficient, indicating the ratio of amino to carboxyl groups, of 2.34±0.01, and the optical density of histochemical staining for free amino groups of proteins was measured at 0.197±0.002 relative optical density units (rel. OD). These findings were statistically significant ($p<0.001$) when compared to placentas with inflammation but without anemia.

Conclusions. The activation of free radical processes appears to be the key factor driving the morphological characteristics of chronic basal deciduitis in iron-deficiency anemia of pregnant women. This is marked by an elevated concentration of peroxynitrites, resulting in enhanced oxidative protein modification and increased activity of limited proteolysis.

The study was conducted in accordance with the principles of the Declaration of Helsinki. The research protocol was approved by the Local Ethics Committee of the respective institution.

The author declares no conflict of interest.

Keywords: placenta, chronic basal deciduitis, free radical processes, nitroperoxides, limited proteolysis, oxidative modification of proteins, iron-deficiency anemia of pregnant women, chemiluminescence, histochemistry.

Деякі гістохімічні особливості білків децидуоцитів базальної пластинки плаценти при хронічному базальному децидуїті на тлі залізодефіцитної анемії у вагітних жінок

В.В. Іліка, О.В. Лазарук, О.В. Гарвасюк, С.В. Наместюк, Н.Я. Козарійчук, А.Я. Велика, М.О. Андрущак

Буковинський державний медичний університет, м. Чернівці, Україна

Вільні радикали та їхній вплив на плаценту є об'єктом активного наукового вивчення, оскільки вони можуть мати значущі наслідки для здоров'я вагітної жінки та розвитку плода.

Мета: гістохімічними методами встановити кількісні характеристики окиснювальної модифікації білків та обмеженого протеолізу в децидуцитах базальної пластинки плаценти у спостереженнях хронічного базального децидуїту на тлі залізодефіцитної анемії вагітних.

Матеріали та методи. Дослідження 82 плацент було проведено хемілюмінесцентним, а також гістохімічними за A. Yasuma та T. Ichikava, з бромфеноловим синім за Mikel Calvo та Бонхегом методами.

Результати. При хронічному базальному децидуїті інтенсивність світіння нітропероксидів у децидуцитах зросла до 170±4,8 умовних одиниць. Кількісні показники коефіцієнта R/B, що відображає співвідношення між аміно- та карбоксильними групами становили 2,34±0,01, а оптична густина гістохімічного забарвлення на вільні аміногрупи білків – 0,197±0,002 відносних одиниць оптичної густини. Ці показники були статистично вищими ($p<0,001$) відносно груп порівняння.

Висновки: активація вільнорадикальних процесів, імовірно, є ключовим чинником формування морфологічних ознак хронічного базального децидуїту при залізодефіцитній анемії вагітних. Це виражається підвищеною концентрацією нітропероксидів, що спричиняє посилену окиснювальну модифікацію білків та підвищену активність обмеженого протеолізу.

Дослідження проведено відповідно до принципів Гельсінської декларації. Протокол дослідження був затверджений Локальним етичним комітетом.

Автор заявляє про відсутність конфлікту інтересів.

Ключові слова: плацента, хронічний базальний децидуїту, вільнорадикальні процеси, нітропероксиди, обмежений протеоліз, окиснювальна модифікація білків, залізодефіцитна анемія вагітних, хемілюмінесценція, гістохімія.

Inflammation of the placenta may be one of the potential causes of placental insufficiency, the main manifestation of which is the disruption of the state of placental proteins [10]. It is important to note that, to date, there has been insufficient research regarding the impact of different types of placental inflammation on the condition of proteins in the placenta. Moreover, free radicals play an active role in inflammatory diseases by intervening at various stages of their initiation, progression, and regulation [13,15,17]. Reactive oxygen species (ROS) significantly increase during hypoxic conditions, which may cause non-selective damage to biological molecules, impair their function, and be considered an inducer of apoptosis [16].

The analysis of domestic and international sources highlights key aspects of the interaction between free radicals and the placenta:

– Oxidative stress: Free radicals can lead to oxidative stress in placental tissues, potentially damaging proteins, lipids, and nucleic acids, thereby threatening the organ's normal function [13].

– Pregnancy complications: Elevated levels of free radicals may be associated with pregnancy complications such as preeclampsia, miscarriage, and other pathologies [7].

– Impact on placental functions: Oxidative stress can affect placental functions, including nutrient transport, hormonal balance regulation, and oxygen transfer to the fetus [2,12].

– Antioxidant defence: Antioxidant systems, which protect cells from free radicals, are crucial for maintaining balance and preventing oxidative stress in the placenta [13].

Therefore, studying placental proteins helps uncover mechanisms that may influence maternal health and fetal development. Special attention to deciduocytes of the basal plate is essential, as structural remodelling in this area may pose a risk of premature placental abruption, leading to serious negative consequences for the course of pregnancy [9].

The aim of this study is to use histochemical methods to establish the quantitative characteristics of oxidative protein modification and limited proteolysis in deciduocytes of the basal plate of the placenta in cases of chronic basal deciduitis against the background of iron-deficiency anemia (IDA) in pregnant women.

Materials and methods of the study

To achieve the aim and address the objectives set in this work, we conducted a morphological study of 82 placentas obtained from women who gave birth at 37 to 40 weeks of gestation. The material was collected during morphological examinations conducted at the Children's Department of the Chernivtsi Regional Clinical Institution «Pathological Anatomy Bureau»

The criteria for dividing the cases into groups were histologically confirmed cases of chronic basal deciduitis, either in combination with IDA in pregnant women or without it. The main study group (Group 1) consisted of 20 placentas with histologically confirmed chronic basal deciduitis in combination with IDA during pregnancy. The comparison groups included 21 placentas with chronic basal deciduitis without IDA during pregnancy (Comparison group 2A) and 21 placentas with IDA during pregnancy without inflammation (Comparison group 2B). The Control group comprised 20 placentas obtained from women with physiological pregnancies. Each group consisted of distinct participants, with no woman included in more than one group, thereby ensuring the acquisition of objective and representative data.

Histological analysis of the placentas was employed to confirm or exclude a diagnosis of basal deciduitis. The presence of IDA pregnancy was diagnosed through a review of the participants' medical records.

The morphological investigations were conducted using the following methods:

1. Chemiluminescent method with luminol on frozen placental sections – utilized to detect nitroperoxides within placental structures. This method is necessary because the natural generation of free radicals by cells produces luminescence that is too weak to be reliably detected without amplification. To enhance luminescence, frozen placental sections were treated with luminol solution for 5 minutes at 37°C. Without washing off the solution, the samples were immediately examined under a LUMAM-R8 microscope, and the luminescence was photographed using a digital camera under standardized conditions for 5 minutes, during which the luminescence remained stable. Luminescence intensity was quantified using a computer-based evaluation tool, with values ranging from «0» (no luminescence) to «255» (maximum intensity), based on the «Brightness» tool and focusing on the maximum recorded value.

2. Histochemical reaction with bromophenol blue for «acidic» and «basic» proteins according to Mikel Calvo, following standardized proce-

dures – employed to quantitatively assess the oxidative modification of proteins (OMP).

3. Histochemical method utilizing the ninhydrin–Schiff reaction for free amino groups of proteins according to A. Yasuma and T. Ichikava – applied to evaluate the degree of limited proteolysis.

4. Histochemical method with bromophenol blue according to Bonheg – used to determine total protein content.

Placental tissue samples were fixed for 24–48 hours in a neutral buffered 10% formalin solution, as per the Lilly method. Following ethanol dehydration, the samples were embedded in paraffin using standard procedures. The histochemical reactions were performed on serial histological sections cut to a thickness of 5 μm.

Subsequently, digital reproductions of images obtained through a Delta Optical Evolution 100 microscope (equipped with plan achromatic objectives) and an Olympus SP-550UZ digital camera were subjected to analysis using the licensed version of ImageJ software (v1.48, W. Rasband, National Institutes of Health, USA) [3], in alignment with the research objectives. To assess the extent of OMP, spectral characteristics were analyzed via computer-based microspectrophotometry, with the colors of the object decomposed into two components – red and blue. A quantitative evaluation of each spectral region was performed within the RGB color system algorithm [4], followed by the calculation of the Red/Blue (R/B) coefficient. This coefficient served as an indicator of the ratio between amino and carboxyl groups in proteins at specific sites, thus offering a measure of OMP.

Further analysis, using computer microdensitometry, focused on determining the optical density of histological images. These images were derived from Bonheg's method for total protein quantification, as well as from A. Yasuma and T. Ichikava's method for assessing limited proteolysis. The optical density of histochemical staining was measured in relative optical density units, spanning from 0 (indicating the absence of staining, absolute transparency) to 1 (indicating maximum staining, absolute opacity), with logarithmic transformations applied to brightness values ranging from 0 to 255.

The statistical analysis of the data was conducted using PAlaeontological STatistics software (free license, v4.16) on a personal computer, adhering to the developers' guidelines [7]. The arithmetic mean and its standard error (M±m) were calculated. To assess differences in mean values, an unpaired Student's t-test was applied, with the distribution of the sample being evaluated for normality using the Shapiro–Wilk test. A significance threshold of $p<0.05$ was employed to accept or reject the statistical hypothesis [11].

All experimental procedures were conducted in accordance with the European Council's Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (18 March 1986), the «Rules of Ethical Principles for Medical Research Involving Human Subjects» as set out in the Declaration of Helsinki (1964–2013), ICH GCP (1996), EEC Directive No. 609 (24 November 1986), and the orders of the Ministry of Health of Ukraine No. 690 (23 September 2009), No. 944 (14 December 2009), and No. 616 (3 August 2012). The Bioethics Commission of the Bukovinian State Medical University confirmed no violations (protocol No. 4, 19 December 2019). All procedures and experiments adhered to the ethical standards of the Declaration of Helsinki 1975, revised in 2008 [5], as well as relevant national legislation.

Results of the study and discussion

For the possibility of data interpretation, considering that oxidatively modified proteins in cells arise under the influence of free radicals, we first conducted chemiluminescent research of nitroperoxides in deciduocytes in basal plate deciduocytes of the placenta (Fig. A).

The present study (Table) demonstrated that in placentas affected by IDA during pregnancy, there was no statistically significant increase in the quantitative parameters of nitroperoxide luminescence intensity ($p>0.05$), which contrasts with the findings of A.V. Goshovska and colleagues [5]. However, their study focused on the cytoplasm of trophoblasts in chorionic villi affected by IDA during pregnancy. Given that chorionic villi, like endothelial cells, are among the structures most sensitive to hypoxic conditions [7], this could explain the discrepancy in our findings.

In cases of chronic basal deciduitis, there was a statistically significant increase in the intensity of nitroperoxide luminescence compared to placentas from physiological pregnancies. This finding is consistent with the work of I.S. Davidenko and colleagues [14], who examined the cyto-

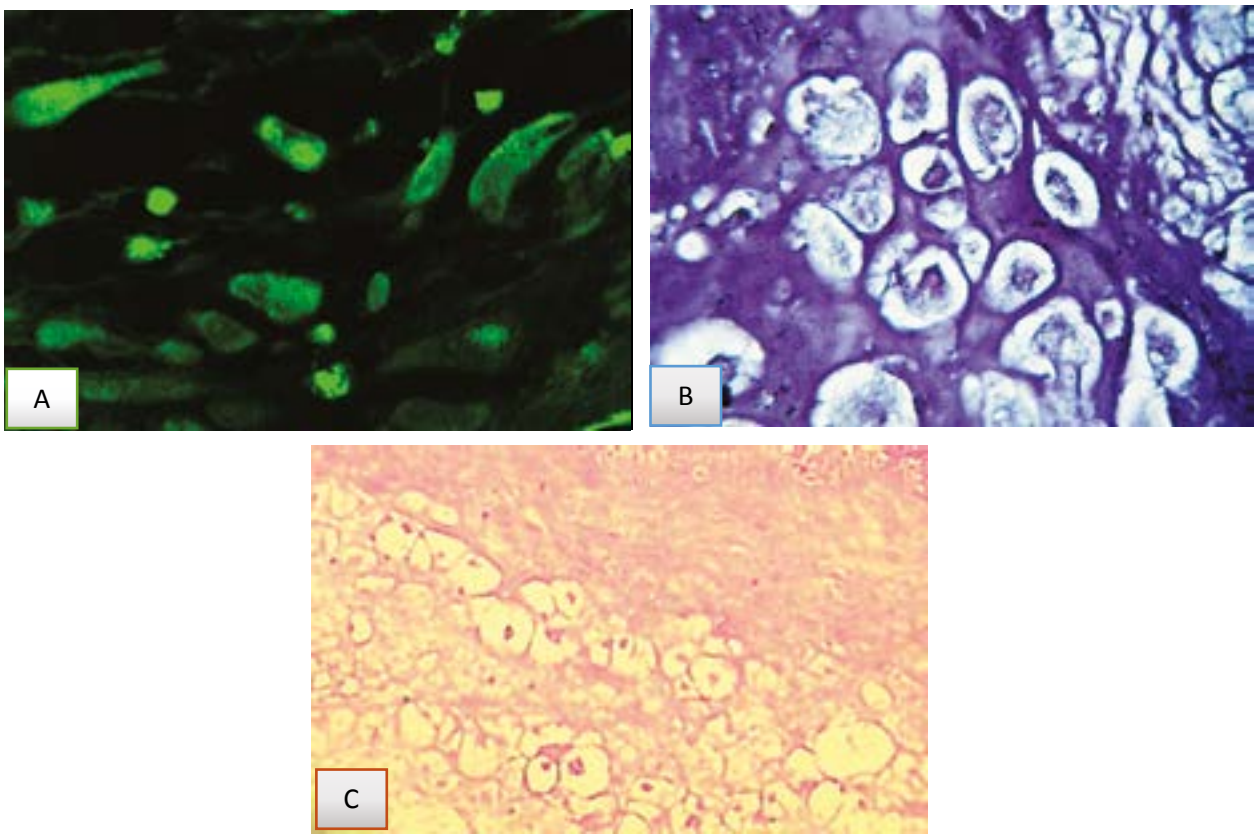

Fig. Observation of chronic basal deciduitis in the context of iron-deficiency anaemia during pregnancy. Deciduocytes of the basal plate: A – chemiluminescent emission of nitroperoxides. Chemiluminescent assay using luminol; B – staining for amino and carboxyl groups of proteins. Histochemical method according to Mikel Calvo; C – specific staining for free amino groups of proteins. Ninhydrin–Schiff reaction by A. Yasuma and T. Ichikava. Magnification: Objective 40×, Eyepiece 10×

plasm of syncytiotrophoblasts in chorionic villi during purulent chorioamnionitis. Furthermore, in cases of chronic basal deciduitis combined with IDA during pregnancy, the mean values were significantly higher ($p<0.001$) than those observed in placentas with inflammation but without anaemia. These results suggest that pregnancy-related anaemia contributes to the elevated concentration of nitroperoxides during inflammation. Several researchers have posited that free radicals may originate from both intracellular and extracellular sources; lymphocytes and fibroblasts also generate free radicals continually, although in small quantities [17]. Given the abundant presence of these cellular elements during inflammation, this may account for the high concentration of radicals in the inflammatory foci of the placental basal plate.

Free radicals have the potential to initiate OMP, a process confirmed through a histochemical method for identifying «acidic» and «basic»proteins using bromophenol blue, according to Mikel Calvo's technique. Subsequent calculation of the R/B ratio further supported these findings, with results detailed in the Table.

Analysis of histochemical samples (Fig. B) demonstrated that decidual cells exhibited clear staining, making them suitable for quantitative analysis. The cell boundaries were well-defined due to staining of the cell membrane and the contrast provided by fibrinoid material surrounding the decidual cells. Additionally, the nuclei, including the nucleoli, were clearly visualized. The R/B ratio analysis in the nucleoplasm indicated a predominance of «basic»proteins, while the nucleoli contained a higher concentration of «acidic» proteins. However, no significant differences in the R/B ratio of the nucleoplasm or nucleoli were observed between the study groups, and therefore, these numerical data are not presented.

Regarding the cytoplasm of decidual cells, it is noteworthy that the specific staining pattern was predominantly granular, with only a few cases showing a diffuse distribution. The spectral characteristics and optical density of the staining varied between individual placental samples and the average values for the study groups.

A review of the tabulated data (Table) reveals that decidual cells in cases of chronic basal

deciduitis are highly susceptible to the effects of free radicals, which intensify OMP processes. This is evidenced by the significantly elevated R/B coefficient values compared to physiological pregnancies and IDA during pregnancy without placental inflammation, with the highest values observed in cases of inflammation combined with IDA during pregnancy ($p<0.001$). To gain a comprehensive understanding of free radical oxidation processes in the placenta, it is important to consider the findings of other researchers who have assessed OMP levels in different placental structures. For example, O.P. Shenderiuk and colleagues [14] reported that in their study of protein properties in the cytoplasm of trophoblasts and the endothelium of chorionic villi during inflammation, they observed a correlation between intensified OMP and the concentration of nitroperoxides in these same locations.

Based on the findings of our study, we suggest that the accumulation of oxidized proteins may serve as an early indicator of tissue damage induced by ROS, potentially representing the earliest marker of oxidative stress.

Recognizing that the intensification of oxidative protein modification may coincide with an increase in limited proteolysis processes [8], we conducted a histochemical reaction to detect free amino groups in proteins, following the method of A. Yasuma and T. Ichikava (Fig. C), in combination with a microdensitometric analysis. In addition to enhanced nitroperoxide luminescence and OMP, we observed an intensification of limited proteolysis processes. This was demonstrated by an increase in relative units of histochemical staining for free amino groups of proteins in decidual cells from the basal plate of the placenta in cases of chronic basal deciduitis ($p<0.001$). In comorbid IDA during pregnancy cases, the quantitative indices were significantly higher compared to placentas with inflammation but without anaemia ($p<0.001$). These results corroborate the views of other researchers [17], who suggest that oxidatively modified proteins may interact with protease systems to regulate their activity. Moreover, structural changes in OMPs can either increase or decrease their susceptibility to proteolytic cleavage. Therefore, oxidative modification directly influences limited proteolysis by modulating the accessibility of specific protein residues to proteases.

To appropriately interpret the data on limited proteolysis, we quantified total protein using the bromophenol blue method as per Bonhag's technique (Table). It is noteworthy

Table

Results of chemiluminescence and histochemical methods (M± m)

Research methods	Control group (n=20)	Group 1 (n=21)	Comparison group 2B (n=20)	Comparison group 2A (n=21)
Histochemical method with bromophenol blue according to Mikel Calvo (R/B coefficient)	1.03±0.01	2.34±0.01 $p_3<0.001$ $p_4<0.001$	1.04±0.01 $p>0.05$	1.91±0.01 $p_1<0.001$ $p_2<0.001$
Chemiluminescent glow of nitroperoxides (unit of luminescence)	34±3.4	170±4,8 $p_3=0.002$ $p_4<0.001$	38±4.8 $p>0.05$	136±4.4 $p_1<0.001$ $p_2<0.001$
Histochemical technique according to method A. Yasuma and T. Ichikava (relative units of optical density)	0.164±0.001	0.197±0.002 $p_3<0.001$ $p_4<0.001$	0.166±0.001 $p>0,05$	0.181±0.001 $p_1<0.001$ $p_2<0.001$
Histochemical method with bromophenol blue by Bonhegg (relative units of optical density)	0.230±0.010	0.230±0.009 $p_1>0.05$ $p_2>0.05$	0.232±0.008 $p>0.05$	0.235±0.009 $p_1>0.05$ $p_2>0.05$

Note: p_1 – the probability of a difference between two means comparing physiological pregnancy and the study group; p_2 – the probability of a difference between two means comparing the IDA during pregnancy group and the study group; p_3 – the probability of a difference between two means comparing chronic basal deciduitis and basal deciduitis combined with IDA during pregnancy; p_4 – the probability of a difference between two means comparing chronic basal deciduitis with IDA during pregnancy and IDA during pregnancy without inflammation.

that no significant alterations in total protein concentration were detected in the decidual cells of the placental basal plate in any of the studied groups ($p>0.05$), indicating effective protein regeneration within the cells. Overall, this suggests that while proteins may lose functionality, potentially leading to denaturation, their total concentration remains stable.

Conclusions

1. In cases of chronic basal deciduitis, relative to placentas from physiological pregnancies and those with IDAP without inflammation, there is an increase in the mean R/B ratio, nitroperoxide luminescence intensity, and optical density of histochemical staining for free amino groups of proteins, without changes in total protein concentration.

2. In placentas from women with IDA during pregnancy, compared to those with inflammation but no anaemia, there is an increase in the mean R/B ratio, nitroperoxide luminescence intensity, and optical density of histochemical staining for free amino groups of proteins, without changes in total protein concentration.

3. The primary factor driving the morphological features of chronic basal deciduitis in IDA is the activation of free radical processes, reflected by an increase in nitroperoxide concentration, the intensification of oxidative protein modification, and the activation of limited proteolysis processes.

The authors declare no conflict of interest.

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Відомості про авторів:
Іліка Віталій Валеріанович — к.мед.н., доц. каф. патологічної анатомії БДМУ. Адреса: м. Чернівці, Театральна площа, 2. https://orcid.org/0000-0002-3714-9883.
Лазарук Олександр Володимирович — к.мед.н., доц. каф. патологічної анатомії БДМУ. Адреса: м. Чернівці, Театральна площа, 2. https://orcid.org/0000-0002-2924-4792.
Гарвасюк Олександра Василівна — к.мед.н., доц. каф. патологічної анатомії БДМУ. Адреса: м. Чернівці, Театральна площа, 2. https://orcid.org/0000-0002-1936-2015.
Наместюк Світлана Валеріївна — - к.мед.н., доц. каф. іноземних мов БДМУ. Адреса: м. Чернівці, Театральна площа, 2. https://orcid.org/0000-0001-5572-0046.
Козарійчук Наталія Ярославівна — к.мед.н., доц. каф. дитячої хірургії, оториноларингології та офтальмології БДМУ. Адреса: м. Чернівці, Театральна площа, 2. https://orcid.org/0000-0002-8884-507X.
Велика Алла Ярославівна — к.хім.н., доц. каф. медичної та фармацевтичної хімії БДМУ. Адреса: м. Чернівці, Театральна площа, 2. https://orcid.org/0000-0001-6560-4822.
Андрущак Маргарита Олександрівна — к.мед.н., доц. каф. інфекційних хвороб та епідеміології БДМУ Адреса: м. Чернівці, Театральна площа, 2. https://orcid.org/0000-0001-5003-9622.

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