

## THE SIGNIFICANCE OF ISCHEMIA FOR THE PROLIFERATIVE ACTIVITY OF THE MUCOSA IN INFLAMMATORY BOWEL DISEASES

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### Introduction.

Ulcerative colitis (UC) and Crohn's disease (CD) are the most common among chronic inflammatory bowel diseases (IBD) [29], which makes their study one of the most urgent problems of modern gastroenterology [1,2]. Development of intestinal inflammatory processes still has unknown links, which may be the sites of influence of targeted therapy [3,4]. The relevance of the issue determines the constant growth of morbidity among the able-bodied population aged 20-40, causing disability of young people [5,6].

UC is known to be a chronic inflammatory disease of the mucous membrane of the large intestine with the development of ulcerative-necrotic changes in the distal parts [7]. The main theory of the development of this disease is autoimmune, in which auto aggression is directed against the mucous membrane of the large intestine. Some authors attribute changes in immunohistological reactivity, genetic predisposition to the disease, dysbiotic shifts, neuropsychiatric disorders, allergic reactions to UC development [8].

CD is the result of the interaction of internal and external factors with the development of local inflammation in various parts of the gastrointestinal tract. Immune mechanisms with a change in the state of the epithelium of the large intestine wall are also not excluded as one of the possible links in CD pathogenesis. The influence of harmful factors of the external environment, which can be associated with peculiarities of the morpho-functional state, is yet to be determined for both diseases.

But despite the origin of the pathological process, the state of the microcirculatory bed remains one of the determining factors in its course. In the presence of small foci of ischemia [9,10], any pathological process is accompanied by dystrophic-necrobiotic consequences, which can also underlie the development or strengthening of the inflammatory process. Based on all of the above, the goal of our study was to determine the impact of the development of mucosal ischemia in the colon on the activity of proliferative processes during inflammation.

### Materials and Methods.

The study used 12 adult WAG rats, divided into two equal groups. Control group rats were fed on a standard diet. Water was provided ad libitum. Experimental group included the rats with chronic colitis induced by oral administration of 2.5% solution (weight/volume) Dextran Sulfate Sodium (DSS) (molecular weight: 40 kDa; PanReac AppliChem, Germany) in drinking water according the scheme: from 1<sup>st</sup> to 5<sup>th</sup> days, from 13<sup>th</sup> to 17<sup>th</sup> days, from 25<sup>th</sup> to 29<sup>th</sup> days the animals from the experimental group were orally administered DSS solution in drinking water; from 6<sup>th</sup> to 12<sup>th</sup> days, from 18<sup>th</sup> to 24<sup>th</sup>, from 30<sup>th</sup>

to 38<sup>th</sup> days they received drinking water [11,12]. On day 39, the animals were removed from the experiment with a guillotine knife.

The presence and extent of the pathological process in the large intestine was assessed by the morphological method by examining paraffin sections stained with hematoxylin and eosin, according to Rego. Immunohistochemical examination (IHC) was performed indirect immunoperoxidase reaction [13] with monoclonal antibodies (mAb) to Ki67 (Thermo scientific, USA). The reaction was visualized using a set of UltraVision LP Detection System HRP Polymer & DAB Plus Chromogen (Thermo scientific, USA).

Serial slides stained according to Rego and IHC that allowed to investigate proliferative activity in ischemic area. Morphometric study was performed using "Olympus DP-soft 3.12" program. Volume of ischemic area was detected. Positive Ki67 expression was diagnosed with nuclear stain in the intermediate and superficial cells. Ki67 staining in basal or parabasal cell was considered as negative.

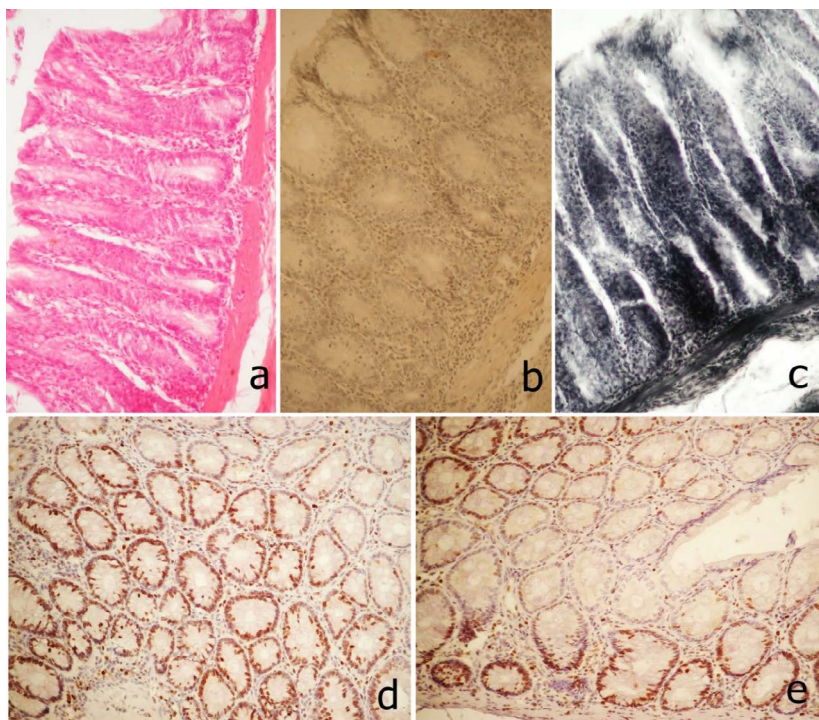
Counting of cells with positive Ki67 was realized in percent number for 1000 cells in investigated area. All values are expressed as means, standard deviation (SD) and standard error of the mean (SEM) for statistical analysis. Statistical comparison was performed using Mann-Whitney test for statistical analysis. Spearman's rank correlation coefficient (r) was counted for measure of the strength of relationship between paired data. The difference between groups was considered statistically significant at  $p < 0.05$ .

The experimental part of the research was carried out in accordance with the general ethical principles adopted by the First National Congress of Ukraine on Bioethics (Kyiv, 2001), the provisions of the European Convention on the Protection of Vertebrate Animals (Strasbourg, 1986) and Council of Europe Directive 86/609/EEC (1986), the Law of Ukraine No. 3447-IV dated 21.02.2006 "On the protection of animals from cruel treatment".

### Results.

Inspection microscopy of preparations stained with hematoxylin and eosin in the experimental (injection of DSS solution) determined alterative-desquamative changes in the surface epithelium and epithelium of intestinal glands (crypt); diffuse polymorphic cellular infiltration in the mucous membrane, which in some places spread to the submucosal base (Figure 1a).

The microscopic signs of peptic ulcer are a complex combination of inflammatory changes and structural abnormalities. The diagnosis of colitis primarily implies changes in the cellular



**Figure 1.** Inflammatory changes in the colon. Diffuse polymorphic cellular infiltration in the mucous membrane, staining with hematoxylin and eosin, magnification x200 (a); absence of ischemic focus (b) and focal ischemic area in the colon mucus (c), staining according to Rego, magnification x200; active even proliferative activity in the colon mucus without ischemia (d) and appearance of uneven focus with reduced proliferation above focus of ischemia (e), immunohistochemical study of Ki-67, magnification x200.

Table 1. Results of morphometric study.

Indicator	Investigated group	
	Inflammatory bowel disease	Control group
Area of ischemia, %	13.09±0.67*	3.79±0.38
Expression Ki67 in all area, (% of positive cells)	49.66±3.28*	24.32±2.46
Expression Ki67 in area without ischemia, (% of positive cells)	57.71±4.68*	25.39±2.02
Expression Ki67 in area with ischemia, (% of positive cells)	18.06±3.33 <sup>#</sup>	13.41±1.88 <sup>#</sup>

\*  $p < 0.05$  significant difference between investigated groups and group of control.

<sup>a</sup>  $p < 0.05$  significant difference between area without and with ischemia

<sup>#</sup>  $p < 0.05$  significant difference between all area indicators and area with ischemia

infiltrate of the lamina propria. These alterations, in turn, can be subdivided into changes in density, composition and distribution. Changes in density (increased density of the cell infiltrate) imply an increase in the number of cells, revealed by histological study of specimens obtained during the experimental study. In the comparison group, the infiltrate is localized in the upper part of the mucosa, while in the experimental group the infiltrate spreads to the underlying sections, capturing the entire mucosa. The diffuse nature of the increase in density of the cell infiltrate is both vertical and lateral. Changes in the composition of the cellular infiltrate are characterized by the accumulation of plasma cells in the deep sections of mucosa and, in particular, between the base of the crypts and the muscularis mucosa (the so-called basal plasmacytosis). In addition to inflammatory infiltration in the lamina propria of the mucous membrane, the content of lymphoid accumulations and lymphoid follicles increases.

An important microscopic sign of acute colitis is the presence of neutrophilic leukocytes in the infiltrate. In combination with signs of damage to the epithelium, they are a morphological

manifestation of activity. In addition to the appearance of neutrophilic leukocytes in the cellular infiltrate, they are found in the surface and crypt epithelium (cryptitis), which leads to erosion and destruction of the crypts.

Investigation of slides stained according to Rego in the experimental and control group determined presence of uneven focal area of black color both in mucus and lamina propria, which has to be recognized as area of ischemia (Figure 1b, 1c). But, if it is insignificant for control group, it is statistically reliable for inflammatory bowel disease group ( $p < 0.05$ ), where such area obtains 13.09±0.67% (Table 1).

Detection of proliferative activity depending on ischemic signs was realized in different level of Ki67 expression. So, lowest level of Ki67 was estimated in mucosa above black stained smooth muscle fibers (Figure 1c) with statistically reliable digits (Table 1). Such areas were insignificant in control group, but their expansion in inflammatory group realized in presence of focus without Ki67 expression (Figure 1e). Most pronounced expression of Ki67 was observed in IBD group in area which no

connected with ischemia (Figure 1d) and was even  $57.71 \pm 4.68\%$  ( $p < 0.05$ ). Strong negative correlation was detected between Ki67 expression and ischemic area ( $r = -0.819$ ).

### Discussion.

According to the literature data, such morphological features as neutrophils in the epithelium of the crypts (cryptitis), in the lumen of the crypts (cryptabscess) in combination with damage and destruction of the crypts are characteristic of active colitis [7,14].

Structural changes in the mucous membrane of the colon are characterized by an uneven surface and a disturbed structure of the crypts [15]. Changes on the part of the crypts include shortening, in which the bases of the crypts are separated from the muscularis mucosa, disappearance of the crypts, and especially expressed budding of the crypts (branching crypts, caudate crypts). Atrophy of the colonic mucosa involves a combination of loss and shortening of the crypts. In addition to erosion, there are ulcerative defects penetrating beyond the lamina propria with the presence of granulation tissue, fibrinoid necrosis and exudate. Part of the mucosal cells is flattened, which can be considered as a sign of restitution, which involves rapid closure of the defect by migrating immature cells from the upper sections of the crypts towards the surface [16,17]. An increase in the proliferative activity of epithelial cells, which is reflected, among other things, in an increase in the number of mitotic figures, correlates well with the degree of damage to the mucous membrane [18]. Another sign is thickening of the muscularis mucosa.

The use of the results of a histological study to assess the activity of UC has been used since the 1950s. The assessment of activity can be given descriptively or using a point scale. Changes in the intensity and composition of the lamina propria cell infiltrate, which are assessed in histological specimens stained with hematoxylin and eosin, make it possible to distinguish between active, inactive colitis and the stage of remission. Disease activity is determined by the presence of neutrophilic leukocytes in the infiltrate in combination with signs of epithelial damage. Inactive chronic colitis is characterized by the presence of structural changes and an increase in the content of mononuclear cells in the lamina propria [19]. And the disease in the stage of remission is characterized by the presence of structural changes without an increase in intensity and disturbances in the composition of the infiltrate of the lamina propria.

To preserve the intestinal tissue architecture an equilibrated balance between proliferation, differentiation and programmed cell loss by apoptosis is needed [20]. In our research ICH with Ki67 was strongly positive in epithelial cells of the colon both in intact tissue and in modeling IBD with significant increasing expression more than twice in inflammatory group. Proliferation is process with numerous background mechanisms in regulation with no sufficient attention to hypoxia in non-ischemic colitis [21]. Simultaneously, local extracellular acidification is associated with several conditions, such as ischemia and inflammatory bowel disease [22]. Morphological detection of such in early stage is difficult but Rego staining is an indicator of mosaic metabolic changes in tissue, partly in myocytes [23,24].

So, our study allows to see morphological connection between development of ischemia and proliferation in IBD.

Ischemic damage to the lining of the small intestine occurs remarkably fast after the onset of hypoperfusion. The lining of the colon, in contrast, is less easily damaged by a decrease in blood supply and its recovery occurs rather slowly compared to the intestine [20]. But previous studies were devoted for acute ischemic changes and less for disturbance of microcirculation as result of continuous inflammation with formation of ischemic focus.

Intestinal inflammation seems invariably to be associated with increased epithelial proliferation with restructuring of mucosal morphology in the flat-mucosa involves considerable adaptation of the connective tissue elements of the lamina propria [17,25]. Whether this occurs in response to changes in ischemia and epithelial renewal is not clear. From one point of view, increased epithelial proliferation occurs in response to different inflammatory stimuli. But simultaneously, we found uneven growth of such activity and even decreasing in area which accords ischemia [26,27].

Despite development of ischemic changes, tissue expression was restricted to cells with a high proliferation rate and regenerative capacity in ischemic area also ( $18.06 \pm 3.33\%$ ). According to literature data [28] this further supports the decisive role in cell division. The results obtained under ischemic conditions demonstrate a comparably high expression. Even in the case of an ischemia injury, the regenerative capacity of the intestine is maintained [29]. Thereby the intestine is capable of preventing or restoring intestinal barrier dysfunction. In combination with previously published results these findings emphasize the substantial role of the intestinal barrier and intestinal permeability for health and disease and reflect the important relevance of an intact intestinal barrier [28].

In conclusion, this study cannot document about ischemia as main link in IBD as we did not see attenuation in clinical, endoscopic disease activity scores, we did not see attenuation in serological and fecal biomarkers of inflammation and maybe chronic inflamed patients in general, are in a conditioned state. But this should be taken into account in future studies of ischemia involving for patients with chronic inflammation.

### Conclusions.

Ki67 was strongly expressed in epithelial cells of the colon both in intact tissue and in modeling IBD with significant increasing expression more than twice in inflammatory group ( $p < 0.05$ ) but spreading of activity process was uneven. Collation of slides with ICH and Rego staining realized in estimation of strong negative correlation between Ki67 expression and ischemia ( $r = -0.819$ ).

### Conflict of Interest Statement.

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Summary.

The state of the microcirculatory bed remains one of the determining factors in course of inflammatory bowel diseases (IBD). The presence of small foci of ischemia could realize in dystrophic-necrobiotic consequences, which can also underlie the development or strengthening of the inflammatory process. Based on above, the goal of our study was to determine the impact of the development of mucosal ischemia in the colon on the activity of proliferative processes during inflammation.

**Materials and methods.** The study was performed on 12 adult WAG rats with modeling IBD by oral administration of 2.5% solution Dextran Sulfate Sodium. Serial slides of the colon were made with stained with hematoxylin and eosin, according to Rego, immunohistochemical examination (IHC) to Ki67. Volume of ischemic area and Ki67 expression were detected. Statistical comparison was performed.

**Results.** Inspection microscopy in the DSS experimental group determined alterative-desquamative changes in the surface epithelium and epithelium of intestinal glands (crypt); diffuse polymorphic cellular infiltration in the mucous membrane, which in some places spread to the submucosal base, that are morphological manifestations of IBD. Foci of ischemia had been detected in that group with  $13.09 \pm 0.67\%$  volume as just microfocal changes were observed in intact animals ( $p < 0.05$ ). Detection of proliferative activity depending on ischemic signs was realized in different level of Ki67 expression. So, lowest level of Ki67 was estimated in mucosa above ischemia ( $18.06 \pm 3.33\%$ ). Most pronounced expression of Ki67 was observed in IBD group in area which no connected with ischemia and was even  $57.71 \pm 4.68\%$  ( $p < 0.05$ ).

**Conclusions.** Ki67 was strongly expressed in epithelial cells of the colon both in intact tissue and in modeling IBD with significant increasing expression more than twice in inflammatory group ( $p < 0.05$ ) but spreading of activity process was uneven. Collation of slides with IHC and Rego staining realized in estimation of strong negative correlation between Ki67 expression and ischemia ( $r = -0.819$ ).

**Key words.** Colitis, experiment, proliferation, ischemia, morphometry.

**Значение ишемии для пролиферативной активности слизистой оболочки при воспалительных заболеваниях кишечника**

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## Резюме.

Состояние микроциркуляторного русла остается одним из определяющих факторов течения воспалительных заболеваний кишечника (ВЗК). Наличие мелких очагов ишемии может реализоваться в дистрофически-некробиотических последствиях, что также может лежать в основе развития или усиления воспалительного процесса. Исходя из изложенного, целью нашего исследования было определение влияния развития ишемии слизистой оболочки толстой кишки на активность пролиферативных процессов при воспалении.

**Материалы и методы.** Исследование выполнено на 12 взрослых крысах линии WAG с моделированием ВЗК путем перорального введения 2,5% раствора декстрана сульфата натрия. Серийные препараты толстой кишки окрашивали гематоксилином и эозином, по Рего, проводили иммуногистохимическое исследование (ИГХ) на Ki67. Определяли объем зоны ишемии и экспрессию Ki67. Было проведено статистическое сравнение.

**Результаты.** При контрольной микроскопии в экспериментальной группе определялись альтеративно-десквамативные изменения поверхностного эпителия и эпителия кишечных желез (крипты); диффузная полиморфная клеточная инфильтрация в слизистой оболочке, местами распространяющаяся на подслизистую основу, что является морфологическим проявлением ВЗК. Очаги ишемии в этой группе заняли  $13,09 \pm 0,67\%$ , тогда как у интактных животных наблюдались только микроочаговые изменения ( $p < 0,05$ ). Выявление пролиферативной активности в зависимости от признаков ишемии выявило разный уровень экспрессии Ki67. Так, самый низкий уровень Ki67 отмечен в слизистой оболочке над ишемией ( $18,06 \pm 3,33\%$ ). Наиболее выраженная экспрессия Ki67 наблюдалась в группе ВЗК в области, не связанной с ишемией, и составляла  $57,71 \pm 4,68\%$  ( $p < 0,05$ ).

**Выводы.** Выраженная экспрессия Ki67 выявлена в эпителиальных клетках толстой кишки как в интактной ткани, так и при моделировании ВЗК со значительным увеличением экспрессии более чем в два раза в воспалительной группе ( $p < 0,05$ ), однако распространение активности процесса было неравномерным. Сопоставление препаратов с окрашиванием по Рего и ИГХ, выявило сильную отрицательную корреляционную связь между экспрессией Ki67 и ишемией ( $r = -0,819$ ).

**Ключевые слова.** колит, эксперимент, пролиферация, ишемия, морфометрия.