We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

Our authors are among the

154
Countries delivered to

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Chapter 7

Astaxanthin as a Modifier of Genome Instability after γ-Radiation

Denys Kurinnyi, Stanislav Rushkovsky,
Olena Demchenko and Mariya Pilinska

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.79341

Abstract

The chapter is devoted to study the effects of astaxanthin on the frequency of chromosomal aberrations and the level of DNA damages in human peripheral blood lymphocytes under ionizing radiation exposure in vitro. To achieve the purpose of the research, a combination of classical cytogenetic methods (G_0- and G_2-radiation sensitivity assays) and method of single-cell electrophoresis (comet assay) was used. The specificity of the modifying effect of astaxanthin on radiation-induced genomic injuries depending on the stage of the cell cycle had been determined. Significant weakening of the negative effect of ionizing radiation on the G_0 stage and the absence of a radioprotective effect on the S and G_2 stages of the cell cycle may be associated with activation by astaxanthin of apoptosis in irradiated cells with a critically high level of the genome damages. The research results not only testify about strong radioprotective effect of astaxanthin but also demonstrate the feasibility of the parallel use of cytogenetic and molecular genetic methods to assess the impact as mutagens as well as factors that modify the effect of mutagens on genome stability.

Keywords: astaxanthin, lymphocytes, γ-radiation, DNA breaks, chromosomal aberrations

1. Introduction

The ecological situation that arose from nuclear accidents in Chornobyl and Fukushima, constant expansion of usage of the ionizing radiation in industry and medicine, and the threats of nuclear terrorism especially aggravated in the last decade are risk factors for the growth of radiation burden on human populations. The abovementioned conditions require the search

IntechOpen

© 2018 The Author(s). Licensee IntechOpen. This chapter is distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. CC BY

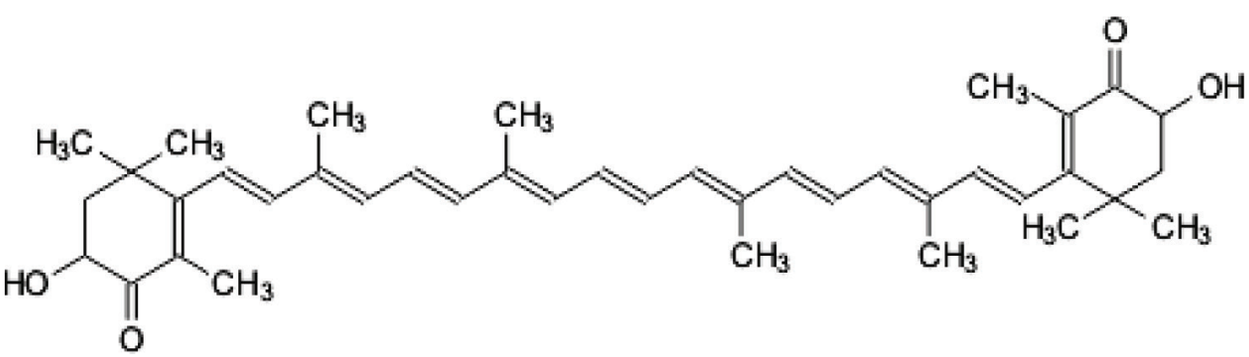
for new safe and effective radioprotectors, preferably of natural origin, for prevention and treatment of radiation-induced damages in humans, especially which cause genome alterations and cancer. For that purpose, carotenoids, due to its chemical and biological properties, are the most promising substances [1].

Astaxanthin is a carotenoid of xanthophyll group, and it is one of the most common red pigments of algae, yeasts, krill, shrimps, crayfish, trout, and salmon [2]. It is known that astaxanthin is the most powerful antioxidant, which has the ability to scavenge free radicals in tens of times higher than α-tocopherol or β-carotene [3], and has anti-inflammatory [2], immunomodulating [4], and anticarcinogenic [5–7] effects.

Since 2015, we have started the investigation of the radioprotective effects of astaxanthin studying parameters of genome damages in human somatic cells. In this chapter, we have concentrated on physicochemical properties of astaxanthin and its biological effects with the main focus on the data from our investigations concerning the impact of astaxanthin on radiation-induced genome damages in human somatic cells and have discussed eventual mechanisms of its action.

2. Physicochemical properties and peculiarities of biological action of astaxanthin

Astaxanthin is a secondary carotenoid, which belongs to the group of xanthophylls and has two additional oxygen atoms on each benzene ring in comparison with β-carotene. This gives astaxanthin a rich red color and greatly increases its antioxidant properties. Unlike β-carotene, astaxanthin is not a vitamin A precursor [8].



Empirical formula: $C_{40}H_{52}O_4$

Molar weight: 596.84 g/M

In contrast to primary carotenoids, which are associated with the structural and functional components of the photosynthetic apparatus, secondary carotenoids, which include astaxanthin, are in the cell in oil droplets, and their main function is to form a protective layer to prevent the damages, which are provoked by stress conditions [9, 10].

Because the astaxanthin molecule contains conjugated double bonds, hydroxyl and keto groups, it has both lipophilic and hydrophilic properties [11]. Astaxanthin has two chiral centers and can exist in three different stereoisomers—3S, 3′S; 3R, 3′S; and 3R, 3′R. The probability of obtaining these isomers of astaxanthin in the process of chemical synthesis is 1:2:1 [12, 13].

Nowadays natural astaxanthin mainly derived from microalgae (hyperproducer *Haematococcus pluvialis*), yeast (*Phaffia rhodozyma*) and animal-consumers included a number of small marine crustaceans (Euphausiacea) and the salmon family (Salmonidae) [2]. Microalgae *Haematococcus pluvialis* produces astaxanthin mainly 3S, 3′S stereoisomeric form; precisely, such molecular structure is considered the most valuable [14].

As shown in experiments *in vitro*, astaxanthin effectively protects cells from nonspecific oxidation by quenching singlet oxygen, effectively inhibits lipid peroxidation in biological samples, and owing to the capture of free radical prevents or stops the chain reaction of oxidation [2, 15, 16]. In addition to direct protective effect, astaxanthin inhibits the activation of the H_2O_2-mediated transcription of the factor NF-kB (the nuclear factor "kappa-b"—a universal transcription factor) that controls the expression of heme oxygenase 1 (HMOX1), one of the markers of oxidative stress, and nitric oxide synthase (iNOS) [17, 18]. Astaxanthin blocks the cytokine production declined by modulating the expression of protein tyrosine phosphatase 1 [18].

Experiments on the determination of astaxanthin toxicity showed a high level of safety—LD_{50} was not established after single administration of substance to rats. The studies confirmed the absence of histopathological changes and the dose-effect dependence upon oral administration of astaxanthin in doses ranging from 4.161–17.076 to 465.0–557.0 mg/kg per day [19].

The accumulated published data have shown the multifaceted positive effect of astaxanthin in mammals by reducing the manifestations of oxidative stress, including during inflammation processes; it can prevent the development of atherosclerotic cardiovascular diseases and participate in the regulation of lipid and glucose metabolism [19–23].

These properties of astaxanthin primarily attributed to its ability to exhibit activity both at the level of the cell membrane and in the area of the cytoplasm, thus affecting the flow of intracellular processes [2]. Due to these unique properties, astaxanthin exhibits significantly higher biological activity in comparison with other antioxidants [24].

Thus, the above data indicate that astaxanthin complies with all the requirements that apply to radioprotectors (low toxicity, high antiradical and antioxidant activity, the ability to act both at the membrane level and in the intracellular space). These properties of astaxanthin suggest that it may have antimutagenic activity and, as consequence, radioprotective effect on the human genome.

3. Investigation of radioprotective properties of astaxanthin

Since 2015, we examined the possibility of modification by astaxanthin and the negative effects of ionizing radiation on the human blood lymphocyte genome in vitro. The decrease in the intensity of radiation-induced genome damages on the chromosomal and molecular

levels was selected as an indicator of radioprotective effect of astaxanthin. The studies were conducted using a combination of the methods of classical cytogenetic analysis (G_0-radiation sensitivity assay and G_2-radiation sensitivity assay) and the method of single-cell electrophoresis (comet assay) [25–29].

The parallel application of two methodological approaches for such a study greatly expanded the experimental possibilities. Thus, due to cytogenetic methods, the state of the chromosomal apparatus of the cell (frequency of different types of chromosome aberrations) is clearly visualized starting from the 48 h of cultivation. The comet electrophoresis is highly sensitive and provides the ability to determine the relative levels of single- and double-strand DNA breaks in individual cell. When conducting cell electrophoresis, the DNA migrates into the agarose gel, forming a structure that resembles a comet (**Figure 1**), and the use of the comet assay can simultaneously estimate the effect of both mutagenic and antimutagenic factors on the stability of the human somatic cell genome, starting from 0 h of cultivation [30, 31]. In addition, the use of single-cell electrophoresis makes it possible to determine the effectiveness of the reparation systems and to assess the correctness of the operation of control mechanisms at checkpoints between all stages of the cell cycle (G_1–S, S–G_2, G_2–M). Moreover, an important feature of the comet assay is the identification of cells in which the apoptosis program has begun or has already been implemented [32–34].

In cells with a lack or a low level of damages, the "tail" is formed also by the release of DNA loops into the gel. Because in the cell during realization of the apoptotic process genomic fragmentation of the high level occurs, a massive yield of DNA fragments into agarose gel is observed (**Figure 1**), and "comets" have the typical elongated "tail" part.

To quantify the migration of DNA into the agarose gel, two indices are used: the percentage of DNA in the "tails" and tail moment (TM). TM simultaneously which takes into account both the

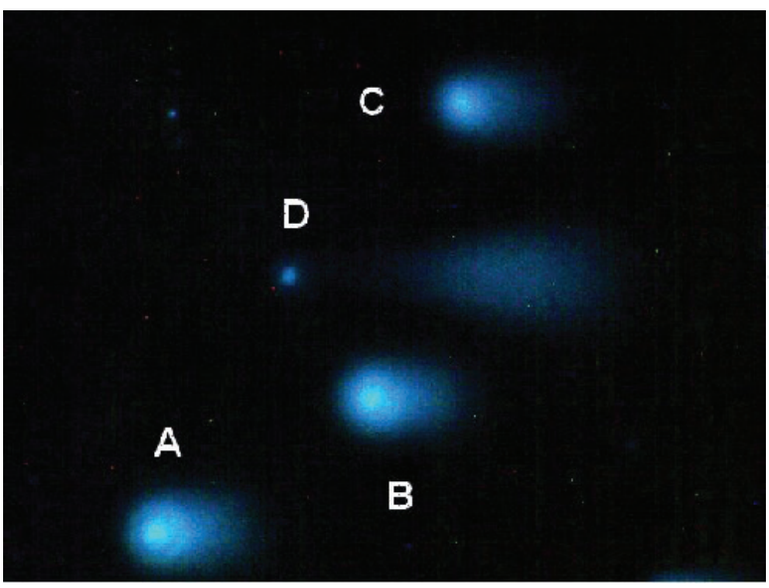


Figure 1. Examples of "comets" obtained in the experiment: (A, B, C) The "comets" arisen from cells with a low level of DNA breaks and (D) "atypical comet" (apoptotic cell) [28].

amount of DNA and the length of the "tail" (TM = "tail" length multiplied by the percentage of DNA in the "tail") is more informative and calculated automatically during the computer analysis.

3.1. The impact of astaxanthin on the level of radiation-induced chromosomal aberrations in human lymphocytes

To evaluate the possible mutagenic activity of astaxanthin, it was tested at concentrations of 2.0, 10.0, 20.0, and 40.0 μg/ml in the culture of human peripheral blood lymphocytes. In the cytogenetic assay, it was found that the frequencies of aberrant cells and the levels of chromosomal aberrations under the astaxanthin exposure in vitro in all tested concentrations did not differ from the corresponding background cytogenetic parameters ($p > 0.05$) [25].

To determine the optimal working concentration of astaxanthin for further research of its radiomodifying capacity, a pilot study of its impact on the culture of human peripheral blood lymphocytes is exposed in vitro to gamma quanta in a dose of 1.0 Gy on G_0 phase of the first mitotic cycle (**Figure 2**).

It is established that astaxanthin in all tested concentrations significantly ($p < 0.01$) reduced the frequencies of radiation-induced chromosome aberrations, but the effectiveness of its modifying action depended on its concentration in the irradiated culture.

The maximum radioprotective effect of astaxanthin (the most effective drop in the frequency of cytogenetic markers of radiation exposure) was observed after administration of astaxanthin before irradiation of cultures at concentrations of 20.0 and 40.0 μg/ml (7.69 ± 1.74 and 7.72 ± 1.80 per 100 cells, respectively). These concentrations did not affect the mitotic activity of the lymphocyte culture, had no mutagenic effect on non-irradiated cells, and effectively (to ~ 70%) reduced the level of aberrant metaphases and the frequency of cytogenetic markers of radiation exposure. So long as significant difference between the values that characterize carotenoid activity in these concentrations ($p > 0.05$) was not observed, for the further studies of the radiomodifying capacity of astaxanthin, the concentrations of 20.0 μg/ml were chosen.

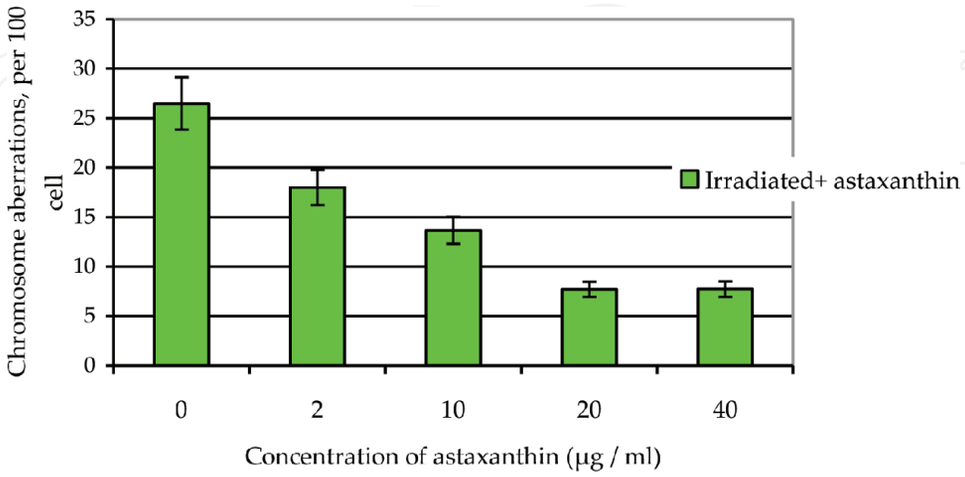


Figure 2. Selection of the optimal concentration of astaxanthin to study its modifying effect on the γ-irradiated culture of human blood lymphocytes.

To analyze the possible dependence of radioprotective properties of astaxanthin from the stage of the mitotic cycle on which the cells were exposed to ionizing radiation, lymphocyte cultures were irradiated at 0, 40, and 46 h, corresponding to G_0, S, and G_2 stages of the first cell cycle. Astaxanthin was added to cultures of lymphocytes at least an hour before irradiation. The obtained data are presented in the **Table 1**.

After irradiation of lymphocyte culture in a dose of 1.0 Gy on the G_0 stage of the cell cycle, the effect of astaxanthin resulted in a significant reduction of the radiation-induced cytogenetic effect, namely, a decrease of almost in 3.5 times both the mean frequency of the aberrant metaphases and the level of chromosome aberrations—up to 7.82 ± 0.72% and 8.48 ± 0.75 per 100 cells, respectively—and exclusively due to aberrations of chromosome type (**Table 1**). The antimutagenic activity of astaxanthin was characterized by significant ($p < 0.001$) decrease in the frequency of classical unstable cytogenetic markers of radiation exposure—dysenteric and ring chromosomes (up to 2.37 ± 0.41 and 0.43 ± 0.18 per 100 metaphases, respectively), as well as the total level of simple acentrics—free double fragments, and acentric rings (up to 4.74 ± 0.62 per 100 metaphases) (**Table 1**, **Figure 3**).

In contrast to the modifying activity shown by astaxanthin in lymphocyte cultures irradiated on the G_0 phase of the cell cycle, the addition of carotenoid on the G_2 phase did not change as the total average frequency of radiation-induced chromosomal damages (72.35 ± 1.17 and 71.54 ± 1.34 per 100 metaphases, respectively, $p > 0.05$) as the spectrum of chromosome aberrations (**Figure 4**). Among chromosomal damages, dominated aberrations of chromatid type represented by single fragments and chromatid exchanges with the total average frequency 58.42 ± 1.47 per 100 metaphases did not differ from such (58.32 ± 1.34 per 100 metaphases) in exposed cultures without adding astaxanthin. Aberrations of the chromosome type were mainly represented by free double fragments; the average group frequencies of it did not differ between themselves (13.12 ± 1.00 and 14.03 ± 0.91 per 100 metaphases, respectively).

In much the same way, astaxanthin did not exhibit modifying effect on radiation-induced cytogenetic effects in lymphocyte cultures irradiated on the S stage of the cell cycle. The total mean group frequencies of radiation-induced chromosomal damages were 19.57 ± 1.11 and 18.46 ± 1.15 per 100 metaphases in exposed cultures without and with the previous addition of astaxanthin, respectively. Among the chromosomal damages, simple aberrations prevailed (single and double fragments) in both variants of the experiment (**Table 1**, **Figure 4**).

Thus, due to the use of cytogenetic methods, the following important aspects of the astaxanthin modifying action were established:

1. The effectiveness of astaxanthin has a dependence on the stage of the cell cycle on which lymphocytes were irradiated.
2. The radioprotective effect of astaxanthin is realized in cells exposed only on G_0 stage of the mitotic cycle which manifests in lowering the frequency of chromosome-type aberrations for the induction of which a large number of double-stranded DNA breaks as the error of repairing of such damages are needed, which permit to suggest the impact of carotenoid on cells with the high level of genomic instability.

	Frequency of the aberrant Metaphases	Chromosome aberrations (per 100 cell)	Frequency of chromosome aberrations								
			Chromatid type			Chromosome type					
			Single fragments	Chromatid exchanges	Total	Double fragments	Dysenteric	Centric rings	Abnormal monocentric	Acentric rings	Total
Unirradiated culture	2.52 ± 0.34	2.57 ± 0.35	1.60 ± 0.28	0.00	1.60 ± 0.28	0.96 ± 0.21	0.00	0.00	0.01 ± 0.01	0.00	0.97 ± 0.22
G_0 (1.0 Gy)	22.93 ± 1.19	24.55 ± 1.22	1.54 ± 0.35	0.00	1.54 ± 0.35	6.47 ± 0.70	12.80 ± 0.95	2.76 ± 0.47	0.49 ± 0.20	0.49 ± 0.20	23.02 ± 1.20
G_0 (1.0 Gy + A)	7.82 ± 0.72	8.48 ± 0.75	0.72 ± 0.23	0.00	0.72 ± 0.23	4.67 ± 0.57	2.37 ± 0.41	0.43 ± 0.18	0.22 ± 0.13	0.07 ± 0.07	7.76 ± 0.71
S (1.0 Gy)	18.30 ± 0.97	19.57 ± 1.11	7.94 ± 0.67	2.27 ± 0.33	10.21 ± 0.37	9.36 ± 0.67	0.00	0.00	0.00	0.00	9.36 ± 0.67
S (1.0 Gy + A)	16.92 ± 1.12	18.46 ± 1.15	6.15 ± 0.67	2.30 ± 0.33	8.45 ± 0.67	10.01 ± 1.0	0.00	0.00	0.00	0.00	10.01 ± 1.0
G_2 (1.0 Gy)	47.06 ± 1.31	72.35 ± 1.17	56.04 ± 1.30	2.28 ± 0.39	58.32 ± 1.29	13.76 ± 0.90	0.27 ± 0.51	0.00	0.00	0.00	14.03 ± 0.91
G_2 (1.0 Gy + A)	46.72 ± 1.48	71.54 ± 1.34	56.47 ± 1.47	1.95 ± 0.41	58.42 ± 1.47	12.94 ± 1.0	0.18 ± 0.41	0.00	0.00	0.00	13.12 ± 1.0

Table 1. Comparison of the mean group values of cytogenetic parameters in irradiated in vitro in dose 1.0 Gy human lymphocyte cultures on G_0, S, and G_2 stages of the cell cycle and under the joint action of γ-radiation and astaxanthin in concentration 20.0 μg/ml.

http://dx.doi.org/10.5772/intechopen.79341

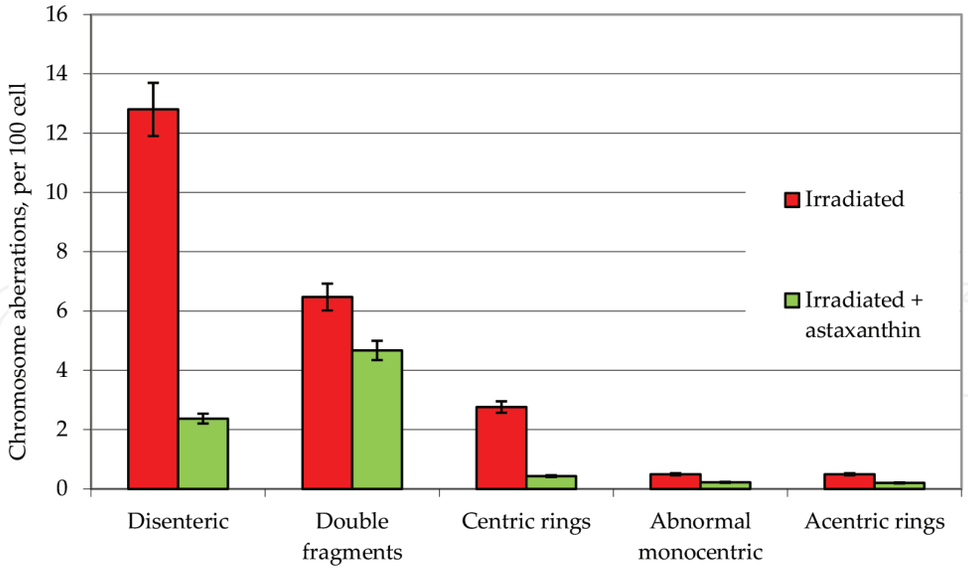


Figure 3. Change in the frequency and spectrum of chromosome-type aberrations under joint action of astaxanthin in the concentration of 20.0 μg/ml and γ-radiation in a dose of 1.0 Gy on the G_0 stage of the cell cycle.

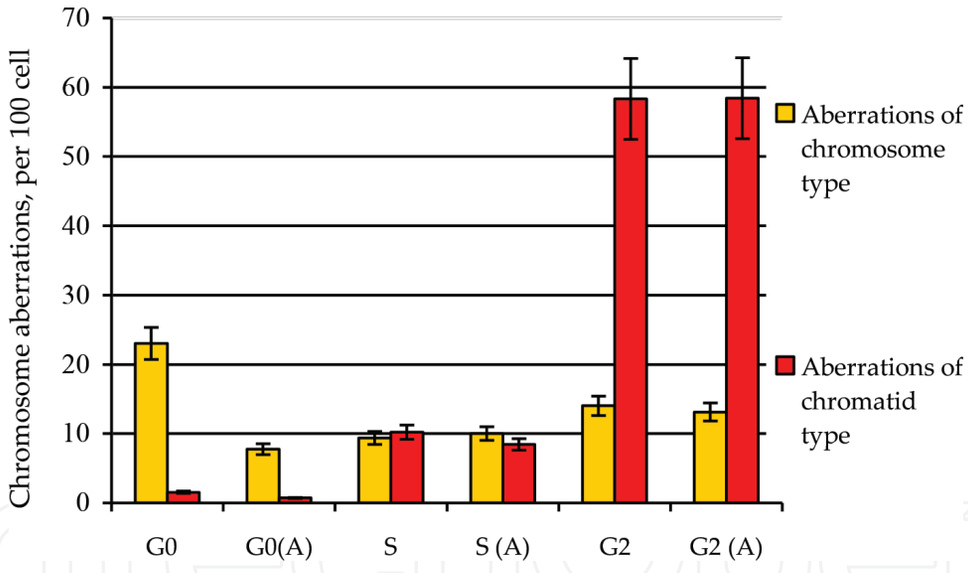


Figure 4. Frequencies and spectra of radiation-induced chromosome aberrations under γ-radiation exposure in dose of 1.0 Gy in vitro and astaxanthin in concentration of 20.0 μg/ml on different stages of the cell cycle. G_0, S, and G_2 (cell cultures irradiated without astaxanthin) and G_0 (A), S(A), and G_2(A) (cell cultures irradiated with supplemented 20.0 μg/ml astaxanthin).

3.2. The impact of astaxanthin on the level of DNA damages in human lymphocytes

For evaluation of the relative level of DNA damages (single- and double-strand DNA breaks), the method of single-cell gel electrophoresis (comet assay) in neutral condition was used. As a parameter of DNA breakage, the TM computed as the %DNA in the comet tail multiplied by the tail length was chosen. For comet assay, we used peripheral blood lymphocytes without

culturing (0 h) and from 48 human-PBL cultures. Some cultures were exposed to γ-ray (emitter IBL-237C, dose rate 2.34 Gy/min) in dose 1.0 Gy at 0, 40, and 46 h of cultivation. Non-irradiated cultures were used as experimental control. Those times were chosen by the reason that lymphocytes, which we can see after 48 h of cultivation on their metaphase stage, are at G_0 (0 h), S (40 h), and G_2 (46 h) phases of the cell cycle. Astaxanthin in the final concentration 20.0 μg/ml, which was defined during our cytogenetic study, was added to the cultures of lymphocytes before irradiation.

Similarly to our cytogenetic data, no significant changes in DNA breakage were detected in non-irradiated samples supplemented with astaxanthin compared with untreated lymphocytes both after 0 and 48 h of cultivation (**Table 2**). This confirms our suggestion that astaxanthin in chosen concentration has no mutagenic activity.

As can be seen from the **Table 2** and **Figure 6**, after γ-irradiation of lymphocytes in dose 1.0 Gy at G_0 phase of the cell cycle, significant increasing in TM was detected (from 2.80 ± 0.54 to 6.55 ± 1.82, $p < 0.05$ and from 4.07 ± 0.60 to 12.86 ± 0.74, $p < 0.05$, after 0 and 48 h of cultivation, respectively).

The effect of astaxanthin on irradiated cells manifested in significant ($p < 0.001$) decrease in the average level of DNA damages in lymphocytes from cultures irradiated at G_0 nearly to the value of non-irradiated control both after 0 and 48 h of cultivation (TM = 3.74 ± 0.82 and 5.27 ± 1.77, respectively) (**Figure 5**).

As expected, significant increase in the level of DNA breaks was detected in lymphocytes after γ-irradiation at 40 h of cultivation (**Table 3**). The mean value of TM was equal to 7.45 ± 0.36 in irradiated and 4.07 ± 0.60 in lymphocytes from intact cultures ($p < 0.01$). Astaxanthin in concentration 20 μg/ml significantly ($p < 0.01$) decreased the DNA damages in lymphocytes from cultures irradiated at 0 and 40 h of incubation nearly to the level of non-irradiated control (TM = 5.27 ± 1.77 and 4.79 ± 0.23, respectively). The treatment of cells with astaxanthin resulted in statistical significant decrease of radiation-induced DNA damages (TM = 3.21 ± 0.48, $p < 0.05$ compared with irradiated samples) likewise after irradiation of lymphocytes at G_0 phase of the cell cycle.

Similar results were obtained after treatment at 46 h of incubation (**Table 4**). Irradiation of lymphocyte cultures at G_2 phase of the cell cycle led to a large amount of DNA breaks and, as outcome, to material increase in TM value (12.06 ± 1.88, $p < 0.001$). The effect of astaxanthin

Treatment	0 h	48 h
	Tail moment (X ± Se)	Tail moment (X ± Se)
Control	2.80 ± 0.54	4.07 ± 0.60
Supplementation with astaxanthin	3.55 ± 1.37	5.93 ± 0.93
Irradiation	6.55 ± 1.82	12.86 ± 0.74

Table 2. The impact of γ-irradiation at G_0 phase and astaxanthin supplementation on DNA damages in human lymphocytes after 0 and 48 h of cultivation.

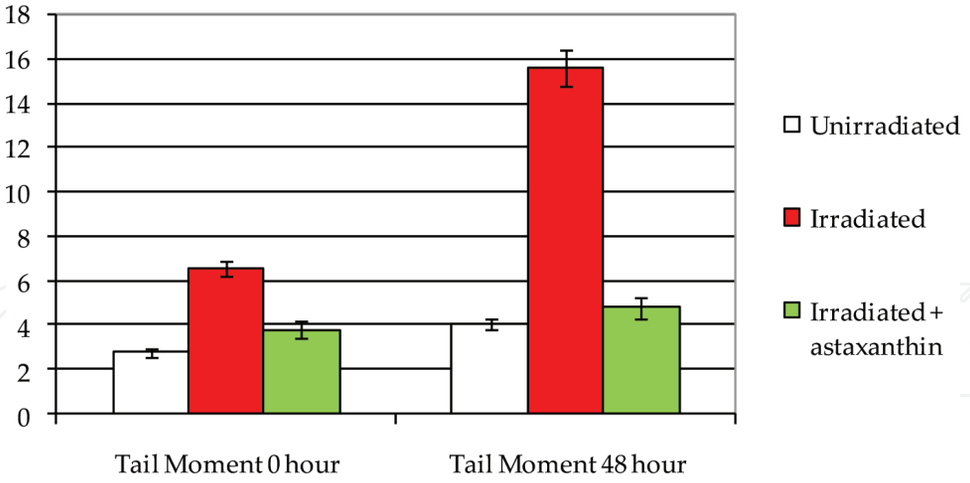


Figure 5. The relative levels of DNA damages irradiated at G_0-phase human lymphocytes non-supplemented or supplemented with astaxanthin cultures.

Treatment	Tail moment	
	X	±Se
Control	4.07	0.60
Control + astaxanthin	5.93	0.93
Irradiation at S phase	7.45	0.36
Irradiation at S phase + astaxanthin	4.79	0.23

Notes: X, mean value; Se, standard error.

Table 3. The tail moment values in human blood lymphocytes after γ-radiation exposure and under combined action of radiation and astaxanthin at S phase of the cell cycle.

Treatment	Tail moment	
	X	±Se
Control	4.07	0.60
Control + astaxanthin	5.93	0.93
Irradiation at G_2 phase	12.06	1.88
Irradiation at G_2 phase + astaxanthin	8.96	2.39

Notes: X, mean value; Se, standard error.

Table 4. The tail moment values in human blood lymphocytes after γ-radiation exposure and under combined action of radiation and astaxanthin at G_2 phase of the cell cycle.

supplementation was somewhat not only weaker than in previous experiments but also significant compared to cultures irradiated on G_2 stage (TM = 8.96 ± 2.39 and 12.06 ± 1.88, respectively, $p < 0.05$).

Our data suggested that astaxanthin decreased the rate of radiation-induced DNA breaks in human lymphocytes regardless of the phase of the cell cycle when the irradiation was performed. However, this conclusion is not consistent with our cytogenetic results: it was observed that astaxanthin is able to decrease frequency of radiation-induced chromosome aberration only if cells were irradiated at G_0 phase of the cell cycle.

For more detailed analysis, we have studied the frequency distribution of individual cells depending on their levels of DNA damages. According to TM, the sampling of "comets" from control variants was divided into ten groups of 10% each. The established values of deciles (TMs were 0.81, 1.28, 1.81, 2.69, 3.80, 5.07, 6.48, 10.19, 15.98) were chosen as boundary indices to form ten groups of cells from irradiated cultures treated or not by astaxanthin and to estimate percentage of "comets" that have TM within the appropriate range. If the value of TM was equal to the boundary index, then "comet" was referred to the next group. The results are shown on **Figure 6**.

When lymphocytes were irradiated at the G_0 phase of the cell cycle, after 48 h of incubation, the increase in the average TM level was caused exclusively by growth of the frequency of the "comets" from the tenth group (TM > 15.98) (**Figure 6A**), which indicates accumulation of cells with a large number of DNA damages with time. Irradiation at the 40th hours of cultivation (**Figure 6B**) resulted in increased levels of the "comets" that belonged to groups 8 and 9 (TM from 6.48 to 15.98). After radiation exposure at 46 h of incubation (**Figure 6C**), the increment of last three groups of "comets" (TM > 6.48) was observed.

It is noteworthy that γ-radiation exposure at 40 and 46 h of incubation did not cause decrease in the frequency of group 1, which includes the "comets" with the smallest DNA release into the "comet" tail (TM from 0 to 0.81). Probably, this situation reflects not so much on the existence in lymphocyte cultures of the populations of radiation-resistant and/or fully recovered cells, as the presence of heavily damaged cells in which the checkpoint has acted on the S phase of the cell cycle, because if the cells are in this phase, then significant decrease of DNA exit under the neutral conditions of electrophoresis is observed [35, 36]. This opinion is confirmed by the lack of increase in frequency of "comets" from the tenth group after radiation exposure at 40 h of cultivation: most of blast-transformed lymphocytes must be on S phase, and cells with the very high level of DNA damages cannot pass S/G_2 checkpoint, and, as a result, they are delayed on this phase.

The supplementation with astaxanthin resulted in significant reduction in the levels of "comets" that belonged only to the ten groups after irradiation at 0 (from 25.07 ± 2.25 to 8.96 ± 1.74%, $p < 0.001$) and at 46 h of cultivation (from 22.38 ± 1.77 to 10.45 ± 1.18%, $p < 0.001$) and groups 9 and 10 after radiation exposure at 40 h of incubation (from 16.56 ± 1.72 to 6.69 ± 1.06, $p < 0.001$ and from 8.60 ± 1.30 to 3.25 ± 0.75%, $p < 0.01$, respectively).

It is known that astaxanthin reveals apoptotic activity in experiments with different cultures of cancer cells [6, 33]. In our studies, the decrease in the frequency of highly damaged cells as a result of astaxanthin treatment may also be caused by activation of apoptotic processes.

The comet assay allowed not only estimating the relative level of DNA damages but also determining the intensity of apoptotic processes [37, 38]. For this purpose simultaneously

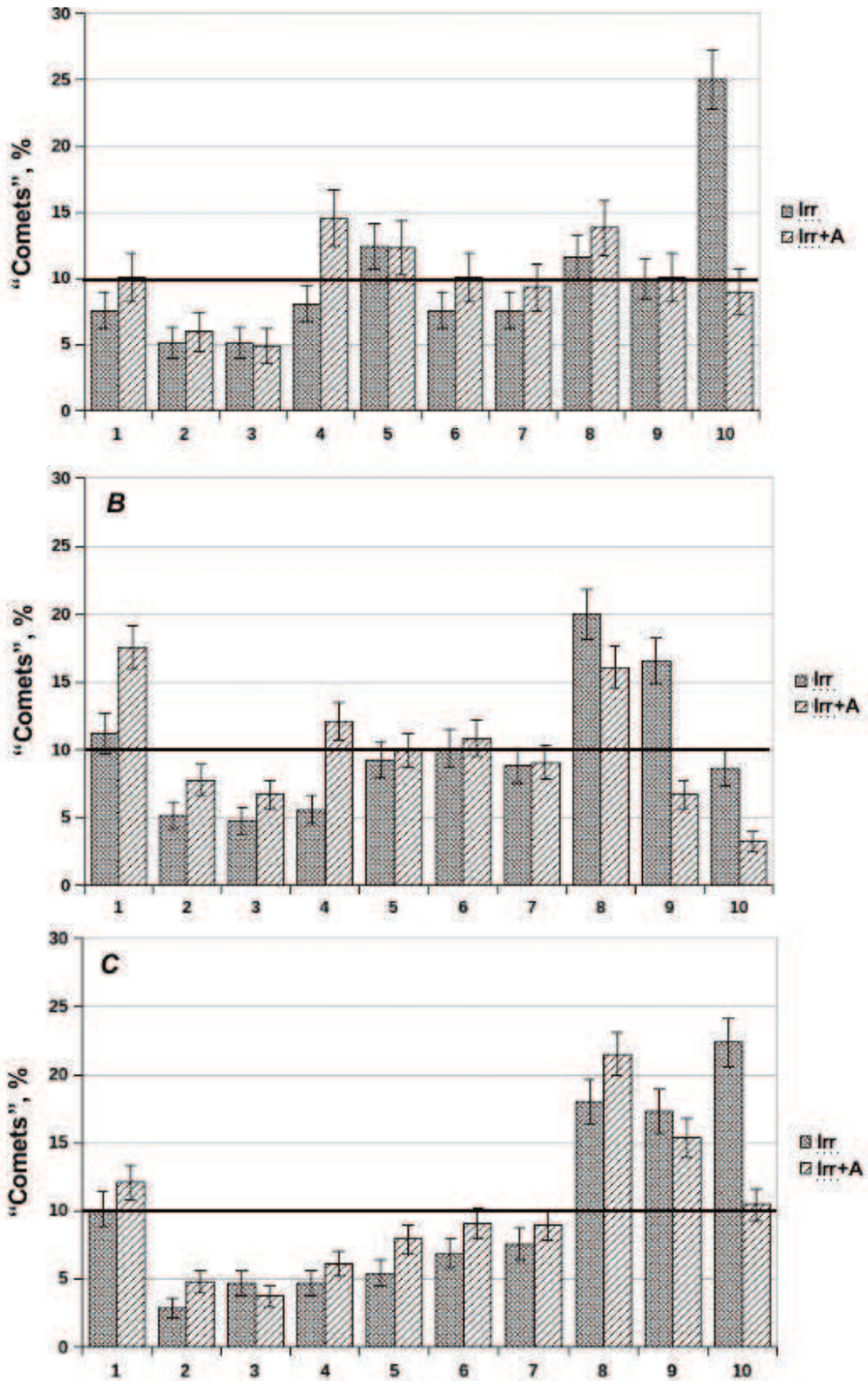


Figure 6. The frequency distribution of "comets" according to the relative levels of DNA damages (see explanation in the text) after irradiation at the 0th hour of cultivation (A), at the 40th hour of cultivation (B), and at the 46th hours of cultivation (C). Irr, irradiated in dose 1.0 Gy cultures; Irr + A, irradiated and supplemented with 20.0 μg/ml astaxanthin cultures. In one to ten groups of "comets," 10% (bold line) is the control value for all groups [28].

with the evaluation of the levels of DNA damages, the count of "atypical comets" (AC) (**Figure 7**) was carried out. It is obvious that AC were formed from the apoptotic cells, because the radiation dose we applied is quite low and cannot induce DNA fragmentation like this [39], while intensive DNA fragmentation occurs exactly during apoptosis [33].

In control cultures after 48 h of cultivation, the AC level was low and did not exceed 1.45 ± 0.53%. The irradiation of lymphocyte cultures at the G_0 stage of the cell cycle with further cultivation led to an increase in the frequency of AC from to 3.11 ± 0.71% ($p < 0.05$), but such effect was not observed after radiation exposure neither at the 40th hour nor at the 46th hours of cultivation (**Figure 7**).

Astaxanthin in concentration of 20 μg/ml per se did not affect the amount of apoptotic cells in non-irradiated cultures of lymphocytes, but the AC level irradiated and treated by astaxanthin cultures was approximately in four times higher than with 48-hour control (7.15 ± 1.13 and 1.69 ± 0.56%, respectively, $p < 0.01$) and in two times higher than with irradiated samples (7.15 ± 1.13 and 3.57 ± 0.81%, respectively, $p < 0.05$). The increase in the frequency of apoptotic cells under the impact of astaxanthin was established exclusively in cultures irradiated at the G_0 stage of the cell cycle and not observed after irradiation in other terms of cultivation.

Thus, similar to cytogenetic effect, the apoptotic activity of astaxanthin was detected only when the irradiated cells were on the G_0 phase of the cell cycle. This may be the cause of the elimination of cells with a large number of DNA breaks and, as consequence, the reduction of the radiation-induced level of chromosomal aberrations we observed earlier.

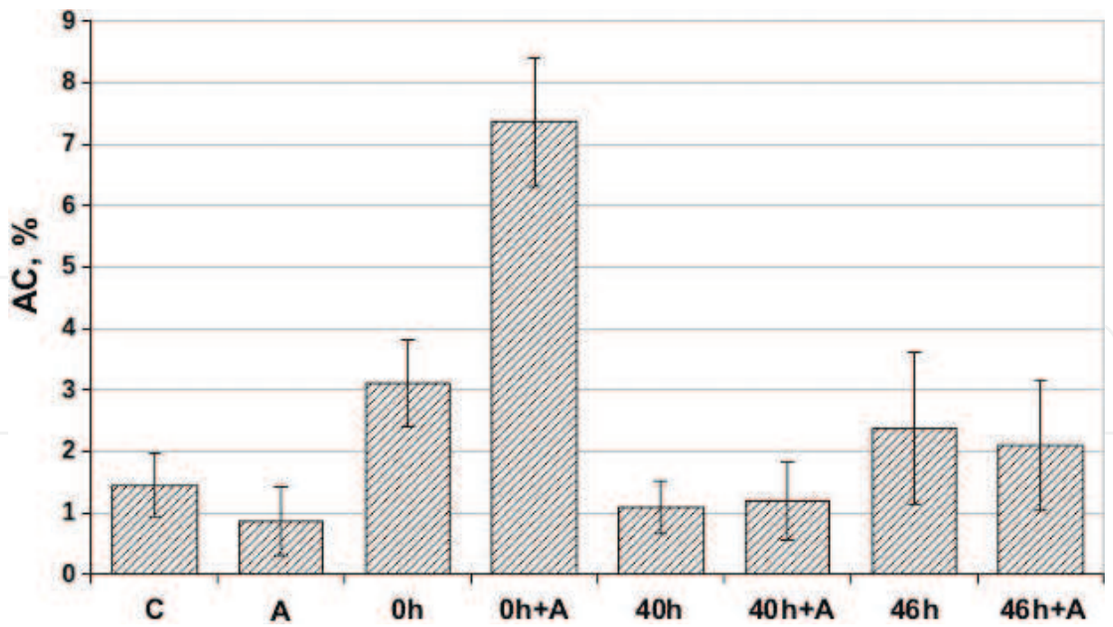


Figure 7. The levels of "atypical comets" (AC%) in cultures of human lymphocytes after 48 hours of cultivation depending on the irradiation terms and the addition of astaxanthin. C, control cultures; A, supplemented with 20.0 μg/ml astaxanthin cultures; 0, 40, and 46 h, cultures irradiated at the 0th, at the 40th, and at the 46th hours of cultivation, respectively; 0 h + A, 40 h + A, and 46 h + A, supplemented with 20.0 μg/ml astaxanthin cultures irradiated at the 0th, at the 40th, and at the 46th hours of cultivation, respectively.

The absence of increase in the frequency of apoptosis after treatment in other terms of cultivation both under the influence of only ionizing radiation and under the combined action of γ-radiation and astaxanthin can be explained by either insufficient time for realization of apoptosis or existence of contingent on the stage of the cell cycle apoptotic pathways, which astaxanthin is unable to activate on the S and G_2 phases of the cell cycle.

Since the increase in the level of apoptosis is not a reason for the decline of the pool of high damaged cells under the astaxanthin influence after irradiation at 40 and 46 h of cultivation, the question remains: what is the cause of such effect of astaxanthin?

It is generally accepted that reactive oxygen species, which are formed by ionizing radiation exposure, cause DNA breaks [40]. Astaxanthin is a power antioxidant and capable to scavenge and quench free radicals and ipso facto to reduce the overall level of DNA damages [3]. According to the data presented in **Figure 7**, the results obtained after irradiation of cells at 46 h of cultivation can be explained by the antioxidant properties of astaxanthin: reducing of oxidative stress leads to a decrease in the number of "comets" of the ten groups and increasing (although not always statistically significant) in the frequencies of cells belonging to other groups (except 3 and 9).

However, such impact of astaxanthin was not observed when cells were irradiated at 40 h of cultivation. It is noteworthy that in this experiment the increase in the frequency of the "comets" of group 1 (from 11.18 ± 1.46 to 17.54 ± 1.62%, $p < 0.01$) was detected. It can be explained by the fact that this group may include cells having a sufficiently large number of lesions enough to trigger mechanisms for the cell cycle arrest on the S phase. Probably, astaxanthin activates S-/G_2-phase checkpoint that leads to an increase in the frequency of the cells from which the "comets" with low DNA are formed (by delay in S phase) and may cause decreasing in the frequency of the "comets" of groups 9 and 10. The results are consistent with the literature data concerning the effects of astaxanthin on the proliferation of tumor cells [7, 17, 41].

4. Conclusion

The obtained results enable us to resume the following astaxanthin effects on irradiated cells that may be clearly observed depending on the phase of the cell cycle and the duration of cells cultivation after irradiation:

1. Stimulation of apoptosis in the irradiated cells resulting in a decrease in the level of cells with a large number of DNA damages (irradiation on the G_0 phase of the cell cycle and cultivation after irradiation for 48 h)
2. Stimulation of the processes that lead to the activation of the checkpoints on the S phase and, accordingly, arrest the division of the most damaged cell population (irradiation on the S phase of the cell cycle and cultivation after irradiation for 8 h)
3. Scavenge of reactive oxygen species resulting in reduction in the total level of DNA breaks (irradiation on the G_2 phase of the cell cycle and cultivation after irradiation for 2 h)

All of these effects are potentially radio- and genoprotective. However, we have previously shown that the protective action of astaxanthin concerning the radiation-induced cytogenetic effect similarly to its apoptotic effect was observed exclusively when irradiated cells were on the G_0 phase of the cell cycle. Moreover, analyzing the ChA spectra (**Figure 3**), we found that supplementation with astaxanthin reduces exactly the levels of classic unstable cytogenetic markers of radiation exposure (dicentric and centric ring chromosomes), and it is known that the cells bearing unstable chromosomal aberrations are eliminated by apoptosis in the first place [42]. So, the radioprotective effect of astaxanthin rather may be due to its ability to stimulate apoptosis in cells that carry a subcritical number of DNA breaks than its potential genoprotective properties (defenses DNA from damages or activates of DNA repair processes).

Author details

Denys Kurinnyi[1]*, Stanislav Rushkovsky[2], Olena Demchenko[1] and Mariya Pilinska[1]

*Address all correspondence to: kurinnyi.d@gmail.com

1 State Institution "National Research Center for Radiation Medicine of the National Academy of Medical Sciences of Ukraine", Kyiv, Ukraine

2 Educational and Research Center "Institute of Biology and Medicine" Taras Shevchenko National University of Kyiv, Kyiv, Ukraine

References

[1] Azqueta A, Collins AR. Carotenoids and DNA damage. Mutation Research. 2012;**733**:4-13

[2] Ambati RR, Phang SM, Ravi S, Aswathanarayana RG. Astaxanthin: Sources, extraction, stability, biological activities and its commercial applications—A review. Marine Drugs. 2014;**12**:128-152

[3] Nishida Y, Yamashita E, Miki W. Quenching activities of common hydrophilic and lipophilic antioxidants against singlet oxygen using chemiluminescence detection system. Carotenoid Science. 2007;**11**:16-20

[4] Lin KH, Lin KC, Lu WJ, Thomas PA, Jayakumar T, Sheu JR. Astaxanthin, a carotenoid, stimulates immune responses by enhancing IFN-γ and IL-2 secretion in primary cultured lymphocytes in vitro and ex vivo. International Journal of Molecular Sciences. 2015;**17**(1). DOI: 10.3390/ijms17010044

[5] Ohno T, Shimizu M, Shirakami Y, et al. Preventive effects of astaxanthin on diethylnitrosamine-induced liver tumorigenesis in C57/BL/KsJ-db/db obese mice. Hepatology Research. 2016;**46**(3):201-209

[6] Song XD, Zhang JJ, Wang MR, Liu WB, Gu XB, Lv CJ. Astaxanthin induces mitochondria-mediated apoptosis in rat hepatocellular carcinoma CBRH-7919 cells. Biological & Pharmaceutical Bulletin. 2011;**34**:839-844

[7] Zhang X, Zhao WE, Hu L, Zhao L, Huang J. Carotenoids inhibit proliferation and regulate expression of peroxisome proliferators-activated receptor gamma (PPARgamma) in K562 cancer cells. Archives of Biochemistry and Biophysics. 2011;**512**:96-106

[8] Mortensen A, Skibsted H. Importance of carotenoid structure in radical scavenging reactions. Journal of Agricultural and Food Chemistry. 1997;**45**:2970-2977. DOI: 10.1021/jf970010s

[9] Kobayashi M, Kakizono T, Nishio N, Nagai S, Kurimura Y, Tsuji Y. Antioxidant role of astaxanthin in the green alga *Haematococcus pluvialis*. Applied Microbiology and Biotechnology. 1997;**48**:351-356

[10] Gong M, Bassi A. Carotenoids from microalgae: A review of recent developments. Biotechnology Advances. 2016;**34**(8):1396-1412

[11] Higuera-Ciapara I, Felix-Valenzuela L, Goycoolea FM. Astaxanthin: A review of its chemistry and applications. Critical Reviews in Food Science and Nutrition. 2006;**46**:185-196

[12] Moretti VM, Mentasti T, Bellagamba F, Luzzana U, Caprino F, Turchini GM, Giani I, Valfrè F. Determination of astaxanthin stereoisomers and colour attributes in flesh of rainbow trout (Oncorhynchus mykiss) as a tool to distinguish the dietary pigmentation source. Food Additives and Contaminants. 2006;**23**(11):1056-1063

[13] Rüfer CE, Moeseneder J, Briviba K, Rechkemmer G. Bioavailability of astaxanthin stereoisomers from wild (*Oncorhynchus* spp.) and aquacultured (*Salmo salar*) salmon in healthy men: a randomised, double-blind study. British Journal of Nutrition. 2008;**99**(5):1048-1054

[14] Shah MM, Liang Y, Cheng JJ, Daroch M. Astaxanthin-producing green microalga *Haematococcus pluvialis*: from single cell to high value commercial products. Frontiers in Plant Science. 2016;**7**:531. DOI: 10.3389/fpls.2016.00531

[15] Shimidzu N, Goto M, Miki W. Carotenoids as singlet oxygen quenchers in marine organisms. Fisheries Science. 1996;**62**:134-138

[16] Hama S, Uenishi S, Yamada A, Ohgita T, Tsuchiya H, Yamashita E, Kogure K. Scavenging of hydroxyl radicals in aqueous solution by astaxanthin encapsulated in liposomes. Biological & Pharmaceutical Bulletin. 2012;**35**(12):2238-2242

[17] Zhang L, Wang H. Multiple mechanisms of anti-cancer effects exerted by astaxanthin. Marine Drugs. 2015;**13**(7):4310-4330

[18] Franceschelli S, Pesce M, Ferrone A, De Lutiis MA, Patruno A, Grilli A, Felaco M, Speranza L. Astaxanthin treatment confers protection against oxidative stress in U937 cells stimulated with lipopolysaccharide reducing O_2^- production. PLoS One. 2014;**9**(2):e88359. DOI: 10.1371/journal.pone.0088359

[19] Stewart J, Lignell A, Pettersson A, Elfving E, Soni G. Safety assessment of astaxanthin—Rich microalgae biomass: Acute and subchronic toxicity studies in rats. Food and Chemical Toxicology. 2008;**46**:3030-3036

[20] Naguib YM. Antioxidant activities of astaxanthin and related carotenoids. Journal of Agricultural and Food Chemistry. 2000;**48**:1150-1154. DOI: 10.1021/jf991106k

[21] Riccioni G, D'Orazio N, Franceschelli S, Speranza L. Marine carotenoids and cardiovascular risk markers. Marine Drugs. 2011;**9**(7):1166-1175

[22] Kishimoto Y, Yoshida H, Kondo K. Potential anti-atherosclerotic properties of astaxanthin. Marine Drugs. 2016;**14**(2):E35. DOI: 10.3390/md14020035

[23] Zou TB, Zhu SS, Luo F, Li WQ, Sun XR, Wu HF. Effects of astaxanthin on reverse cholesterol transport and atherosclerosis in mice. BioMed Research International. 2017; **2017**:4625932. DOI: 10.1155/2017/4625932

[24] Yuan JP, Peng J, Yin K, Wang JH. Potential health promoting effects of astaxanthin: A high-value carotenoid mostly from microalgae. Molecular Nutrition & Food Research. 2011;**55**:150-165. DOI: 10.1002/mnfr.201000414

[25] Pilinska MA, Kurinnyi DA, Rushkovsky SR, Dybska OB. Genoprotective properties of astaxanthin revealed by ionizing radiation exposure *in vitro* on human peripheral blood lymphocytes. Problems of Radiation Medicine and Radiobiology. 2016;**21**:141-148

[26] Kurinnyi DA, Rushkovsky SR, Dybska OB, Dubrovina GV. Astaxanthin modifies clastogenic effects of ionizing radiation in vitro in peripheral blood lymphocytes of the persons recovered from acute radiation sickness. Experimental Oncology. 2016;**38**(4): 280-282

[27] Kurinnyi DA, Rushkovsky SR, Demchenko OM, Pilinska MA. Study the impact of astaxanthin on developing of genomic instability in human peripheral blood lymphocytes irradiated *in vitro* on G_2 phase of cell cycle. Problems of Radiation Medicine and Radiobiology. 2017;**22**:208-216

[28] Kurinnyi DA, Rushkovsky SR, Demchenko OM, Pilinska MA. Peculiarities of modificationby astaxanthin the radiation-induced damages in the genomeof human blood lymphocytes exposed *in vitro* on different stages of the mitotic cycle. Cytology and Genetics. 2018;**52**(1):40-45

[29] Kurinnyi DA, Rushkovsky SR, Pilinska MA. The lack of modifying effect of astaxanthin on cytogenetic effects in irradiated *in vitro* human peripheral blood lymphocytes on stage G_2 of the cell cycle. Dopov. Nac. akad. nauk Ukr. 2017;**4**:91-95

[30] Dhawan A, Anderson D, editors. The Comet Assay in Toxicology. Cambridge, UK: Royal Society of Chemistry; 2016. 610p. DOI: 10.1039/1757-7187. Print ISSN: 1757-7179 Series ISSN: 1757-7179

[31] Olive PL, Banáth JP. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. Radiation Research. 2012;**178**:35-42

[32] Gopalakrishna P, Khar A. Comet assay to measure DNA damage in apoptotic cells. Journal of Biochemical and Biophysical Methods. 1995;**30**(1):69-73

[33] Roser S, Pool-Zobel BL, Rechkemmer G. Contribution of apoptosis to responses in the comet assay. Mutation Research. 2001;**497**:169-175

[34] Olive PL. Impact of the comet assay in radiobiology. Mutation Research. 2009;**681**(1):13-23

[35] Afanasieva K, Zazhytska M, Sivolob A. Kinetics of comet formation in single-cell gel electrophoresis: Loops and fragments. Electrophoresis. 2010;**31**:512-519

[36] Afanasieva K, Chopei M, Lozovik A, Semenova A, Lukash L, Sivolob A. DNA loop domain organization in nucleoids from cells of different types. Biochemical and Biophysical Research Communications. 2017;**483**(1):142-146

[37] Olive PL, Banáth JP. The comet assay: a method to measure DNA damage in individual cells. Nature Protocols. 2006;**1**:23-29

[38] Liao W, McNut MA, Zhu WXG. The comet assay: A sensitive method for detecting DNA damage in individual cells. Methods. 2009;**48**:46-53

[39] Ahnström G, Erixon K. Measurement of strand breaks by alkaline denaturation and hydroxyapatite chromatography. In: Friedberg EC, Hanawalt PC, editors. DNA Repair. A Laboratory Manual of Research Procedures. New York, NY: Marcel Dekker; 1981. pp. 403-418

[40] Von Sonntag C. Free-Radical-Induced DNA Damage and Its Repair. A Chemical Perspective. Springer-Verlag; 2006. p. 528

[41] Kim JH, Park JJ, Lee BJ, Joo MK, Chun HJ, Lee SW, Bak YT. Astaxanthin inhibits proliferation of human gastric cancer cell lines by interrupting cell cycle progression. Gut and Liver. 2016;**10**(3):369-374

[42] Belloni P, Meschini R, Czene S, Harms-Ringdahl M, Palitti F. Studies on radiation-induced apoptosis in G_0 human lymphocytes. International Journal of Radiation Biology. 2005; **81**:587-599