

STUDY OF MODIFYING EFFECTS OF ASTAXANTHIN ON CYTOGENETIC MANIFESTATIONS OF BYSTANDER RESPONSE IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES *IN VITRO*

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Aim: To study the possible impact of astaxanthin on the cytogenetic manifestations of the simultaneous development of radiation-induced (RIBE) and tumor-induced bystander effect (TIBE) in human peripheral blood lymphocytes. **Materials and Methods:** Peripheral blood lymphocytes from healthy persons and patients with chronic lymphocytic leukemia were cultured separately or cocultured with or without previous irradiation *in vitro* by ^{137}Cs at a dose of 0.5 Gy. The cells were cultured with and without 20 $\mu\text{g/ml}$ astaxanthin. **Results:** In the presence of astaxanthin, the decrease of chromosomal instability both in the variant with separate TIBE and with simultaneous development of TIBE and RIBE was observed as the reduction in the frequency of simple chromosome-type aberrations, namely, double fragments. The average level of chromatid-type aberrations did not change under the action of astaxanthin. Although the total chromosome instability in bystander cells diminished, this did not lead to the elimination of the RIBE and TIBE development in the presence of astaxanthin. **Conclusion:** In the setting of experiment, astaxanthin did not reduce the frequency of chromatid-type aberrations in bystander cells due to RIBE and TIBE but reduced the frequency of simple aberrations of chromosomal type, not associated with the development of bystander response phenomenon.

Key Words: astaxanthin, TIBE, B-cell CLL, RIBE, ^{137}Cs γ -quanta, chromosome instability, chromatid-type aberrations, chromosome-type aberrations.

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Radiation-induced and tumor-induced bystander effects (RIBE and TIBE, respectively) represent the manifestations of the universal bystander response phenomenon, namely the response of the normal recipient cells interacting with damaged donor cells. RIBE and TIBE can play negative role in the development of human cancer, contributing, in particular, to the occurrence of secondary malignancies [1–3]. However, particularly dangerous for cancer patients may be their compatible genotoxic impact, which is quite real when using radiation therapy [4–6] and confirmed at the cytogenetic level in the *in vitro* experiments. In particular, TIBE leads to increased manifestations of RIBE due to the synergy between them [7, 8].

Based on the above, it is relevant to find such conditions of therapeutic irradiation of cancer patients that would help to protect normal cells without preventing the destruction of malignant tumors. For this purpose, radioprotectors and radiomitigators of natural or artificial origin are used, the action of which is aimed at the prevention and treatment of acute and long-term effects of ionizing radiation caused by radiation-induced mutagenesis [9, 10].

Since oxidative stress is believed to play a leading role in the mechanism of RIBE and TIBE, one of the promising areas of the research is the study of the ability of certain biologically active substances with powerful antioxidant and antiradical potential to modify

these bystander effects. Mosse *et al.* [11] used melatonin and melatonin to modify RIBE, as they have high antiradical activity. The use of these antioxidants diminished the manifestation of the RIBE phenomenon and improved the survival of HPV-G human keratinocytes treated with medium taken from the cells irradiated at the doses of 0.5 and 1.0 Gy. Konopacka *et al.* [12, 13] in the *in vitro* experiments showed that such free radical and peroxide scavengers as DMSO and vitamin C reduced the frequency of chromosome aberrations and micronuclei and the level of apoptosis in non-irradiated bystander cells. Shemetun *et al.* [3, 14–15] found that at the cytogenetic level the development of RIBE can be modified by the antioxidant composition (water-soluble forms of vitamins E, C, A), the effect of which at a concentration of 40 $\mu\text{g/ml}$ before X-irradiation of target cells — human peripheral blood lymphocytes (PBL) — at a dose of 1.0 Gy eliminated induction of RIBE in bystander cells. However, Olivares *et al.* [16] using micronucleus test in the *in vitro* and *in vivo* experiments did not obtain the protective effect regarding the development of RIBE under the influence of rosemary acid — powerful natural antioxidant that exhibited radioprotective and gene-protective effects in directly X-irradiated mouse target cells.

The aim of our research was to study the possible impact of astaxanthin — naturally occurring powerful antioxidant, carotenoid from the xanthophyll group — on the cytogenetic manifestations of simultaneous RIBE and TIBE development. Radioprotective property of astaxanthin was established by cytogenetic markers of human genome damage in PBL irradiated *in vitro* with gamma quanta ^{137}Cs at a dose of 1.0 Gy, exclusively at the G_0 stage of the cell cycle [17].

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Abbreviations used: CLL – chronic lymphocytic leukemia; PBL – peripheral blood lymphocytes; RIBE – radiation-induced bystander effect; TIBE – tumor-induced bystander effect.

MATERIALS AND METHODS

To determine the background level of spontaneous chromosome aberrations in bystander cells (control), the conventional separate cultivation of human PBL was used; to model the simultaneous development of TIBE and RIBE in the absence and in the presence of astaxanthin our own system was applied — co-cultivation of PBL obtained from individuals of both sexes [3] As a model of malignant cells-inductors the hematopoietic cells from 7 untreated patients (5 males, 2 females) with B-cell chronic lymphocytic leukemia (CLL) — non-irradiated (for TIBE identification) and irradiated *in vitro* to identify TIBE + RIBE were used; as a model of intact cells-bystanders normal PBL of 7 healthy individuals (5 females, 2 males) were applied. All participants denied conscious contact with ionizing radiation and other mutagenic factors. Individuals from both groups participated in voluntary cytogenetic observation after signing the informed consent on the use of their biologic materials for scientific purposes. The study was authorized by the Commission on Bioethical Expertise and Research Ethics of National Research Center for Radiation Medicine of the National Academy of Medical Sciences of Ukraine.

Samples of venous blood obtained from individuals with CLL were irradiated with ^{137}Cs γ -quanta (emitter IBL-237C, power 2.34 Gy/min) at a dose of 0.5 Gy prior to culturing. Astaxanthin (Sigma, USA) was used, which was added before the incubation of the PBL culture (at the early presynthetic stage of the first cell cycle — G_0) to the final concentration of 20 $\mu\text{g/ml}$.

For cytogenetic analysis, heparinized whole blood (~ 0.3 ml from each individual) was cultured by semi-micromethod in our modification. PBL cultures were incubated in RPMI-1640 with L-glutamine (Sigma, USA) without embryonic calf serum and antibiotics, with phytohemagglutinin (PHA, Difco-P, Sigma, USA) for 48 h (the final 2 h with Colcemid, Sigma, USA), which allowed analyzing cells mainly from the first culture mitosis. After hypotonic treatment (0.075 M KCl solution) and fixation (absolute ethanol and glacial acetic acid in a ratio of 3: 1) fixed cell sediments were obtained, which were stored at -20°C until slides of metaphase chromosome were prepared. The slides were stained with Giemsa dye (Merck, Germany) for traditional cytogenetic analysis of uniformly painted chromosomes with group karyotyping. Cytogenetic analysis was performed on encrypted slides at magnification $\times 1000$. In cytogenetic analysis, the all aberrations of chromatid and chromosome types were taken into account.

For each experiment point, the percentage of aberrant metaphases and the frequency of chromosome aberrations per 100 metaphases were calculated. Data for individual points of the experiment were combined into groups according to the study design, followed by the calculation of mean values and statistical errors. The difference between the mean values in the

different study groups and variants was determined. The null hypothesis was tested at a significance level of $p \leq 0.05$ using the Student's *t*-test.

RESULTS AND DISCUSSION

Prior to the research concerning the modification of bystander response phenomenon we confirmed the absence of astaxanthin impact on the level of chromosomal instability in PBL of healthy persons — the mean-group frequency of chromosome aberrations under its action (1.23 ± 0.29 per 100 metaphases) did not differ significantly from that of the intact PBL (1.52 ± 0.30 per 100 metaphases, $p > 0.05$). The individual values of chromosome aberrations frequency in these persons under the astaxanthin exposure ranged from 0.93 to 2.00 per 100 metaphases, which corresponded to the population age norm.

The frequency of chromatid and chromosome type aberrations in the setting of the development of bystander response manifestations are represented in the Figure.

During TIBE manifestation under the co-cultivation of intact PBL with non-irradiated blood cells of patients with CLL in the presence of astaxanthin, the mean frequency of chromosome aberrations in the bystander cells (2.20 ± 0.43 per 100 metaphases) did not differ significantly from the corresponding data under TIBE development without astaxanthin (3.31 ± 0.50 per 100 metaphases, $p > 0.05$). Individual frequencies of chromosome aberrations ranged from (1.98 ± 0.88) to (3.00 ± 1.71) per 100 metaphases. Chromatid-type aberrations, which are considered as markers of chromosomal instability, were represented only by single fragments with mean frequency (1.86 ± 0.39 per 100 metaphases) that exceeded the corresponding control value (0.96 ± 0.26 per 100 metaphases, $p < 0.01$), indicating the induction of TIBE in human intact PBL co-cultured with blood cells of patients with CLL even in the presence of astaxanthin. However, this value did not differ from the level of chromatid-type aberrations under the development of TIBE in the absence of astaxanthin (2.32 ± 0.42 per 100 metaphases, $p > 0.05$).

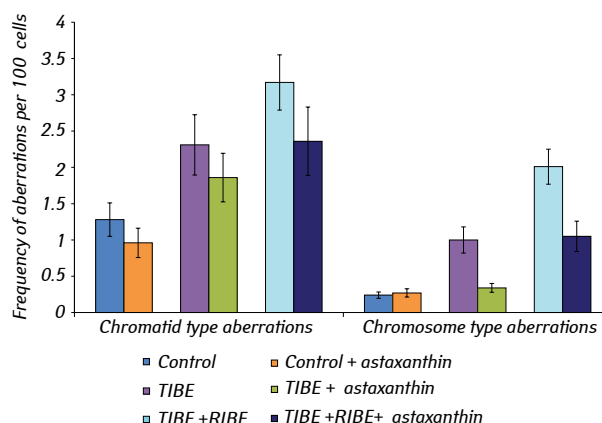


Figure. Frequency of chromatid and chromosome type aberrations under development of bystander response manifestations in different experimental variants

Chromosome-type aberrations were represented exclusively by double fragments, which mean frequency (0.34 ± 0.17 per 100 metaphases) did not differ from the control value (0.18 ± 0.11 per 100 metaphases, $p > 0.05$), but were significantly lower than the corresponding value under the development of TIBE without astaxanthin (0.92 ± 0.27 per 100 metaphases, $p < 0.01$).

In the variant of the experiment “TIBE + RIBE + astaxanthin”, which modeled the impact of astaxanthin on the simultaneous development of both manifestations of bystander response phenomenon, the mean frequency of chromosome aberrations (3.41 ± 0.54 per 100 metaphases) was lower ($p < 0.01$) than in the variant of the experiment “TIBE + RIBE” without astaxanthin (5.18 ± 0.51 per 100 metaphases), however, exceeded the control values in the bystander cells ($p < 0.01$) under their separate cultivation without astaxanthin (1.52 ± 0.30 per 100 metaphases) and with it (1.23 ± 0.29 per 100 metaphases).

Comparative analysis of the registered chromosome aberrations spectra showed that under the effect of astaxanthin the decrease of chromosomal instability both in the variant with separate TIBE development and with simultaneous development of TIBE and RIBE was due to reduced frequency of simple chromosome-type aberrations, namely — double fragments (by 63% and 53% respectively). The average level of chromatid-type aberrations (markers of bystander response development), decreased by 20% and 26%, respectively, which is similar to the control value (25%). Thus, although the total chromosome instability in bystander cells decreased by 34%, it did not eliminate the development of TIBE and RIBE, which are characterized by increased levels of chromatid-type aberrations, the frequency of which did not change under the action of astaxanthin ($p > 0.05$).

Since the leading factor in the development of bystander response is the effect of oxidative stress which at cytogenetic level causes the induction of chromatid-type aberrations, it can be assumed that astaxanthin had no antioxidant impact on the studied cells. However, the data obtained indicate that the presence of astaxanthin in co-cultures of PBL from healthy individuals (cells-bystanders) with non-irradiated (TIBE) or irradiated *in vitro* (TIBE + RIBE) blood cells of patients with CLL (cells-inductors) is capable to influence the level of chromosomal instability in bystander cells, possibly, via other mechanisms [18].

In conclusion, co-cultivation of normal human PBL with non-irradiated or irradiated *in vitro* blood cells of patients with CLL in the presence of astaxanthin did not reduce the frequency of chromatid-type aberrations in bystander cells induced by oxidative stress during the development of TIBE and RIBE, however affected the level of chromosomal instability in bystander cells by reducing the frequency of simple

aberrations of chromosomal type, not associated with the development of these phenomena.

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ДОСЛІДЖЕННЯ МОДИФІКАЦІЇ АСТАКСАНТИНОМ ЦИТОГЕНЕТИЧНИХ ПРОЯВІВ ЕФЕКТУ СВІДКА *IN VITRO* В ЛІМФОЦИТАХ ПЕРИФЕРИЧНОЇ КРОВІ ЛЮДИНИ

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Мета: Дослідити можливий вплив астаксантину на цитогенетичні прояви одночасного розвитку радіаційно- та пухлинно-індукованого ефекту свідка в лімфоцитах периферичної крові людини. **Матеріали та методи:** Лімфоцити периферичної крові здорових людей та хворих на хронічну лімфоцитарну лейкемію культивували окремо або разом після попереднього опромінення *in vitro* ^{137}Cs в дозі 0,5 Гр

або без попереднього опромінення. Клітини культивували за наявності астаксантину в дозі 20 мкг/мл або без нього.

Результати: За наявності астаксантину відзначено зниження частоти простих аберацій хромосомного типу (парних фрагментів) як при розвитку пухлинно-індукованого ефекту свідка, так і за одночасного розвитку радіаційно- та пухлинно-індукованого ефекту свідка. Середній рівень аберацій хроматидного типу статистично значуще не змінювався.

Висновки: Застосування астаксантину не запобігало розвитку радіаційно- та пухлинно-індукованого ефекту свідка, оскільки не спричиняло зниження частоти аберацій хроматидного типу, що утворюються внаслідок дії оксидативного стресу при розвитку ефекту свідка і є маркерами його індукції. Разом з тим встановлено, що астаксантин знижує рівень хромосомної нестабільності в клітинах-свідках за рахунок зменшення частоти простих аберацій хромосомного типу, що не асоційовано з розвитком ефекту свідка.

Ключові слова: астаксантин, радіаційно-індукований ефект свідка, пухлинно-індукований ефект свідка, В-клітинна хронічна лімфоцитарна лейкемія, хромосомна нестабільність, аберації хроматидного типу, аберації хромосомного типу.