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Comprehensive morphological study of free radical processes in chronic chorioamnionitis on the background of iron deficiency anemia in pregnancy

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ABSTRACT

Aim: To establish the features of free radical processes in the endotheliocytes of the chorionic plate of the placenta in chronic chorioamnionitis against the background of iron deficiency anemia of pregnant women using both chemiluminescent and histochemical methods of research.

Materials and Methods: 82 placentas from parturients at 37 – 40 weeks of gestation were studied. Including, for comparison, the placenta during physiological pregnancy and the observation of iron deficiency anemia of pregnant women without inflammation of the placenta. The number of observations in specific study groups is given in the tables. To achieve the objective and solve the tasks set in this study, there were carried out the following histochemical, chemiluminescent, morphometric and statistical methods of material processing.

Results: In case of chorioamnionitis against the background of anemia in pregnancy, the R/B ratio (R/B - ratio between amino- (blue) and carboxyl (red) groups of proteins) in the method with bromophenol blue according to Mikel Calvo was 1.56 ± 0.021 , indicators of chemiluminescence of nitroperoxides were 133 ± 4.5 , relative optical density units of histochemical staining using the method according to A. Yasuma and T. Ichikawa was -0.224 ± 0.0015 .

Conclusions: With chronic chorioamnionitis, the intensity of the glow of nitroperoxides, the average indicators of the R/B ratio, and the optical density of histochemical staining for free amino groups of proteins are increased compared to placentas of physiological pregnancy and anemia of pregnant women. Comorbid i anemia of pregnant women causes increasing of the intensity of the glow of nitroperoxides, the average values of the R/B ratio, and the optical density of histochemical staining for free amino groups of proteins comparing to placentas with inflammation without anemia. The key factor in the formation of morphological features of chronic chorioamnionitis with comorbid anemia is the intensification of free radical processes, which is reflected by the increase in the concentration of nitroperoxides in the center of inflammation, with the subsequent intensification of the processes of oxidative modification of proteins, which is followed by the increasing activity of the processes of limited proteolysis.

KEY WORDS: chorioamnionitis, oxidative modification of proteins, limited proteolysis, free radical processes, iron deficiency anemia in pregnancy

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INTRODUCTION

In case of both inflammation of the placenta and iron deficiency anemia in pregnancy (IDAP), one or other signs of placenta insufficiency are almost always registered, which can have adverse consequences for the fetus [1-3].

In addition, there is a strong cross-relationship between hypoxia and inflammation. Inflammation creates hypoxic conditions due to increased metabolic activity; hypoxia at the site of inflammation can cause vascular damage, blood coagulation and blood flow blockage, and subsequently tissue necrosis [4]. In this regard, there are studies in the modern literature aimed at studying the influence of the inflammatory process on the clinical course of IDAP, however, no information about morphological manifestations in the placenta with comorbidity of these conditions was found in the studied scientific literature.

It is also known that free radical processes are activated both during inflammation and during IDAP. This can potentially cause their mutual aggravation and the development of more severe pathology of the placenta. An imbalance between the occurrence of reactive oxygen species and the ability of antioxidants to inhibit oxidative damage leads to damage to macromolecules, and therefore to impaired placental cell function, defects in the exchange of nutrients between the mother and the fetus [5-6]. The cases described in the national and international literature do not cover all pathomorphological changes in the placenta caused by free radicals both in inflammation and in iron deficiency anemia. No data was found when combining these states either. For this purpose, this paper is aimed at a comprehensive study of the state of free radical processes in individual structures of the placenta using modern histochemical and fluorescent methods.

AIM

To establish the features of free radical processes in the endotheliocytes of the chorionic plate of the placenta in chronic chorioamnionitis against the background of iron deficiency anemia of pregnant women using both chemiluminescent and histochemical methods of research.

MATERIALS AND METHODS

82 placentas from parturients at 37 – 40 weeks of gestation were studied. Including, for comparison, the placenta during physiological pregnancy and the observation of iron deficiency anemia of pregnant women without inflammation of the placenta. The number of observations in specific study groups is given in the table (Table 1).

To achieve the objective and solve the tasks set in this study, there were carried out the following histochemical, chemiluminescent, morphometric and statistical methods of material processing:

1. Histochemical methods. Pieces of placenta were fixed for 24 to 48 hours in neutral Lilly-buffered 10% formalin solution, followed by ethanol dehydration and paraffin pouring according to standard procedures. On serial histological sections of 5 μ m thick there were performed 3 methods:
 - with bromophenol blue according to the Mikel Calvo method to determine the degree of oxidative modification of proteins (OMB). Performed in the modification of I.S. Davydenko [7-9], which included 4 stages: 1) histochemical stage (staining with bromophenol blue according to Mikel Calvo); 2) optical stage (application of microscopic optics); 3) photographic stage (application of digital photo documentation); 4) computer microspectrometric stage (color analysis on digital microphotographs).
 - by the method of A. Yasuma and T. Ichikawa (technique using ninhydrinoschiffian reaction to free amino groups of proteins);
 - with bromophenol blue according to Bonheg (method for total protein morphometric).
2. Chemiluminescence method (determination of nitroperoxides in the studied structures). To enhance luminescence, frozen sections of the placenta were treated for 5 minutes (37 °C) with a solution of luminol mixed with stabilizers according to the prescription. Immediately, without washing off the solution, the preparations were studied in a LUMAM-R8 microscope with immediate photofixation of the glow by a digital camera under standard conditions for 5 minutes, when the glow remains stable (the same).
3. Morphometric research methods. In order to objectify quantitative studies, computer morphom-

etry of objects in histological and histochemical preparations was performed. First, digital copies of the optical image of the sections of the microscopic preparations were first obtained using a Delta Optical Evolution 100 microscope (planachromatic lenses) and an Olympus SP-550UZ digital camera using different microscope lenses depending on the purposes of the analysis (eyepiece 10x, lens 10x – 20x – 40x).

The obtained digital copies of the images were analyzed using a licensed copy of the computer program Image J (1.48, W. Rasband, National Institutes of Health, USA) according to the intended purpose. Thus, to measure the degree of oxidative modification of proteins, spectral characteristics were determined using computer microspectrophotometry (decomposition of the color of the object into two components - red and blue, with a quantitative assessment of each part of the spectrum) in the RGB color evaluation system according to the algorithm [8]. Using computer microdensitometry, optical density was determined on photocopies of histological images obtained by the method of Bonheg (for total protein) and A. Yasuma and T. Ichikawa (for evaluation of limited proteolysis). The optical density of histochemical staining was measured in relative units of optical density in the range from 0 - no staining, absolute transparency, to 1 - maximum staining, absolute opacity, based on logarithmic transformations of the brightness value in gradations from 0 to 255.

On the obtained images of samples of the chemiluminescent method of research, the intensity of luminescence was determined by computer evaluation on a scale of 256 gradations - from 0 (the indicator of the absence of luminescence) to 255 (the maximum intensity of luminescence), which made it possible to carry out quantitative measurements of luminescence (units of luminescence).

4. Statistical methods of material processing. Statistical processing of digital data was carried out on a personal computer depending on the specific task of a certain fragment of the study using a free-to-use licensed copy of the computer program PALaeontological STatistics (PAST) v3.15 [10].

The arithmetic mean and its error ($M \pm m$) were calculated. Differences in mean values were tested using an unpaired Student's t-test after positive testing of the sample for normality of distribution in it by the Shapiro-Wilk test. The critical value of the level of significance (p) was accepted at $p \leq 0.05$, which could indicate the influence of iron deficiency anemia in pregnancy on the course of free radical processes during inflammation of the placenta. If a value of $p > 0.05$ was obtained, the difference between the values was considered unreliable.

Table 1. The results of histochemical research methods for “acidic” and “basic” proteins, as well as for total protein with bromophenol blue according to Mikel Calvo, for free amino groups of proteins according to the method of A. Yasuma and T. Ichikawa, chemiluminescence study of nitroperoxides ($M \pm m$)

Research groups	Physiological pregnancy (n=20)	Observation of iron deficiency anemia of pregnant women without inflammation of placenta (n=21)	Observation of placenta inflammation during pregnancy without anemia (n=20)	Observation of placenta inflammation in iron-deficiency anemia of pregnant women (n=21)
Histochemical technique for “acidic” and “basic” proteins with bromophenol blue according to Mikel Calvo (R/B ratio)	0,81±0,018	0,98±0,023 p<0,001	1,38±0,016 p ₁ <0,001 p ₂ <0,001	1,56±0,021 p ₃ <0,001 p ₄ <0,001
Chemiluminescent glow of nitroperoxides (unit of lum.)	18±4,8	35±4,7 p=0,01	112±4,2 p ₁ <0,001 p ₂ <0,001	133±4,5 p ₃ =0,001 p ₄ <0,001
Histochemical technique according to the method of A. Yasuma and T. Ichikawa on free amino groups of proteins (relative optical density units)	0,164±0,0019	0,168±0,0018 p>0,05	0,205±0,0021 p ₁ <0,001 p ₂ <0,001	0,224±0,0015 p ₃ <0,001 p ₄ <0,001
Histochemical technique for total protein with bromophenol blue according to Bonheg (relative optical density units)	0,234±0,0108	0,230±0,0091 p>0,05	0,233±0,00104 p ₁ >0,05 p ₂ >0,05	0,230±0,0092 p ₁ >0,05 p ₂ >0,05

Note. P₁ is the probability of the difference between the two means between physiological pregnancy and the studied group; P₂ is the probability of the difference of two means between the group of placentas with IDAP and the studied group; P₃ is the probability of the difference between the two means between inflammation and inflammation in combination with IDAP. P₄ is the probability of the difference between the two means between inflammation in combined with IDAP and IDAP without inflammation.

Correlation analysis was performed using the Pearson (r) method.

All studies were performed in compliance with the Council of Europe Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (March 18, 1986) “Ethical Principles for Medical Research Involving Human Subjects”, approved by the Declaration of Helsinki (1964 - 2013), ICH GCP (1996), EEC Directives #609 (dated 11/24/1986), Orders of the Ministry of Health of Ukraine #690 dated 09/23/2009, #944 dated 12/14/2009, #616 dated 08/03/2012. The Commission on Bioethics of the Bukovinian State Medical University of Ukraine found no violations (Minutes #4 of December 19, 2019). All the procedures and experiments of this study respect the ethical standards of the Helsinki Declaration of 1975, as revised in 2008 (5), as well as the national law. Informed consent was obtained from all the patients included in the study.

RESULTS

In order to study the processes of oxidative modification of proteins in the endotheliocytes of the chorionic plate of the placenta, the R/B ratio was determined by the microspectrometric method on digital copies of the images after the histochemical technique for “acidic”

and “basic” proteins with bromophenol blue according to Mikel Calvo. The R/B ratio served as an indicator of the ratio between amino and carboxyl groups in proteins of a specific localization, that is, it was a measure of OMP.

Upon visual assessment, it is established that endotheliocytes are quite clearly stained, well recognized due to their characteristic localization, that is, they are suitable for quantitative research. The cytoplasm of endotheliocytes is mostly stained diffusely, although sometimes individual, darker granular formations can be observed. Endotheliocyte nuclei are not visualized - the image of the cell nucleus merges with the cytoplasm. This distinguishes the endotheliocyte from many other types of cells. Therefore, the ratio of “acidic” and “basic” proteins in an endotheliocyte cannot be histochemically studied separately for the cytoplasm and the cell nucleus, that is, only together, in general.

The average quantitative indicators of the R/B ratio in the studied groups are presented in the Table 1.

Since OMP in cells occurs under the action of free radicals, in order to determine nitroperoxides, as the largest long-lived free radicals capable of overcoming relatively significant diffusion distances and therefore able to cause real pathology in the depth of the tissue, and not only on the surfaces [11-12], a chemiluminescent research method (fig. 3) was conducted with luminol, followed by measurement of luminous intensity

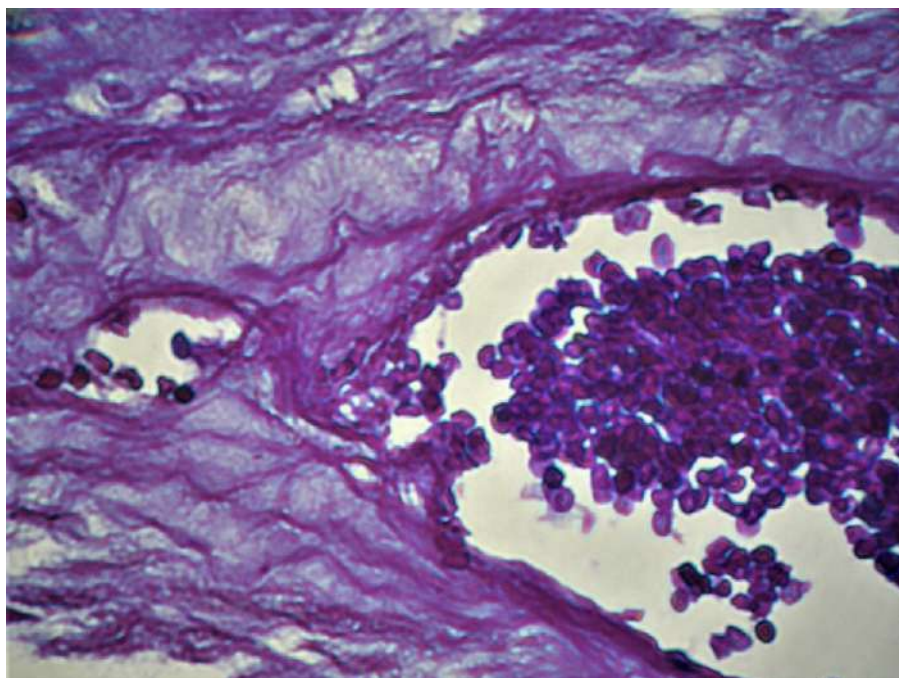


Fig. 1. Observation of chronic chorioamnionitis against the background of iron deficiency anemia in pregnancy. Endothelium of the chorionic plate. Staining for amino and carboxyl groups of proteins. Histochemical technique according to Mikel Calvo. Magnification: Lens 40x, eyepiece 10x.

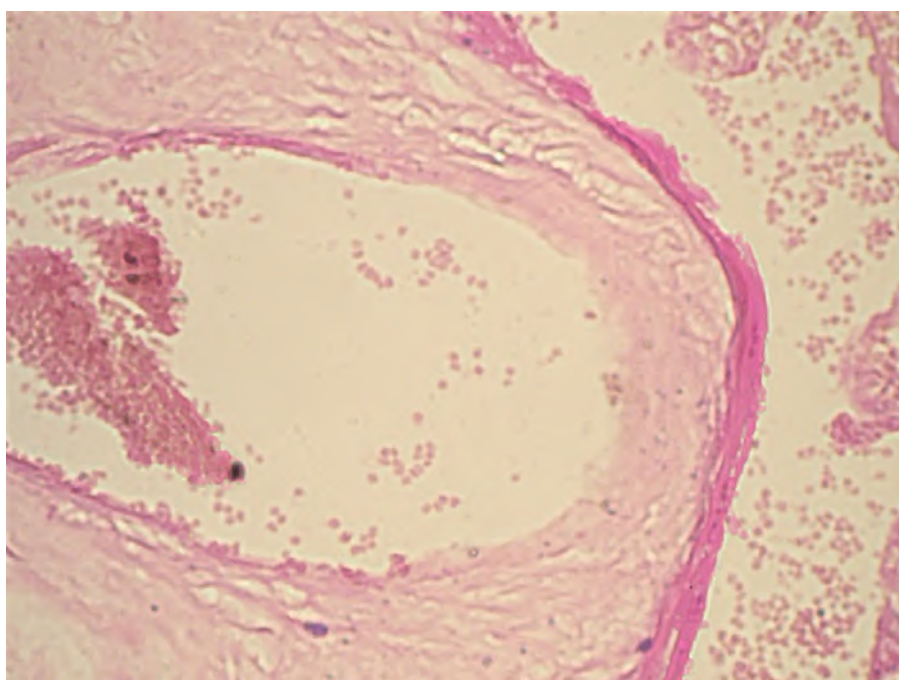


Fig. 2. Observation of chronic chorioamnionitis against the background of iron deficiency anemia in pregnancy. Endothelium of the chorionic plate. Specific staining for free amino groups of proteins. Ninhydrin-Schiff reaction according to the method of A. Yasuma and T. Ichikawa. Magnification: Lens 40x, eyepiece 10x.

(relative units of luminosity). Photomicrographs show that chemiluminescence after luminol treatment was irregular among endotheliocytes, but differences in luminescence depending on the zone of the chorionic plate of the placenta were not detected. Outside the endothelium of blood vessels, there are faintly visible foci of luminescence, which may indicate the ability of nitroperoxides to overcome comparatively larger diffusion distances far beyond the boundary of the foci of their origin.

The average quantitative indicators, according to the results of the studies in the endotheliocytes of the

blood vessels of the chorionic plate, are shown in the Table (Table 1).

It is known that during many pathological processes, as a rule, the level of free oxygen radicals increases, which, by oxidizing the amino groups of proteins, change the properties of these macromolecules [12]. Intensification of the processes of oxidative modification of proteins may be accompanied by enhanced processes of limited proteolysis.

To study these processes, a histochemical technique was performed according to the method of A. Yasuma and T. Ichikawa, which allows using the ninhydrin-Schiff

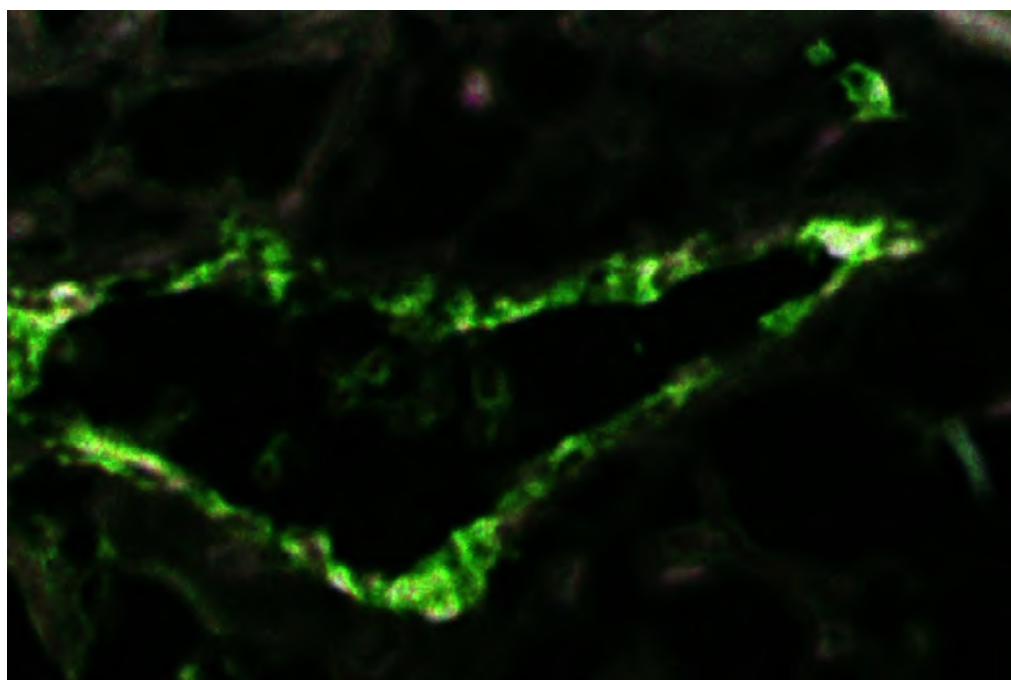


Fig. 3. Observation of chronic chorioamnionitis against the background of iron deficiency anemia in pregnancy. Endothelium of the chorionic plate. Chemiluminescence of nitroperoxides. Chemiluminescence study with luminol Magnification: Lens 40x, eyepiece 10x.

reaction to specifically stain free NH₂ (amino groups) of proteins. In order to be able to interpret the obtained data regarding limited proteolysis, quantitative determination of total protein was additionally carried out by histochemical method with bromophenol blue according to Bonheg. The results of the study are shown in the table (Tabl. 1).

Analyzing the obtained results of the study, highlighted in the Table, we can note that in case of IDAP, the R/B ratio and the intensity of the nitroperoxide glow increase with a statistically probable discrepancy ($p < 0.05$), in the absence of changes in the quantitative indicators of the optical density of histochemical staining for free amino groups of proteins according to the method of A. Yasuma and T. Ichikawa, and for total protein with bromophenol blue according to Bonheg ($p > 0.05$).

In case of chronic chorioamnionitis, the R/B ratio, the intensity of the nitroperoxide glow, and the relative units of the optical density of histochemical staining for free amino groups of proteins significantly increase on average compared to the placentas of physiological pregnancy and iron-deficiency anemia of pregnant women without placenta inflammation ($p < 0.001$). However, the indicators of the optical density of histochemical staining for total protein with bromophenol blue according to Bonheg remain without statistically significant differences compared to the groups of comparisons ($p > 0.05$).

In the observations of chronic chorioamnionitis against the background of iron deficiency anemia in pregnancy, a sharp increase in quantitative indicators was observed when using all research methods

($p \leq 0.001$), with the exception of an increase in total protein indicators in the histochemical method for total protein with bromophenol blue according to Bonheg ($p > 0.05$).

Below there are microphotographs of placenta sections stained with bromophenol blue according to Mikel Calvo for "acidic" and "basic" proteins. (fig. 1), according to the method of A. Yasuma and T. Ichikawa on free amino groups of proteins (fig. 2), as well as photocopies of preparations obtained by chemiluminescence of nitroperoxides with luminol (fig. 3).

DISCUSSION

Morphological assessment of oxidative stress in placental tissue structures should be comprehensive and include many pathohistological research methods. First of all, considerable attention should be paid to the determination of radicals, in particular nitroperoxides, which, due to their longer existence, are able to overcome greater diffusion distances and therefore are able to cause real pathology in the middle of the tissue, not only on its surface [13] and are subject to quantitative studies.

In our study, it is determined that in the placentas from parturient women who were diagnosed with iron deficiency anemia, an increase in the level of nitroperoxides was observed in the endotheliocytes of the blood vessels of the chorionic plate of the placenta among all the studied structures (35 ± 4.7 units of lum. at physiological pregnancy indicators - 18 ± 4.8 units of lum., $p = 0.01$). The results close to ours were obtained by A.V. Hoshovska et al. [13] in the study of nitroperoxides in the cytoplasm of the trophoblast of chorionic villi in case of IDAP.

At the same time, based on the following research results, the relationship between free radicals and the inflammatory process should not be underestimated, because free radicals actively participate in the development of the inflammatory reaction, starting from the alteration phase and ending with reparation processes [14]. Thus, studying nitroperoxides in the endotheliocytes of the blood vessels of the chorionic plate of the placenta in chronic chorioamnionitis (112 ± 4.2 units of blood, $p < 0.001$, with normal values - 18 ± 4.8 units of blood, $p < 0.001$;) we noticed an increase in brightness intensity compared to the norm. The same trend was observed by I. S. Davydenko et al. [15], who used the chemiluminescent technique with luminol to determine nitroperoxides in the syncytiotrophoblast cytoplasm of the chorionic villi of the placenta in purulent chorioamnionitis. After it was carried out, the average quantitative indicators were statistically higher than the indicators of physiological pregnancy.

With regard to comorbid IDAP during inflammation, we received data on the intensification of the chemiluminescent glow of nitroperoxides (133 ± 4.5 unit of lum., $p = 0.001$).

Let's try to find a logical explanation of the obtained quantitative indicators of the chemiluminescent glow of nitroperoxides during inflammation.

This is primarily due to the fact that the endothelium is one of the tissues most sensitive to the effects of hypoxia [16] and is the first to be activated during inflammation. When endothelial cells and smooth muscle cells are stimulated, there is an excess of radicals, in particular superoxide anion radical (O_2^-), which easily reacts with free nitric oxide (NO), forming a highly reactive and toxic compound - peroxynitrite (ONO^2). This can be one of the main reasons for the development of a vasoconstrictor effect, disruption of micro- and macrocirculation processes, metabolic disorders in tissues and their acceleration, and, ultimately, acceleration of the processes of apoptosis and necrosis in cells [17].

In addition, the sources of reactive radicals can be both intracellular structures (mitochondria, xanthine oxidase), and extracellular ones - neutrophils and macrophages. Lymphocytes and fibroblasts also constantly generate small amounts of superoxide radicals. And since these cellular elements are constantly present in large quantities during inflammation, this confirms the high concentration of radicals in the foci of inflammation [11].

A high concentration of nitroperoxides in the center of inflammation should be considered as a trigger for the intensification of the processes of oxidative modification of proteins, since reactive oxygen species can be considered as the main inducers of OMP [18].

We used the histochemical technique for "acidic" and "basic" proteins with bromophenol blue according to

Mikel Calvo. The essence of this research method is that the oxidation of amino groups of proteins leads to a change in the ratio between amino and carboxyl groups in them, and when staining histological sections with bromophenol blue, proteins are colored in different colors. In particular, basic proteins are colored blue, acidic proteins are colored red.

We evaluated colors using computer microspectrophotometry on digital copies of images in the RGB color evaluation system. The final result of the study was the R/B ratio, which is actually a quantitative expression of the ratio between amino and carboxyl groups of proteins and an indicator of the degree of oxidative modification of proteins.

During the histochemical study of placentas with IDAP, there was an increase in the R/B ratio in the endotheliocytes of the chorionic plate of the placenta ($p < 0.05$). The obtained results complement the conclusions of scientists who studied the process of OMP in other structures of the placenta with IDAP [19].

Taking into account the data we obtained earlier about the high level of nitroperoxides during placenta inflammation, the increase in the R/B ratio is most correctly associated with the increase in the intensity of OMP processes. Thus, our results showed that in the group of placentas with chronic ChA, in the endotheliocytes of the chorionic plate of the placenta with a high statistical probability ($p < 0.001$), the processes of OMP increased (1.29 ± 0.019 units of lum.) and even more in inflammation against the background of IDAP.

To confirm cause-and-effect relationships, we conducted a correlation analysis using the Pearson method between observations of inflammation in comorbid iron-deficiency anemia of pregnant women. Thus, strong correlations were established between the intensity of the nitroperoxide glow and OMP processes according to the R/B ratio, where $r = 0.793$ ($p < 0.05$), which can confirm the induction of OMP processes by free radicals when these states are combined.

Increased formation of free radicals under the influence of hypoxia caused by iron-deficiency anemia of pregnant women with a natural increase in the processes of oxidative modification of proteins should be accompanied by an increase in free amino groups of proteins. This was confirmed by the histochemical method of research according to the method of A. Yasuma and T. Ichikawa in combination with microdensitometry, both in chronic inflammation of the chorionic plate (0.205 ± 0.0021 , $p < 0.001$) and against the background of IDAP (0.224 ± 0.0015 , $p < 0.001$). We obtained similar results when studying the processes of limited proteolysis in the fibrinoid of both the chorionic and basal plates of patients with inflammation against the background of anemia of pregnancy [20].

The peculiarity of this technique is that it detects only free groups of proteins. Therefore, this technique shows the number of unhidden (free) amino groups of proteins rather than the total amount of protein. If there is a loss of proteins of a higher degree of structural organization (denaturation of proteins), the hidden amino groups of proteins become free - available for oxidation by ninhydrin. Therefore, in order to be able to interpret the obtained data on limited proteolysis, there was conducted a quantitative determination of total protein (using the method with bromophenol blue according to Bonheg) [20]. The results of the study are shown in the Table (Table 1). At the same time, we did not find any signs of changes in the concentration of total protein in the endotheliocytes of the chorionic plate of the placenta ($p > 0.05$), which indicates the fact of sufficient regeneration of proteins in the cells. As a result, this causes the protein to lose its ability to perform its functions and protein denaturation occurs.

In order to find a relationship between the intensity of the brightness of nitroperoxides and the processes of limited proteolysis, the correlation coefficient was determined using the Pearson method. Thus, according to the indicators of the optical density of histochemical staining, $r = 0.704$ ($p < 0.05$), and the correlation of limited proteolysis with OMP processes with statistical probability ($p < 0.05$) was $r = 0.783$.

The obtained results are an addition to the hypothesis about the intensification of free radical processes during inflammation of the placenta, which manifests itself with a certain regularity: an increase in the intensity of the glow of nitroperoxides, which indicates a high concentration of them in the center of inflammation, an increase in the R/B ratio, which is a reflection of OMP processes, and an increase in optical density histochemical staining for free amino groups of proteins as an

indicator of the activity of limited proteolysis processes.

This gives reason to believe that the key factor in the formation of morphological features of inflammation of the placenta is the intensification of free radical processes, and iron deficiency additionally significantly modifies these processes, which in turn enhances proteolysis [20].

CONCLUSIONS

1. With iron-deficiency anemia in pregnancy, the intensity of the glow of nitroperoxides and the average indicators of the R/B ratio increase compared to placentas of physiological pregnancy.
2. With chronic chorioamnionitis, the intensity of the glow of nitroperoxides, the average indicators of the R/B ratio and the optical density of histochemical staining for free amino groups of proteins increase compared to the placentas of physiological pregnancy and iron-deficiency anemia of pregnant women without inflammation.
3. Comorbid iron-deficiency anemia in pregnant women increases the intensity of the glow of nitroperoxides, the average indicators of the R/B ratio and the optical density of histochemical staining for free amino groups of proteins compared to placentas with inflammation without anemia.
4. The key factor in the formation of morphological features of chronic chorioamnionitis with comorbid iron deficiency anemia is the intensification of free radical processes, which is reflected by the increase in the concentration of nitroperoxides in the center of inflammation, with the subsequent intensification of the processes of oxidative modification of proteins, which is followed by the increasing activity of the processes of limited proteolysis.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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