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Effect of local ozone therapy on inflammatory markers in experimental ulcerative colitis

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ABSTRACT

Aim. To evaluate the effect of rectal insufflations of medical ozone on markers of inflammation in experimental ulcerative colitis.

Materials and methods. The experimental study was performed *in vivo* on 49 white, sexually mature male Wistar rats weighing 250 ± 15 g. The model of ulcerative colitis was reproduced using two-stage oxazolone administration (Sigma-Aldrich, USA). A group of animals received rectal insufflations of medical ozone at a dose of 1.0 mg / l once a day in the volume of 10 ml of ozone / oxygen mixture. The cycle of insufflations lasted 10 days. The ozone / oxygen mixture was obtained using an automated ozone therapy device with an ozone destructor UOTA-60-01"Medozon" (Medozon LLC, Moscow, Russian Federation). According to the disease activity index (DAI) score, the disease activity index was evaluated. The intensity of neutrophil phagocytosis in the blood was detected using polystyrene latex particles. The ability of neutrophils to reduce nitroblue tetrazolium (NBT) was determined using spontaneous and induced NBT tests. The interleukin-17 (IL-17) concentration in the serum was determined by enzyme-linked immunosorbent assay (ELISA) using a test system for rats manufactured by Bender MedSystems (Austria).

Results. Under the conditions of ozone therapy by rectal insufflations in experimental ulcerative colitis, we demonstrated improvement in the clinical presentation of the disease, intensity of phagocytosis, phagocytic index, and spontaneous and induced ability of neutrophils to reduce NBT with normalization of the functional reserve of cells and the level of proinflammatory IL-17 on day 6 of the experiment.

Conclusion. The results obtained allow to verify pronounced anti-inflammatory and immunomodulatory effects of ozone and consider it as one of the most relevant treatment strategies for inflammatory bowel diseases.

Keywords: experiment, ulcerative colitis, inflammation, medical ozone

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Влияние локальной озонотерапии на маркеры активности воспалительного процесса при экспериментальном язвенном колите

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РЕЗЮМЕ

Цель. Оценить влияние ректальных инсуффляций медицинского озона на маркеры активности воспалительного процесса при экспериментальном язвенном колите.

Материалы и методы. Экспериментальное исследование проводили в условиях *in vivo* на 49 белых половозрелых самцах крыс линии Вистар массой 250 ± 15 г. Модель язвенного колита воспроизводили с помощью двухэтапного введения оксазолона (Sigma-Aldrich, США). Группе животных проводили инсуффляции медицинского озона ректально в дозе 1,0 мг/л, 1 раз/сут, в объеме 10 мл озоно-кислородной смеси (ОКС). Курс 10 сут. ОКС получена на озонотерапевтической автоматической установке с деструктором озона УОТА-60-01 «Медозон» (ООО «Медозон», Москва, Россия). В соответствии со шкалой DAI (disease activity index) оценивали индекс активности болезни. Детекцию активности фагоцитоза нейтрофилов крови проводили с использованием частиц полистирольного латекса. НСТ-редуцирующую способность нейтрофилов определяли с применением спонтанного и индуцированного вариантов НСТ-теста. Определение концентрации интерлейкина (IL) 17 в сыворотке определяли методом иммуноферментного анализа с помощью тест-системы для крыс фирмы Bender Medsystems (Австрия).

Результаты. В условиях ректального применения озонотерапии при экспериментальном язвенном колите показано улучшение клинической картины заболевания, нормализация показателей интенсивности фагоцитоза, фагоцитарного числа, спонтанной и индуцированной НСТ-редуцирующей способности с нормализацией показателя функционального резерва клеток и уровня провоспалительного IL-17 на 6-е сут эксперимента.

Заключение. Полученные результаты позволяют констатировать наличие выраженного противовоспалительного и иммуномодулирующего эффектов озона и рассматривать последний в качестве одного из актуальных направлений терапии воспалительных заболеваний кишечника.

Ключевые слова: эксперимент, язвенный колит, воспаление, медицинский озон

Конфликт интересов. Авторы заявляют об отсутствии явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic disease of the colon which is characterized by the presence of pronounced inflammation in the mucosa. The incidence of UC in the European population ranges from 0.6 to 24.3 per 100,000 people, and the prevalence reaches 505 cases per 100,000 people [1]. The etiology of UC is multifactorial and understudied. A special role in the pathogenesis of UC is assigned to gut microbiome imbalance [2]. A key defect of innate immunity in UC is hyperactivation of proinflammatory signaling pathways, which leads to impaired recognition of bacterial molecular patterns

by dendritic cells of the colonic mucosa. Infiltration of the colonic mucosa tissue by lymphocytes with signs of plasmocytic differentiation mainly to the Th2-phenotype is accompanied by overexpression of proinflammatory cytokines (tumor necrosis factor- α (TNF-α), interleukin (IL)-6, IL-17), intercellular adhesion molecules (ICAM), and signaling molecules with formation of characteristic morphological changes.

One third of patients with UC are known to be refractory to background therapy, and many patients develop a wide range of adverse effects that limit its use [3]. This dictates the need for search for new, pathogenetically grounded approaches to UC treatment, including the use of genetically engineered immunobiological drugs with anti-cytokine effect, cell adhesion molecule inhibitors, stem cells, and relevant efferent therapy techniques. Among the latter, the use of therapeutic concentrations of medical ozone is of special interest due to its pronounced anti-inflammatory, immunomodulatory, bactericidal, and antioxidant effects. The successful use of ozone therapy in surgery, dermatocosmetology, and ENT disorders is known, and there are some publications on the use of ozone therapy in gastroenterology.

The aim of the study was to evaluate the effect of rectal insufflations of medical ozone on inflammatory markers in experimental UC.

MATERIALS AND METHODS

The study was conducted at the site of the experimental biological clinic (vivarium) of the South Ural State Medical University of the Ministry of Health of the Russian Federation. All procedures were carried out in strict accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETSIN 123, March 18, 1986) and in compliance with the Directive 2010/63/EU of the European Parliament and of the Council of 22.09.2010 and were approved by the local Ethics Committee at South Ural State Medical University (Protocol No. 4 of 22.05.2020) [4]. Experimental studies were carried out *in vivo* on 49 white, sexually mature male Wistar rats weighing 250 ± 15 g. By simple randomization, the male rats were divided into 2 groups: group 1 (n = 9) included intact animals, group 2 (n = 54) – animals with UC.

The model of UC was reproduced by two-stage oxazolone administration (Sigma-Aldrich, USA) with clinical and morphological verification [5]. The first stage was characterized by development of skin sensitization following application of 150 μl of 3% oxazolone solution to the pre-treated interscapular area. At the second stage, the 3% oxazolone solution was rectally injected to a depth of 7 cm. The study was performed on days 2, 4, and 6. After receiving the experimental model of UC, 21 animals were randomly selected for ozone therapy (group 3). Daily, the animals of group 3 received rectal insufflations of medical ozone at a dose of 1.0–1.2 mg / l once a day in the volume of 10 ml of ozone / oxygen mixture. The cycle lasted 10 days. The ozone / oxygen mixture was obtained using an automated ozone therapy device with an ozone destructor UOTA-60-01"Medozon" (Medozon LLC, Moscow, Russian Federation). Blood sampling for the study was carried out under general anesthesia (the drug Zoletil-100 (tiletamine hydrochloride, Virbac Sante Animale, France) at a dose of 20 mg / kg). Blood was collected by a left ventricular puncture in vacuum blood collection tubes "Vacuette" (Greiner Bio-One, Austria) with an anticoagulant (K3-EDTA or heparin) for immunological studies.

The clinical examination was performed by calculating the disease activity index (DAI), according to the modified score for evaluating the studied pathology in rats [6]. The score includes 3 parameters, such as body weight, stool consistency, and the presence of blood in the stool. For the evaluation, a 4-point scale from was used (from 0 to 4). The scores were summed, the minimum index value being 0, the maximum – 12.

The granulocyte population was isolated by ficoll (Pharmacia, Sweden) – verografin (SPOFA, Czech Republic) density gradient centrifugation. Functional assessment of the phagocytic activity of blood neutrophils was performed using polystyrene latex particles (diameter 1.5 μm). The results were considered using immersion microscopy with the calculation of the percentage of cells that engulfed at least one polystyrene latex particle (activity of phagocytosis (AP)), the number of engulfed latex particles (units) in 100 cells (intensity of phagocytosis (IP)), and the number of engulfed latex particles (units) per one phagocyte (phagocytic index (PI)).

The ability of neutrophils to reduce NBT was determined using spontaneous and induced NBT tests, with the calculation of the functional reserve of cells according to the method proposed by A.N. Mayanskiy and M. E. Vixman [7]. The result was expressed in units. The interleukin-17 (IL-17) concentration in the serum was determined on the automated immunoassay analyzer "Personal LAB" using a test system for rats (Bender MedSystems, Austria). The results were expressed in pg / ml.

Statistical processing of the results was carried out using the Statistica 8.0 software package. The data in the tables are presented as the median and interquartile range *Me* (Q_{25}–Q_{75}). The groups were compared using the nonparametric Kruskal – Wallis and Mann – Whitney tests with the Bonferroni correction. The differences were considered statistically significant at $p \leq 0.016$.

RESULTS

The clinical status in experimental UC in the animals of group 2 was evaluated according to the modified Disease Activity Index (DAI) score and showed elevation of DAI on day 2, with an increase on day 4 and 6 (Table 1). Changes in the ethological status were manifested through reduced motor activity, grooming, and feed consumption.

The evaluation of the ability of neutrophils to engulf particles (Table 2) in experimental UC showed that on day 2 of the observation in group 2, the activity of phagocytosis (AP), the intensity of phagocytosis (IP), and the phagocytic index (PI) increased. The maximum values for these parameters were observed on day 4, with a tendency to a decrease in AP and IP on day 6 compared with the intact group.

When assessing the ability of blood neutrophils to reduce NBT in animals with experimental UC, it was found that on day 2, the activity and intensity of the spontaneous and induced NBT tests increased, along with the functional reserve of neutrophils, estimated by the activity and intensity of the NBT test. Day 4 of the experiment was characterized by high values for spontaneous NBT test and functional reserve, however, on day 6, the parameters of spontaneous and induced NBT tests and functional reserve did not differ from those in the intact group. The results of the study on the concentration of proinflammatory IL-17 in the serum of animals with experimental ulcerative colitis (EUC) at runtime are presented in Table 3. The level of IL-17 in group 2 increased by day 4 and reached the maximum value by day 6 of the experiment compared with the group of intact animals.

Under the conditions of rectal ozone therapy in animals with EUC, positive changes in the ethological status were recorded in the form of increased motor and stress-protective activity and higher degree of feed consumption. Against the background of ozone therapy, starting from day 2 of the observation, no visible signs of intestinal bleeding with a lack of blood in the stool, as well as more formed and firmer stool were noted. These signs were manifested through the integrated parameter of DAI assessment, the values of which are presented in Table 1. Therefore, in group 3, the DAI values on day 2 did not differ significantly from those in group 2, with a significant decrease on days 4 and 6. However, despite a significant decrease, the DAI values in these groups on days 4 and 6 did not reach the values in the group of intact animals, which indicates incomplete restoration of the colonic mucosa against the background of local medical ozone administration.

The effect of rectal insufflations of medical ozone in EUC on the functional activity of blood neutrophils was studied (Table 2). Starting from day 2, as well as on days 4 and 6, an increase in AP and IP was revealed compared with the intact group. Compared with group 2, starting from day 2 and on day 4, significantly lower values for IP and PI were recorded, which decreased to the level in the intact group on day 6 of the experiment.

Under the conditions of rectal ozone therapy, on day 2 of the experiment, the parameters of spontaneous and induced NBT test (activity) significantly increased, and the parameters of the functional reserve decreased compared with the intact group. Compared with group 2, induced NBT test and functional reserve values in group 3 decreased. On days 4 and 6, the parameters of spontaneous and induced NBT test (activity) decreased compared with group 2 and reached the level in the intact group. On days 4 and 6, the functional reserve values did not differ from those in the intact group.

Under the conditions of rectal ozone therapy, an increase in IL-17 in the blood (Table 3) was noted on day 4 of the experiment compared with the intact group and group 2. Complete normalization of the parameter was observed on day 6 compared with the intact group.

Table 1

Effect of ozone therapy on the changes in the disease activity index in experimental ulcerative colitis, *Me* (Q_{25}–Q_{75})

Parameter	Group 1 Healthy animals (n = 9)	Group 2 Rats with EUC, day 2 (n = 9)	Group 2 Rats with EUC, day 4 (n = 9)	Group 2 Rats with EUC, day 6 (n = 9)	Group 3 Rats with EUC + ozone rectally, day 2 (n = 9)	Group 3 Rats with EUC + ozone rectally, day 4 (n = 9)	Group 3 Rats with EUC + ozone rectally, day 6 (n = 9)
DAI, units	0	5.0 (2.0–8.0)*	8.0 (7.0–12.0)*	12.0 (10.0–13.0) *	6.0 (4.0–7.0)*	3.0 (2.0–3.0) * #	2.0 (1.0–2.0) * #

Note: the differences between the groups are obtained using the Kruskal – Wallis test and the Mann – Whitney test with the Bonferroni correction (here and in Table 2, 3). Statistically significant differences with group 1 – * , with the corresponding observation day in group 2 – #.

Table 2

Effect of rectal ozone therapy on the functional activity parameters of blood neutrophils in experimental ulcerative colitis, *Me* (Q_{25}–Q_{75})

Parameter	Group 1 Healthy animals (n = 9)	Group 2 Rats with EUC, day 2 (n = 9)	Group 2 Rats with EUC, day 4 (n = 9)	Group 2 Rats with EUC 6 days (n = 9)	Group 3 Rats with EUC + ozone rectally, day 2 (n = 9)	Group 3 Rats with EUC + ozone rectally, day 4 (n = 9)	Group 3 Rats with EUC + ozone rectally, day 6 (n = 9)
Phagocytosis activity, %	34.1 (31.0–45.0)	54.0 (44.0–57.0)*	60.8 (48.0–66.0)*	46.3 (36.0–65.0)*	51.60 (36.0–57.5)*	56.3 (44.0–62.0)*	43.5 (35.0–64.0)*
Phagocytosis intensity, units	0.72 (0.5–0.85)	4.5 (3.83–5.5)*	7.2 (5.7–13.1)*	5.8 (4.5–11.5)*	2.3 (1.32–3.7) *#	1.7 (0.7–1.9)*#	0.9 (0.4–1.2)#
Phagocytic index, units	1.5 (1.2–1.9)	8.2 (7.3–9.3)*	13.8 (12.5–16.0)*	13.6 (9.2–15.3)*	5.6 (4.8–6.2)*#	4.3 (2.3–4.6)*#	1.20 (1.2–4.0)#
Sp. NBT test, activity, %	4.0 (4.0–5.0)	9.0 (8.0–10.0)*	14.0 (13.0–16.0)*	7.5 (5.0–12.0)	6.5 (5.0–10.0)*	4.0 (3.0–5.0)#	3.5 (2.0–4.5)#
Sp. NBT test, intensity, units	0.05 (0.04–0.07)	0.18 (0.16–0.19)*	0.20 (0.19–0.21)*	0.08 (0.03–0.12)	0.14 (0.13–0.15)*	0.12 (0.02–0.15)*#	0.06 (0.05–0.08)
Ind. NBT test, activity, %	5.0 (4.0–6.0)	23.5 (9.0–24.0)*	9.5 (8.0–13.0)	6.0 (4.0–7.0)*	8.0 (7.0–10.0)*#	5.5 (5.0–6.0)#	5.0 (4.0–6.0)#
Ind. NBT test, intensity, units	0.04 (0.03–0.05)	0.26 (0.10–0.26)*	0.06 (0.04–0.2)	0.05 (0.03–0.05)*	0.10 (0.09–0.10)*#	0.05 (0.03–0.05)#	0.04 (0.03–0.05)#
Functional reserve (activity of the NBT test)	1.53 (0.70–1.6)	1.8 (1.4–2.1)*	1.70 (1.3–2.0)*	1.64 (0.53–4.3)	0.78 (0.66–0.9)*#	1.6 (0.8–1.9)	1.58 (0.98–1.7)
Functional reserve (intensity of the NBT test)	1.4 (0.95–1.5)	1.62 (1.52–1.7)*	1.73 (1.3–2.2)*	1.4 (0.3–1.5)	0.75 (0.5–0.85)*#	1.2 (0.8–1.3)	1.5 (0.58–1.6)

Table 3

Effect of rectal ozone therapy on the changes in the level of IL-17 (pg / ml) in the blood serum in experimental ulcerative colitis, *Me* (Q_{25}–Q_{75})

Parameter	Group 1 Healthy animals (n = 9)	Group 2 Rats with EUC, day 2 (n = 9)	Group 2 Rats with EUC, day 4 (n = 9)	Group 2 Rats with EUC 6 days (n = 9)	Group 3 Rats with EUC + ozone rectally, day 2 (n = 9)	Group 3 Rats with EUC + ozone rectally, day 4 (n = 9)	Group 3 Rats with EUC + ozone rectally, day 6 (n = 9)
IL-17, pg / ml	6.3 (3.3–7.4)	5.7 (4.3–8.2)*	15.3 (9.6–22.3)*	17.3 (9.3–64.7)*	5.4 (4.2–7.3)	10.3 (9.52–14.25)#	6.62 (5.6–6.8)#

DISCUSSION

In EUC at runtime, an increase in DAI indicated the presence of inflammatory changes in the colonic wall. Neutrophils and then monocytes migrated to the area of primary alterations, causing local tissue damage due to the release of enzymes, inflammatory mediators, and reactive oxygen species (ROS), which was accompanied by an increase in the ability of blood neutrophils to engulf particles and reduce NBT on day 2. Chemotaxis of lymphocytes to the lesion site was accompanied by

an increase in clonal expansion of lymphocyte subpopulations and a rise in the secretory activity of the latter, which was accompanied by an increase in proinflammatory cytokines, including IL-17, in the blood.

IL-17 is known to be a secretory product of a special subpopulation of memory T cells identified as CD4 CD45RO, the synthesis of which is controlled by IL-23. In some inflammatory bowel diseases, IL-23 is essential in the final differentiation of Th0 to Th17 after exposure to IL-1β, IL-6, and TNF-α, while IL-12 induces polarization of the Th1-dependent immune response with production of IFN-γ, TNF-α, and other cytokines [8]. At the same time, the hematopoietic and proinflammatory activity of IL-17 is mediated by the ability of the latter to stimulate production of TNF-α, IL-1β, PGE2, GM-CSF, IL-6, IL-10, IL-12, IL-1 receptor antagonist (IL-1RA), and stromolysin.

Improvement in the clinical presentation (decrease in the severity of symptoms, decrease in DAI) and normalization of IP, PI, and spontaneous and induced NBT test activity with the restoration of the functional reserve of cells on day 6 of the experiment under the conditions of rectal ozone therapy indicate a positive effect of the latter on inflammation in the intestinal wall due to anti-inflammatory and immunomodulatory effects of ozone. The local effect of average therapeutic concentrations of medical ozone directly on pathologically altered areas of the colonic tissues consists in direct ozonation of bioorganic compounds, as well as an indirect effect of ozonolysis products that form a reserve of ROS with a possibility of their subsequent permanent use in aerobic metabolism for maintaining the relevant level of energy substrates of colonocytes.

ROS act as “molecular phages”, contributing to purification of an ulcerated lesion and activating the chemotaxis of neutrophils and monocytes in the lesion [9]. Hydrotrioxides formed following ozone oxidation of organic substances associated with unsaturated fatty acids are extremely unstable compounds that decompose in a cell with release of molecular oxygen, thereby exerting a metabolic effect on the cell and a non-specific bactericidal effect. It is also known that administration of low concentrations of ozone / oxygen mixture launches free radical reactions in the cell. Subthreshold levels of ROS, in turn, are able to strengthen the antioxidant system in the cell according to the feedback principle [9].

We believe that changes in the functional activity of blood neutrophils under the conditions of local ozone therapy in EUC are due to a decrease in destructive processes in the pathological focus, a decrease in the secretion of inflammatory mediators, and restriction of phagocyte activation, both in the lesion area and in circulating neutrophils in the blood. This assumption is confirmed by a decrease in the concentration of the proinflammatory cytokine IL-17 to the values of the intact group against the background of rectal ozone therapy.

CONCLUSION

Therefore, the obtained results demonstrate pronounced anti-inflammatory and immunomodulatory effects of rectal insufflations of medical ozone in EUC, which allows to consider it as one of the promising areas in the therapy for inflammatory bowel diseases.

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Authors contribution

Osikov M.V. – conception of the study and final approval of the manuscript. Davydova E.V. – analysis and interpretation of data. Kaygorodtseva N.V. – collection of material.

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