

REDOX DEPENDENT FEATURES OF TUMORS, ADIPOSE TISSUE, NEUTROPHILES AND PLATELETS IN PATIENTS WITH METASTATIC COLORECTAL CANCER

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Aim: To study the levels of redox-forming molecules generated in tumor, adipose tissue (AT), neutrophils and platelets of patients with colorectal cancer (CRC) with normal body weight or obesity. **Materials and Methods:** 83 samples of tumor tissue and 83 samples of the AT taken at the distance of 5 cm from the tumor as well as 83 blood samples have been analyzed in the patients with CRC with metachronous lesions in liver. The comparison group consisted of 18 patients who were surgically treated for ulcer. The rates of free radical generation were measured by electron paramagnetic resonance. **Results:** Free radical generation rate in tumor tissue and AT increased and nitrogen oxide (NO) levels decreased in patients with body mass index ≥ 25 kg/m² as compared to the patients with normal body weight ($p < 0.05$). The superoxide generation activity in neutrophils of CRC patients exceeded about 7-fold the values obtained in neutrophils from patients with ulcer while the NO levels produced by neutrophils of CRC patients decreased significantly ($p < 0.01$). The same trends held true for the platelets. **Conclusions:** In obese patients with CRC, superoxide-generating activity increases in cancer cells, AT, neutrophils and platelets. The obesity should be considered as the additional oncological risk representing the potent predictor for the events realized in obese patients such as oxidative stress, inflammation, insulin resistance and endothelial dysfunction. **Key Words:** colorectal cancer, obesity, neutrophils, platelets, free radical generation, NO.

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Colorectal cancer (CRC) ranks third in males and second in females by its incidence worldwide. More than one million CRC cases are diagnosed globally every year with more than half million deaths being registered. Among CRC risk factors are obesity, smoking, increased consumption of alcohol, red meat, and starch, decreased consumption of fruits and vegetables. The heme iron in foods such as red meat facilitates the increasing rate of DNA damage acting as prooxidant that increases oxidative stress in the cells [1]. Recent data suggest the total antioxidant activity of the consumed food rather than the specified content of each of the antioxidant substances is critical for decreasing CRC risk.

Within the recent decade, the role of reactive oxygen species (ROS) in carcinogenesis has been thoroughly studied [2]. In the setting of the uncontrolled ROS production coupled with decreased activity of cellular antioxidant enzyme systems, both ROS and their derivatives may react with cell macromolecules resulting in modulation of gene expression. In view of this, ROS could be considered as the potential therapeutic target. It has been evident that free-radical nitrogen oxide (NO) acts as the transcellular messenger involved in plenty of physiological and pathological processes. It should be stressed the NO also binds to the Fe²⁺ with high affinity (free iron, iron in iron-sulfur centers, ferrous

heme) [3]. NO-ferrous heme binding in fact mediates various biological processes such as smooth muscle relaxation, neuromodulation, inhibition of platelet aggregation and adhesion. The interruption of blood supply in the adipose tissue (AT) followed by the complete or partial restoration of blood flow in the setting of the surgical resection of the tumor results in ischemia-reperfusion oxidative injury in the adipose tissue. This state is further associated with the chronic inflammation in the tissues subjected to the surgical stress.

Based on these assumptions, we have attempted to study the levels of redox-forming molecules generated in tumor, AT, neutrophils and platelets of CRC patients with normal body mass or obesity.

MATERIALS AND METHODS

83 samples of tumor tissue and 83 samples of the AT taken at the distance of 5 cm from the tumor as well as 83 blood samples have been analyzed in the patients with metastatic CRC (mCRC) with metachronous lesions in liver. The patients were treated in the National Cancer Institute. Two groups of patients were delineated with body mass index (BMI) ≤ 25 kg/m² ($n = 37$) and BMI ≥ 25 kg/m² ($n = 46$). The comparison group consisted of 18 patients who were surgically treated for ulcer. The study was approved by the Institutional Ethics Committee in accordance with the requirements of the Declaration of Helsinki 2008. All patients gave their informed consent on the use of the clinical samples for research purposes.

The samples for electron paramagnetic resonance (EPR) study were prepared from tumor tissue and AT. Neutrophils and platelets were isolated from the blood samples by density gradient centrifugation.

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Abbreviations used: AT – adipose tissue; BMI – body mass index; CRC – colorectal cancer; EPR – electron paramagnetic resonance; FA – fatty acids; mCRC – metastatic CRC; NO – nitrogen oxide; ROS – reactive oxygen species.

The rates of free radical generation and NO levels by tumor cells, AT, neutrophils and platelets were measured by EPR using spin traps TEMPONE-H and DETK (Sigma, USA), respectively).

Briefly, 200 μ l of tissue homogenate or 10^5 cells were transferred to the paramagnetically “clear” quartz cuvette. Then 20 μ l of the corresponding spin trap was added and the EPR spectra were recorded within 2-min intervals detecting the rates of free radical generation and NO levels [4]. Free fatty acids (FA) were measured according to [5].

The data were statistically processed using the statistical package R (r-project.org). The significance of the difference was assessed based on Student *t*-test. The data are presented as $M \pm SE$. *P*-values < 0.05 were considered statistically significant. The survival of patients was analyzed by Kaplan — Meier method.

RESULTS AND DISCUSSION

Fig. 1, *a* demonstrates the increased free radical generation rate in tumor tissue as compared to that in the intestinal tissue of the patients with ulcer (group of comparison). Moreover, the free radical generation rate in tumor tissue increased in patients with BMI ≥ 25 kg/m² as compared to that in patients with BMI ≤ 25 kg/m². Therefore, increased AT mass seems to be associated with more intense free radical generation in tumor tissue. It is known that increased availability of lipids and carbohydrates in case of obesity is coupled with the increased energy requirement with

activation of electron transport chain in mitochondria, oxygen utilization and free radical generation [6, 7]. When NO levels in tumor tissue of mCRC patients were measured, the same trend in the association with obesity, although non-significant, has been noticed (Fig. 1, *b*). This is in line with the data on the association between obesity and NO levels as well as dysregulation of NO synthase system demonstrated elsewhere [8, 9].

The free radical generation rates in AT of mCRC patients with/without obesity paralleled those in tumors (Fig. 2, *a*). Namely, the free radical generation rate in AT increased in patients with BMI ≥ 25 kg/m² as compared to that in patients with BMI ≤ 25 kg/m². This fact suggests the ensuing oxidative stress in AT of mCRC patients. The trend for NO levels in AT of mCRC patients relative to their obesity is reverse. In patients with BMI ≥ 25 kg/m², NO levels decreased as compared to the patients with normal body weight (Fig. 2, *b*). This could be explained by the decreased bioavailability of NO due to peroxynitrite (ONOO⁻) production and effects of the endogenous inhibitors such as dimethylarginine known to increase in the obese persons [10].

When superoxide generation activity was assessed in neutrophils of mCRC patients, such activity exceeds about 7-fold the values obtained in donor' neutrophils (from patients with ulcer). Nevertheless, the superoxide generation activity did not depend on whether the patients were obese or not (Fig. 3, *a*). The levels of NO produced by neutrophils in mCRC patients decrease significantly (5.6-fold) as compared to neutrophils of patients

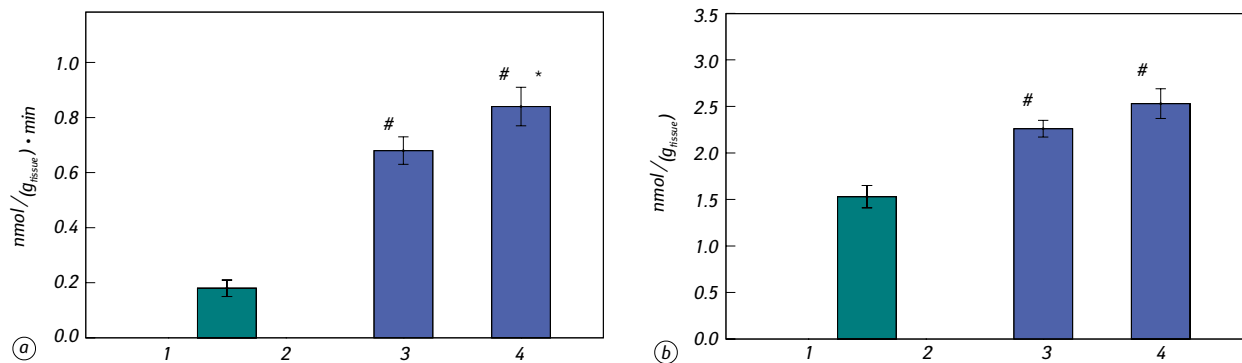


Fig. 1. Free radical generation rates (*a*) and NO levels (*b*): 1 — in the intestinal tissue of patients with ulcer; 2 — in tumor tissue in patients with BMI ≤ 25 kg/m²; 3 — in tumor tissue in patients with BMI ≥ 25 kg/m². **p* < 0.05 compared to patients with BMI ≤ 25 kg/m²; #*p* < 0.01 compared to patients with ulcer

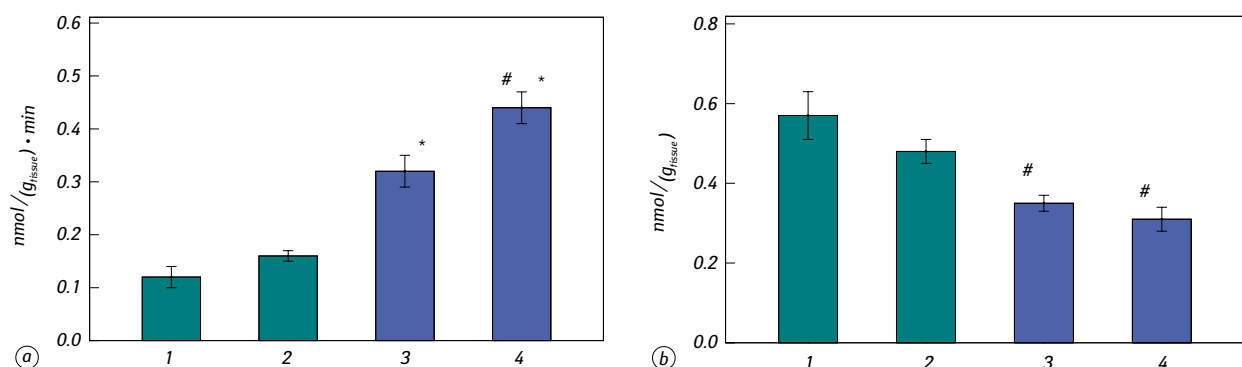


Fig. 2. Free radical generation rates (*a*) and NO levels (*b*) in AT: 1 — patients with ulcer, BMI ≤ 25 kg/m²; 2 — patients with ulcer, BMI ≥ 25 kg/m²; 3 — mCRC patients, BMI ≤ 25 kg/m²; 4 — mCRC patients, BMI ≥ 25 kg/m². **p* < 0.01 compared to patients with ulcer; #*p* < 0.05 compared to patients with BMI ≤ 25 kg/m²

with ulcer (Fig. 3, *b*). It is known that at the early stages of tumor growth, neutrophils are cytotoxic towards tumor cells due to generation of free radicals and NO at high levels while later protumoral effects of tumor-associated neutrophils became evident [11]. It should be also noted that within the first days of high-lipid diet, neutrophils migrate to AT [12]. Meanwhile, tumor-associated neutrophils inhibit protumoral $\gamma\delta$ 17T-cells [13]. Therefore, the dual effects of neutrophils in tumor microenvironment should be taken into consideration.

Analysis of superoxide generation activity in platelets of mCRC patients demonstrated the significant increase in mCRC patients (Fig. 4, *a*) while NO levels decreased much profoundly in the obese patients (Fig. 4, *b*). Moreover, three groups with different superoxide generation activity could be delineated in platelets of mCRC patients, namely with ≤ 1.7 nmol/ 10^3 cell·min ($n = 8$), ≤ 1.9 nmol/ 10^3 cell·min ($n = 16$) and ≤ 2.1 nmol/ 10^3 cell·min ($n = 22$). Kaplan — Meyer analysis of survival in these three subgroups revealed the significant differences. 5-year overall survival in the subgroup with high superoxide generation activity in platelets was 39% vs 56% and 62% in subgroups with less superoxide generation activity (Fig. 5, *a*). The same difference in 5-year survival was evident in subgroups delineated by the different NO levels (Fig. 5, *b*).

Therefore, the dysfunctional AT facilitates the increased superoxide generation activity in platelets of mCRC patients. In human platelets, NADPH-oxidases represent the major sources for free radicals upon

platelet activation [14]. It is known that obesity increases platelet activation and neutrophil count. It should be also noted that activated platelets adhering to endothelial cells secrete pro- and antiangiogenic factors and produce ROS resulting in the formation of procoagulant phenotype of platelets that increases thrombotic risk. The compensatory increase in platelet count in the blood of CRC patients due to the decreased NO generating activity is associated with increasing predisposition to metastasis and worsened prognosis [15].

Taking into account the changes in redox state in tumors, AT, neutrophils and platelets, one should envisage the increased content of free FA in blood of mCRC patients. In fact, the content of free FA increased substantially in plasma of mCRC patients as compared to patients with ulcer. Moreover, the content of free FA in the obese patients with mCRC was significantly higher than in patients with BMI ≤ 25 kg/m² (Fig. 6). The increased content of free FA should affect undoubtedly their metabolism. This could be of importance for tumor progression taking into account that FA are not only the structural components of the membranes but may also serve as the secondary messengers and represent the energy source. It is also significant that the products of lipid peroxidation could modulate cell function affecting gene expression and proliferation [16].

To sum up, in obese patients with mCRC, superoxide-generating activity increases in cancer cells, AT, neutrophils and platelets. In contrast, NO levels increases only

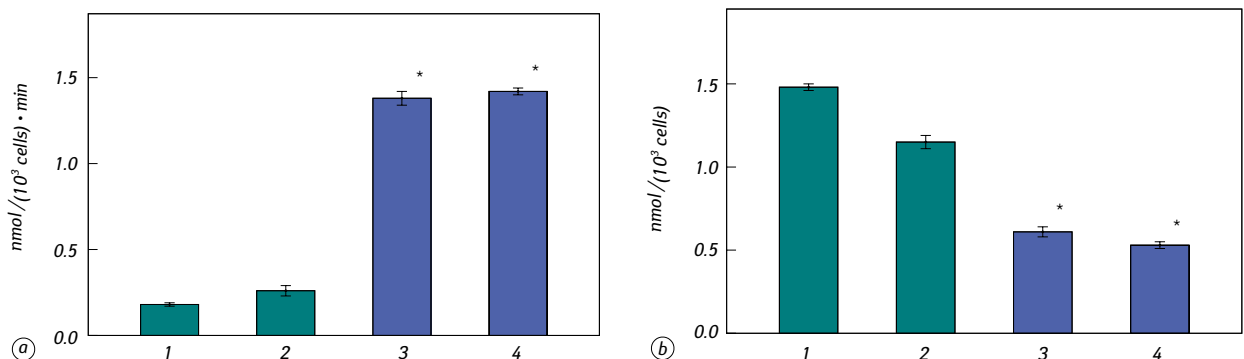


Fig. 3. Free radical generation rates (*a*) and NO levels (*b*) in peripheral blood neutrophils: 1 — patients with ulcer, BMI ≤ 25 kg/m²; 2 — patients with ulcer, BMI ≥ 25 kg/m²; 3 — mCRC patients, BMI ≤ 25 kg/m²; 4 — mCRC patients, BMI ≥ 25 kg/m². * $p < 0.01$ compared to patients with ulcer

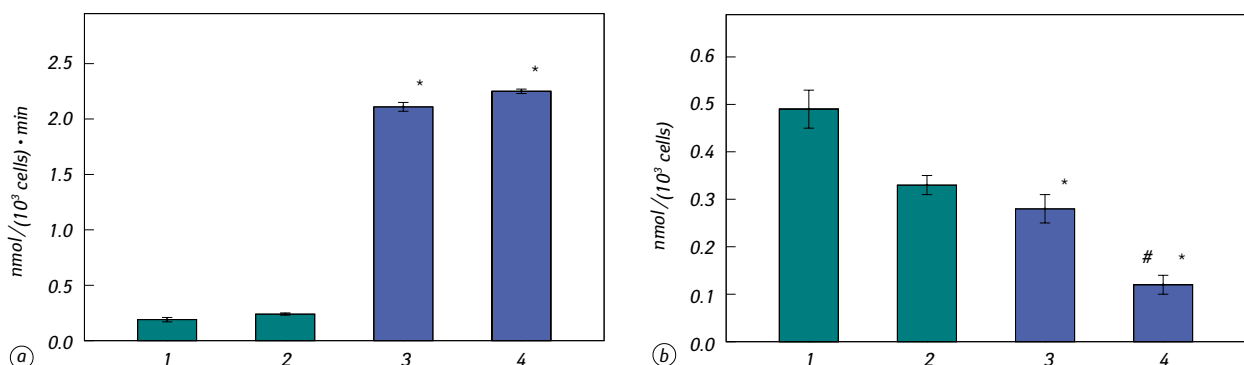


Fig. 4. Superoxide generation (*a*) and NO production (*b*) in platelets: 1 — patients with ulcer, BMI ≤ 25 kg/m²; 2 — patients with ulcer, BMI ≥ 25 kg/m²; 3 — mCRC patients, BMI ≤ 25 kg/m²; 4 — mCRC patients, BMI ≥ 25 kg/m². * $p < 0.01$ compared to patients with ulcer; [#] $p < 0.01$ compared to patients with BMI ≤ 25 kg/m²

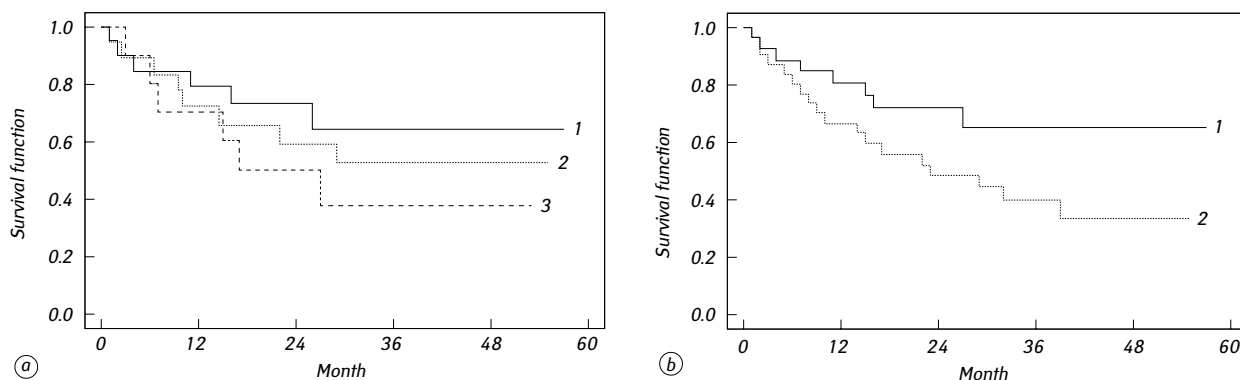


Fig. 5. Survival distribution functions depending on (a) superoxide-generating activity of NADP-N-oxidase of platelets: 1 — ≤ 1.7 nM/ 10^3 cells·min; 2 — ≤ 1.9 nM/ 10^3 cells·min; 3 — ≤ 2.1 nM/ 10^3 cells·min; $p = 0.35$; (b) NO-generating activity of platelets 1: ≥ 0.3 nM/ 10^3 cells·min; 2: ≤ 0.3 nM/ 10^3 cells·min. The survival curves were compared using a log-rank test

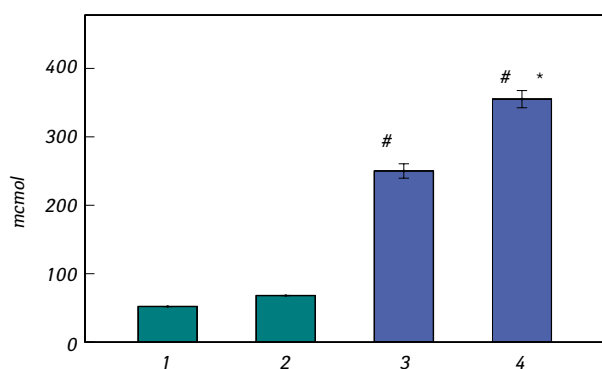


Fig. 6. Free FA in blood plasma у плазмі крові: 1 — patients with ulcer, BMI ≤ 25 kg/m 2 ; 2 — patients with ulcer, BMI ≥ 25 kg/m 2 ; 3 — mCRC patients, BMI ≤ 25 kg/m 2 ; 4 — mCRC patients, BMI ≥ 25 kg/m 2 . * $p < 0.01$ compared to patients with ulcer; # $p < 0.01$ compared to patients with BMI ≤ 25 kg/m 2

in tumor while NOS activity decreases in AT, neutrophils and platelets. Therefore, increased AT mass in cancer patients is associated with the intensified generation of free radicals by mitochondria while the excess of energy substrates in obesity enhances mitochondrial dysfunction and oxidative signaling. The increased generation of redox-forming molecules results also in the increased content of free FA in the blood. The latter become the major energy source for cancer cells. Moreover, the free FA are the important secondary messengers. Therefore, the obesity should be considered as the additional cancer risk representing the potent predictor for the events realized in obese patients such as oxidative stress, inflammation, insulin resistance and endothelial dysfunction. One could speculate that the molecular processes and pathways inherent to the obesity, in particular NOS as regulator of the energy metabolism may be considered as the most probable therapeutic target to decrease the associated cancer risks.

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РЕДОКС-ЗАЛЕЖНІ ОСОБЛИВОСТІ ЖИРОВОЇ ТКАНИНИ, ПУХЛИНИ, НЕЙТРОФІЛІВ ТА ТРОМБОЦИТІВ У ХВОРИХ НА МЕТАСТАТИЧНИЙ КОЛОРЕКТАЛЬНИЙ РАК

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Мета: Дослідити рівні редокс-формуючих молекул, які генеруються у пухлині, жировій тканині, нейтрофілах та тромбоцитах хворих на колоректальний рак з нормальною та надмірною масою тіла. **Матеріали та методи:**

Проведено дослідження 83 зразків тканини аденокарцином, 83 зразків жирової тканини, відпрепарованої на відстані 5 см від пухлини, та 83 зразків крові у хворих на метастатичний колоректальний рак з метакронним ураженням печінки. Рівні швидкості генерування вільних радикалів визначали методом електронно-парамагнітного резонансу. **Результати:** У хворих на метастатичний колоректальний рак за наявності ожиріння виявлено зростання рівнів супероксид-генеруючої активності та зниження рівнів NO в жировій тканині, нейтрофілах та тромбоцитах. **Висновки:** У хворих на колоректальний рак з ожирінням виявлено зростання рівнів супероксид-генеруючої активності мітохондрій клітин пухлини, жирової тканини, а також нейтрофілів та тромбоцитів. Ожиріння визначає додатковий онкологічний ризик і є потужним предиктором подій, які накопичуються і реалізуються при ожирінні, а саме: окиснювальний стрес, запалення, резистентність до інсуліну, дисфункція ендотелію.

Ключові слова: колоректальний рак, ожиріння, нейтрофіли, тромбоцити, вільні радикали, NO.