

DRAG REDUCING POLYMERS ATTENUATE ADVERSE EFFECTS OF ISCHEMIA-REPERFUSION UPON RESECTION OF LIVER METASTASES MODELED BY MC38 MOUSE COLON ADENOCARCINOMA

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Background: The resection of metastases within healthy parenchyma improves significantly the long-term outcome in metastatic colorectal cancer. Until now, the resection technique involves Pringle maneuver, which allows reducing blood loss during transection of liver parenchyma. However, the classical Pringle maneuver has restrictions due to ischemia/reperfusion (I/R) effect, in particular increasing risk of tumor recurrence after liver surgery. **Aim:** To study the pathological impact of surgical intervention and I/R effect on healthy liver tissue in the experimental setting by evaluating the markers of redox-homeostasis and oxidatively induced mutagenesis, and also to assess the current possibilities of their correction by application of drag-reducing polymers (DRPs). **Materials and Methods:** MC38 mouse colon adenocarcinoma cells were transplanted intrahepatically to C57Bl/6 mice. The influence of warm ischemia on metastatic potential of MC38 cells, the speed of superoxide radicals (SR) generation and 8-hydroxydeoxyguanosine content were studied. **Results:** In case of modeled liver metastases, the surgery initiates an increase in the rate of SR generation into the remaining liver tissue and, consequently, provokes its ischemic injury. The application of DRPs protects liver tissue under I/R conditions. **Conclusions:** The warm I/R can promote metastatic lesions in the healthy part of the organ with underlying increase in the rate of SR generation and oxidatively induced damage of guanine in DNA. The hemorheological effects of DRPs ensure increase of microcirculatory perfusion and oxygenation of liver tissues with the reduction of the rate of SR generation and decrease of 8-hydroxydeoxyguanosine as a marker of oxidatively induced mutations in DNA of hepatocytes. The intraperitoneal administration of nanomolar doses of DRPs prevents the activation of the growth of dormant metastatic MC38 cells in the liver. Further experimental and clinical study of these substances will allow reducing the risks of activation of uncontrolled tumor growth in the liver due to the pathological effect of post-operative I/R.

Key Words: ischemia/reperfusion, liver metastases, metastatic colorectal cancer, drag reducing polymers.

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The resection of metastases within healthy parenchyma remains the main method that significantly improves the long-term treatment outcome in the patients with metastatic colorectal cancer (MCRC). Until now, the resection technique involves clamping portal triad within the hepatoduodenal ligament applying the Pringle maneuver (PM), which allows reducing blood loss during transection of liver parenchyma [1]. Nevertheless, the classical PM has restrictions due to ischemia/reperfusion (I/R) effect, in particular increasing risk of tumor recurrence after liver surgery [2]. The experimental and clinical studies have shown that surgical stress and ischemic liver damage due to the long-term PM application and impaired blood flow/outflow from a specific area of liver parenchyma can lead to uncontrolled tumor growth [3]. The response to inflammation due to the damage of hepatocytes and development of acute liver failure also can potentiate the risks of tumor progression [4]. Despite

the fact that liver resection in patients with MCRC allows improving survival in comparison with cases when only the modern palliative chemotherapy is used, the recurrent metastatic lesions upon resection are recorded in 50–60% of cases that, in turn, becomes the main problem in the treatment strategy [5].

Yamashita *et al.* [6] proved that the degree of ischemia due to the impaired blood supply in hepatic parenchyma within the first 30 days after resection (liver infarction) could be considered as a negative independent predictor of prognosis in patients with MCRC. The authors demonstrated a direct correlation between the infarction severity in the resection area (assessed by CT imaging) and the duration of relapse-free survival.

In response to the surgical stress, tumor-associated neutrophils generate pathologically high levels of superoxide radicals (SR) [7]. The latter cause oxidation of molecular structures including DNA molecules resulting in mutations. 8-Hydroxydeoxyguanosine (8-oxodGu) is the commonly used biomarker of mutagenesis consequent to oxidative stress [8].

So far, no adequate methods for controlling the above-indicated processes in clinical setting exist. Nevertheless, some promising studies proved the possibility of correcting these pathological changes

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Abbreviation used: 8-oxodGu – 8-hydroxydeoxyguanosine; DRPs – drag-reducing polymers; I/R – ischemia/reperfusion; MCRC – metastatic colorectal cancer; PM – Pringle maneuver; RBC – red blood cells; SR – superoxide radicals.

under the experimental conditions [9]. In particular, Tohme et al. [10] have demonstrated the effectiveness of drag-reducing polymers (DRPs) in protecting the liver from I/R injury and decreasing the metastatic spread of tumor cells. Toms et al. [11] described the ability of low concentrations of soluble DRPs to reduce the turbulent flow in tubular structures. A number of experimental animal studies have shown that even nanomolar concentrations of DRPs are able to adapt liver against ischemic damage by improving tissue perfusion and oxygenation, and reducing vascular resistance [12, 13]. The presence of DRPs in blood circulation leads to the increase in concentration of red blood cells (RBC) near the vascular walls, thus reducing the pathological effects of leukocytes and platelets on the microcirculation of the parenchymal organ [14]. The rheological effects of DRPs allow for reducing the interaction between platelets, leukocytes and vascular endothelium, which is crucially important for effective suppression of inflammation [15].

Based on the information above, the purpose of our study was to assess the pathological impact of surgery and I/R effect on healthy liver tissue in the experimental setting by assessing the markers of redox homeostasis and oxidatively induced mutations as well as evaluating the possibilities of their correction.

MATERIALS AND METHODS

MC38 mouse colon adenocarcinoma cells provided by Prof. Orel (National Cancer Institute, Kyiv, Ukraine) were applied for modeling metastatic liver injury in mice following two subcutaneous passages. C57Bl/6 mice aged 8 weeks from animal-breeding facility of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, the National Academy of Sciences of Ukraine were used in the experiments.

30 mice were divided into four groups: the intact control group ($n = 6$); group A ($n = 8$) — animals with transplanted MC38 cells; group B1 ($n = 8$) — animals with transplanted MC38 cells with simultaneous warm ischemia; group B2 ($n = 8$) — animals treated in the same way as B1 with intraperitoneal administrations of DRPs immediately after I/R.

The metastatic process in the liver was modeled according to the procedure described by Tohme *et al.* [3]. The animals were anesthetized and operated as follows: through the section 2–2.5 cm in length in the wall of abdominal cavity, the cells ($5 \cdot 10^4$ in 0.1 ml) were subcapsularly injected into the middle lobe of liver (with U-40 insulin syringe with the needle of 12.7 mm ($1/2''$) $\times$ 27 G). The modified PM was performed as follows: the vascular Dieffenbach Bulldog Clamp was applied onto the “pedicle” supplying the middle hepatic lobe for 30 min. Then the abdominal wall was sutured. On the 7th day of tumor growth, the liver with tumor-affected area was resected. The fragments of liver tissue were sampled for determining *ex tempore* 8-oxodGu level.

Polyethylene oxide with a molecular weight of 4 million Da was used as DRP (Polysciences, Inc. Germany). For applying DRPs, the preparation pre-dissolved in Ringer’s solution (100 μ l, concentration 10–1000 ppm) was administered intraperitoneally to the animals of group B2 immediately after ischemia of the median liver lobe.

The rate of SR generation was studied by EPR with Spin Traps technique. 8-oxodGu level in liver tissue was measured upon solid-phase extraction followed by spectrophotometric identification by absorption spectra and quantification on the 7th postoperative day [16].

For histological study, the fragments of the resected tumor with surrounding tissues, up to 4 mm in thickness, were fixed in buffered 10% formalin pH 7.4 for 16 h. The fixed specimens were impregnated in wax in histioprocessor Histos-5 (Milestone, Italy) followed by embedding with heated paraffin in the molds of HESTION TEC-2800 Embedding Center (Australia) and transferring the specimens to the refrigeration module of HESTION TEC-2800 Cryo Console (Australia). The histological sections, 5 μ m in thickness, prepared in Microm HM325 (Germany) were stained with hematoxylin-eosin. The histological sections were analyzed by morphometry calculating the volume of viable tissue. The microphotos were taken under OLYMPUS CX 41 (Japan) microscope equipped with QuickPHOTO MICRO 2.3 software. The degree of necrosis of sinusoidal hepatocytes was assessed by the conventional method and calculated by a formula $A/B \times 100$ where A — number of necrotic cells, B — total number of cells being counted in 10 fields of view. The results obtained were statistically analyzed using SPSS 20.0 statistics (IBM, Armonk, NY, USA). The concentrations of 8-oxodGu were compared at different stages of the disease using One-way ANOVA. The two-way statistical tests were considered statistically significant when $p < 0.05$.

RESULTS

The morphological study of tumor and adjacent tissues demonstrated significant dystrophic and necrobiotic changes both in healthy liver parenchyma and tumor tissue (Fig. 1). The tumor is represented by polymorphic cells with the nuclear-cytoplasmic ratio > 1 and stroma in the form of connective tissue bundles (Fig. 1, a). In B1 group, preserved tumor parenchyma remained predominantly around the vessels, and the expressed necrobiotic changes were noted in most cases (Fig. 1, b). In B2 group (warm ischemia), the large necrotic areas were evident with preserved parenchyma predominantly around the blood vessels with dystrophic and necrobiotic changes (Fig. 1, c). The volume of viable tumor tissue in specimens of group B2 was about 28%.

The rate of SR generation in liver with transplanted MC38 carcinoma (group A) increased sharply lesions as compared with the control values (Fig. 2). The formation of liver hypoxia conditions due

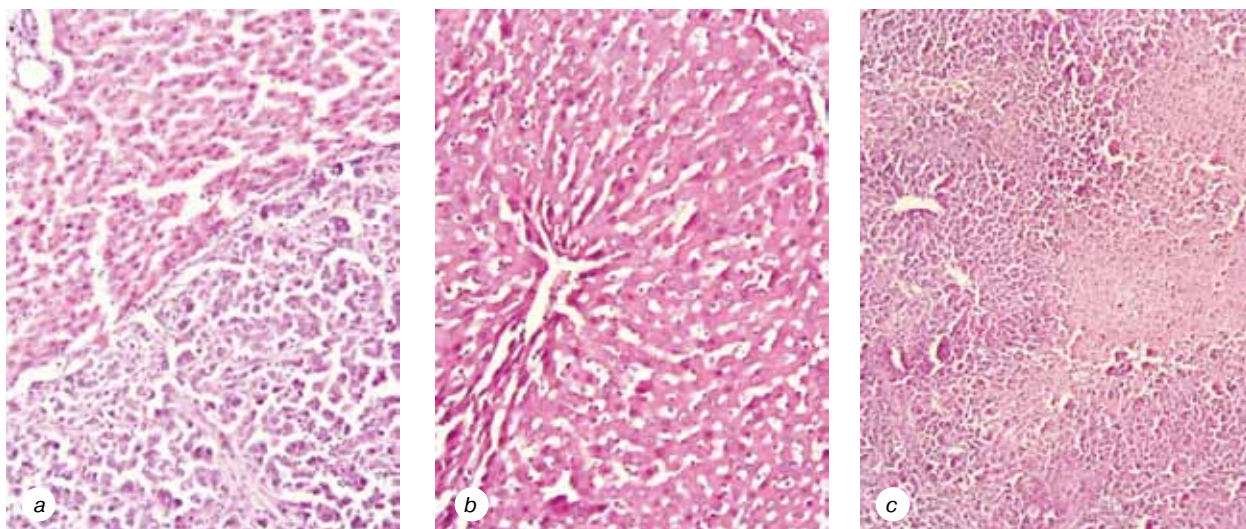


Fig. 1. Morphological changes of liver parenchyma associated with tumor growth and warm ischemia: (a) — group A, H&E, $\times 200$; (b) — group B1, H&E, $\times 400$; (c) — group B2, H&E, $\times 100$

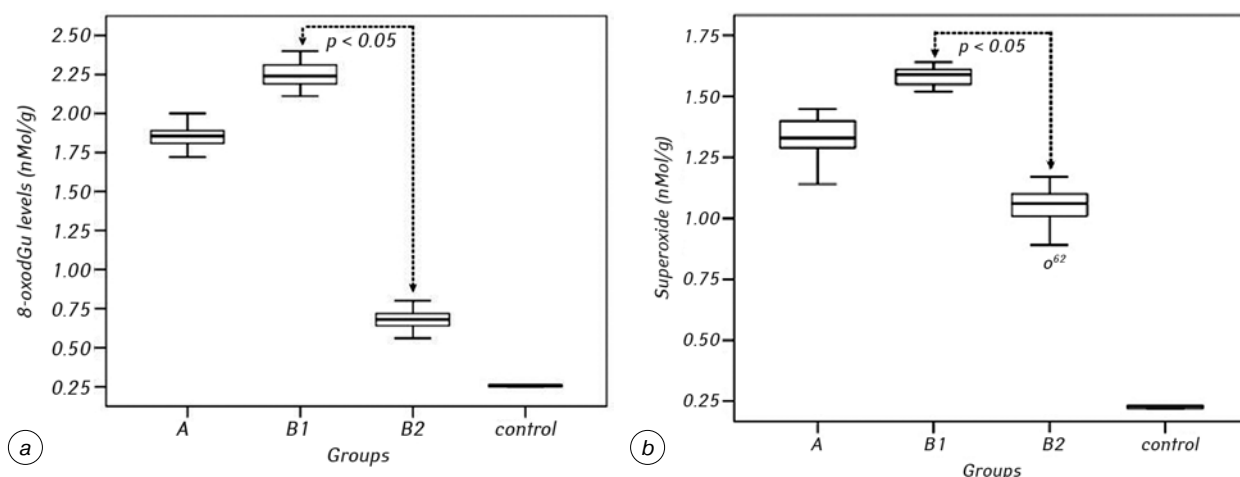


Fig. 2. Box-plot graph showing 8-oxodGu (a) and SR (b) levels in the liver of control and experimental groups of animals on the 7th postoperative day

to restriction of blood flow to the organ following PM (group B1) was accompanied by further intensification of the oxidation processes (SR generation rate of 1.58 ± 0.35 nmol/g of crude tissue), whereas DRP injection (group B2) allowed reducing it significantly (1.04 ± 0.86 nmol/g of crude tissue, $p < 0.05$) (see Fig. 2).

In the cells of liver parenchyma in the control group of animals, 8-oxodGu content was determined at the level of 0.25 ± 0.05 nmol/g of tissue. The development of metastatic nodules (group A) caused significant increase of the guanine oxidation marker in DNA to the values of 1.85 ± 0.71 nmol/g of tissue. The warm ischemia of the middle liver lobe in the setting of tumor growth (group B1) led to even greater intensification of the oxidation processes and further increased 8-oxodGu level to 2.25 ± 0.74 nmol/g. The injection of nanomolar concentrations of DRPs (group B2) into the abdominal cavity of rats (after the modified PM) led to the definite stabilization of 8-oxodGu level at 0.68 ± 0.63 nmol/g of tissue, $p < 0.05$ (see Fig. 2).

We have analyzed I/R effect on the growth of tumor after intracapsular grafting of MC38 cells. In B1 group, I/R resulted in significant increase in tumor lesions number counted on the 7th day as compared to group A ($p < 0.05$) (Fig. 3). The use of DRPs in B2 group en-

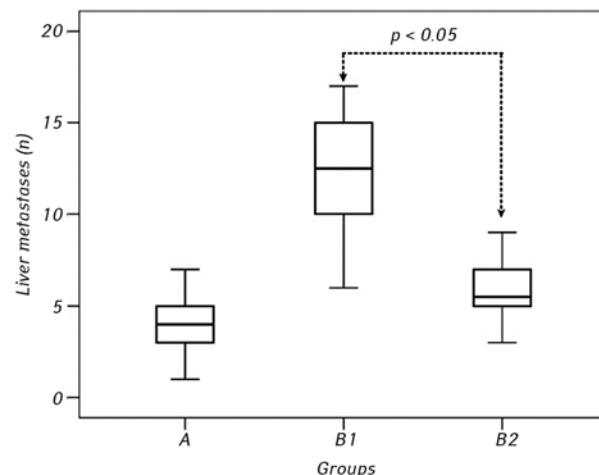


Fig. 3. Box-plot graph showing I/R effect on the number of macroscopically detected metastatic lesions in the liver on the 7th postoperative day

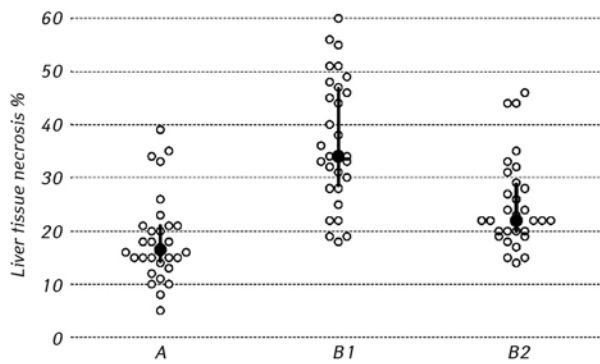


Fig. 4. Box-plot graph showing the extent of necrosis in liver parenchyma in the studied groups

abled us to decrease by half the number of macroscopically detected metastatic lesions as compared to B1 group ($p < 0.05$) (see Fig. 3).

Furthermore, the administration of DRPs also decreased the necrotic area in the affected liver tissue (Fig. 4). A single DRP injection led to the reduction of the extent of necrobiotic alterations in liver tissues caused by PM. The DRPs enabled the reduction of necrosis of normal liver parenchyma.

Therefore, DRP in our experimental setting allowed to mitigate the negative I/R component in PM, especially concerning the growth potential of tumor cells.

DISCUSSION

Liver warm ischemia promotes increasing risk of tumor recurrence after liver surgery [2]. SR represent a key factor that initiates the induction of cell apoptosis playing an important role in the development of acute liver failure after its resection. In addition, liver surgery could affect the rheological features of blood changing the hemodynamics of blood flow in the microcirculatory bed vessels [17]. Apart from that, the persistent oxidative stress contributes to pathological progression of liver fibrosis increasing risk of liver failure.

As we have demonstrated previously, the basic point is that growth and development of malignant tumors are accompanied by a steady increase of SR generation owing to dysfunction of electron-transport chain of mitochondria [18]. The experimental studies have defined the dominant molecular pathways capable of mediating I/R effects and their role in activating the growth of dormant tumor cells.

It is known that during resection of liver with accompanying PM, the conditionally-healthy parenchymatous organ is affected by I/R implying the short-term and remote sequelae [19]. Tohme *et al.* [10] have shown that small doses of DRPs under the experimental conditions can reduce significantly the above I/R pathological effects, thus reducing the impact of surgery on tumor recurrence. The biorheological effect of DRPs is explained by the ability to re-distribute cells of the immune system, which usually infiltrate the endothelium of vascular wall in the microcirculatory bed. In the normal physiological conditions, RBCs preferentially flow in the center of blood vessels, whereas leukocytes and platelets

tend to flow at the marginal plasma layer [20]. The administration of DRPs results in re-distribution of RBCs in the vascular lumen. Such changes allow reducing the interaction of leukocytes, neutrophils and platelets with vascular endothelium receptors after the pathological I/R effect. DRP application decreases hypoxic, pro-inflammatory and pro-tumorigenic effects of the tumor. Neutrophils have shown the ability to participate in the activation of metastatic tissue growth of intestinal adenocarcinoma due to pathological damaging effect of I/R [21]. Platelets can cause the same effect; they start microthrombosis of liver sinusoids, thus initiating the “arrest” of circulating adenocarcinoma cells in the blood and provoking formation of new sites of metastases [22].

In vivo, the effects of DRPs have not been yet sufficiently investigated. It is reported that the nano-concentrations of DRPs enabled reducing blood flow microturbulence in the areas of bifurcation of microvascular bed improving the speed of parietal flow of blood cells [23]. The overall therapeutic effect of DRPs consists in raising blood flow rate, increasing the number of functioning capillaries and improving the volume of microvascular blood flow [24]. The improvement in oxygen delivery and uptake by tissues reduces the damage caused by ischemia and may be advantageous for the reduction of metastasizing.

Our results demonstrate the therapeutic effect of DRPs with decreasing rate of SR generation in liver tissue and decreased oxidative damage of nitrogenous bases in DNA of hepatocytes. The decreased extent of necrotic changes was also recorded with accompanying reduction of microthrombosis level in liver sinusoids and less severe inflammation of ischemic tissues.

To sum up, the warm post-operative I/R promotes the spread of metastatic lesions in the liver with the accompanying increase in the rate of SR generation and oxidatively induced damage of DNA. The hemorheological effects of DRPs results in increased microcirculatory perfusion and oxygenation of liver tissues, with the decrease in the rate of SR generation and oxidatively induced damage. We believe that further experimental and clinical studies of these substances will allow us to reduce the risks of activation of uncontrolled tumor growth in the liver due to the pathological effect of the postoperative I/R.

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