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Effect Of α- Tocopherol and Ascorbic Acid on the Genotoxicity by Gamma-Irradiation in *Drosophila melanogaster*

Drosophila melanogaster'de Gama-radyasyonu ile oluşan Genotoksisiteye α-Tokoferol ve Askorbik Asitin Etkisi

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ABSTRACT

Determination of the radioprotective effects of non-toxic and effective natural antioxidants is an important issue. Vitamin E is known for its efficient scavenging of free radicals, which are generated by irradiation. Additionally vitamin C can be act as pro-oxidant or antioxidant, although the effect is concentration dependent. We investigated the antimutagenic effects of the vitamin E and C administration (separately and together) under gamma- irradiation in *Drosophila melanogaster*. We used two different genetic toxicology tests including SLRL (Sex Linked Recessive Lethality) and Translocation. Radiation and vitamin applications were administered only to males which were crossed with females which had not taken vitamin and radiation beforehand. Then, offspring of the males were scanned to determine individuals carrying lethal chromosomes generated via irradiation. Then we investigated whether vitamin administration reduced the number of individuals having the lethal chromosomes generated by irradiation. Results indicate that vitamin E affected the lethal frequency significantly only when administered alone and before irradiation. Vitamin C did not preventive effect at all. Vitamin C and E administration with together did not reduce the lethality compare the control.

Key Words

Drosophila melanogaster, antigenotoxicity, SLRL, vitamin E, vitamin C.

ÖZ

Toksik olmayan ve etkili doğal antioksidanların radyoprotektif etkilerinin belirlenmesi önemli bir konudur. E Vitamini, radyasyon ile oluşan serbest radikalleri etkili bir şekilde temizlemesiyle bilinmektedir. Bununla birlikte, C vitamini konsantrasyona bağlı olarak prooksidan veya antioksidan görevi görebilir. Bu çalışmada, *Drosophila melanogaster*'de gama-ışınlamasına karşı E ve C vitamini uygulamasının (ayrı ayrı ve birlikte) antimutajenik etkilerini araştırdık. Bu amaçla, SLRL (Eşeye Bağlı Resesif Letalite) ve Translokasyon genetik toksikoloji testlerini kullandık. Her iki test sisteminde, sadece erkek bireylere radyasyon ve vitamin uygulamaları yapıldı. Bu erkekler, daha önce vitamin ve radyasyon almamış dişilerle çaprazlandı ve radyasyon yoluyla üretilen letal kromozomları taşıyan bireyleri belirlemek için bu erkeklerin yavruları tarandı. Ardından, vitamin uygulamasının, radyasyonla oluşmuş letal kromozom taşıyan birey sayısını azaltıp azaltmadığını araştırdık. Sonuçlar, E vitamininin letal frekansını yalnızca tek başına ve ışınlamadan önce uygulandığında önemli ölçüde azalttığını göstermiştir. C vitamini hiçbir şekilde önleyici etki göstermemiştir. C ve E vitaminlerinin birlikte verilmesinin, kontrole kıyasla letaliteyi azaltmadığı tespit edilmiştir.

Anahtar Kelimeler

Drosophila melanogaster, antigenotoksisite, SLRL, E vitamini, C vitamini.

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INTRODUCTION

The main effect of ionising radiation is inducing DNA damage directly and also causing lipid peroxidation by the formation of highly reactive free radicals that are removing hydrogen atoms from fatty acids [1]. Along with many chemical pollutants and dietary carcinogenic, ionizing radiations can trigger free radical reactions in organisms which lead to formation of ROS (reactive oxygen species). Reactive oxygen species can in turn cause mutations in DNA, protein oxidation and lipid peroxidation. ROS and lipid peroxides can induce many types of diseases, including Parkinson's disease, cataracts, atherosclerosis, nephrotic syndroms, diabetes, infectious diseases, ailments associated with aging and cancer [2,3]. Apart from these effects, ROS can also alter the balance of endogeneous protective systems, such as enzymatic antioxidants (GPx, SOD, CAT) defence systems [4]. Most of all, although spontaneous mutation rates in nature across many species is quite low, ionizing radiation via reactive oxygen species significantly increases that rates [5,6]. Organisms have evolved various defence mechanisms against the harmful effects of ROS. The enzymes SOD (superoxide dismutase), CAT (catalase) , GPx (glutathione peroxidase) and the antioxidant vitamins have been studied extensively for their role in these defences. The antioxidant vitamins E (α- tocopherol) and C (ascorbic acid) have gained considerable attention for their role in those defences [3,7,8].

There are numerous reports of the mutation protective effect of α- tocopherol in various organisms both prokaryotic and eukaryotic [9-11]. But results of negative or even mutagenic effects have also been reported [12].

It is universally certain that vitamin E is indispensable component of antioxidant response [13]. Basically, it functions to protect membrane phospholipids from oxidative damage. In particular, α- tocopherol is the most effective chain- breaking lipid-soluble antioxidant in biological membranes. It contributes to stabilization at the cell membrane and protect to cell components from oxygen free radical damage and lipoperoxidation products. Additionally, Vitamin E is a well-known antioxidant, effective in scavenging free radicals generated by radiation exposure.

Vitamin E analogs, collectively known as tocols, have been subject to active investigation for a long time as radioprotectors in patients undergoing radiotherapy and in the context of possible radiation accidents or terrorism scenarios [14].

There are also various investigations that show the mutation preventory role of ascorbic acid [15,16]. However, when ascorbic acid acts together with vitamin E, it has positively effect to peroxidation chain-breaking [17-19]. More importantly, ascorbic acid has been demonstrated to involve in α - tocopherol regeneration after vitamin E reaction with oxyradicals [20].

Drosophila melanogaster is a well understood, high-throughput model organism being used more than 110 years to study the different biological aspects related to the toxicity of chemicals and natural compounds, genetics, embryonic development, human disease and behaviour studies. These flies are routinely used in genotoxicity tests and continue to be used [21-23].

In this research we, present results of the protective effects of vitamin E and C on ionizing radiation in *Drosophila*. We believe these data provided contributory knowledge concerned the widely contradictory results obtained previously by various authors. We used different mutagenicity test systems, SLRL and Translocation, with *Drosophila melanogaster* to assess the protection levels, in a design measuring the effects imposed before and after the vitamin treatment, mutagenicity source being the gamma radiation given in different doses.

MATERIALS and METHODS

Drosophila stocks; standard and the test stocks

Oregon; This is the standard highly homogeneous wild type laboratory stock. It has been kept our laboratory for more than ten years.

Basc (Muller-5); Classical marker stock for sex-linked recessive lethality testing with *white apricot* (w^a,1-1.5) and *Bar* (B,1-57.0) as markers. Detailed description on the genetic markers is found in [24].

Oster ; (*bw; st* p^p); Stock for translocation test with markers on 2nd (*brown* bw,2-104.5) and 3rd (*scarlet* st, 3-44.0 and *peach-pink* 3.48.0) chromosomes.

Vitamins and chemicals

L-ascorbic acid (sodium salt, Sigma CAS No. 134-03-2); d-α-tocopherol (acetate, Sigma CAS No. 7695-91-2); detergent (Tween-80, Sigma CAS No. 9005-65-6)

Culture maintenance

All the experimental stock strains were kept at 25°C ± 1 constant living temperature and 60% rh in half pint bottles with instant medium (Carolina, Formula 4-24), at consecutive periods of 12 dark-12 light hours in fly chambers.

Radiation treatment

All irradiations were done with a ^{60}Co Gammacell- 220 type (¥) gamma irradiator located at the Department of Chemistry of Hacettepe University. Flies were exposed to irradiation in three replicate foodless tubes of 2.5x7.5 cm. dimension, each with 10 males per experiment. All replicates of all the experiments were in the same standing distance to the gamma source.

LD_{50} determination

To find out the appropriate (highest, in our case) irradiation dose which did not cause sterility, LD_{50} value was determined with 100 males of *Oregon Drosophila melanogaster. Oregon* males of 5 to 7 days (d) of age were exposed to different doses of gamma-irradiation. Number of dead flies was daily recorded, counting being terminated on the 10th to 12th d after exposure [25]. The LD_{50} of gamma-irradiation was evaluated to be 1.21 kGy at which all the flies lost fertility. The highest dose at which all the flies were fertile was 30 Gy and this was used in all the experiments concerning γ - irradiation.

Preparations of vitamin solutions and LC_{50} determination

Males of *Drosophila melanogaster* strains were fed on the instant medium containing vitamins C and E. Deionized water solutions of vitamin C were added to the mediums. Because lipid solutions of vitamin E make the medium sticky, and thus detrimental to the flies, vitamin E solutions were prepared in a harmless detergent Tween-80 for LC_{50} determination, 100 *Oregon* males of 4 to 5 d of age were first stored for 4 to 5 hours. They were then introduced to vials containing with different doses (in g/ml) of vitamin C or E. For vitamin C, dead flies were recorded consecutively on 1, 2 and 3 d after treatment.

Probit analyses were used to determine LC_{50} value. The LC_{50} of vitamin C for males was 0.23 g/ml. 10% and 15% concentrations of this value were used in the experiments. No LC_{50} value could be assessed for vitamin E as there was no lethal effect of vitamin E at any dose, including highest ones. We prepared a stock solution following the method by Enosco and Verdone Smith [26]. First 1 ml of Tween-80 (1% solution) and 100 mg of vitamin E were mixed. Then 1 ml of this solution was added to 100 ml of distilled water. Final solution was stirred until no traces of oil left. The concentrations used in our study were the 1 and 2.5% of the final solution added to the medium.

Genetical tests

Following tests were carried out after the method described by Würgler et al. [27]. The experimental groups consisted of the following and all experiments were performed in three replicates:

- Only Vitamin administration (no irradiation)
- Irradiation before vitamin administration
- Irradiation before combined vitamin administration
- Irradiation after vitamin administration
- Irradiation after combined vitamin administration
- Only Irradiation (no vitamin)

SLRL- Sex linked recessive lethality test

Drosophila melanogaster has been one of the most preferred organisms to evaluate genetic damage induced by radiation in gametes. The SLRL test is an important method that detects mutations at approximately 600-800 loci corresponding to 80% of *D. melanogaster*'s X chromosome. The method has a high sensitivity for direct-acting agents and simple promutagens, and a high specificity, which means that in general a positive result has considerable value for prediction of potential genotoxicity in mammals [28].

Here *Oregon* males of 3 to 5 d age were treated in particular to each experimental restriction as defined above. Then each treated male was crossed to 3 virgin *Basc* untreated females. Every virgin F_1 female from each such a cross were backcrossed individually to 3 *Basc* males from the stock. Resulting F_2 generations were scored for the presence of lethals as recommended by Würgler et al. [27]. The chromosome would be lethal if the normal shaped reddish eye phenotypic class was completely absent in F_2 progeny of a treated wild type *Oregon* male.

Translocation test

The translocation test screens for the presence of translocations induced by a putative mutagen. Translocations known as exchange of fragments between non-homologous chromosomes can be induced by applied chemical and physical agents. Here, we test whether

gamma radiation-induced translocations are reduced by vitamins E and C using marker genes on chromosomes II and III [29]. *Oregon* males of 3 to 5 d age treated as above were individually crossed to 3 *Oster* (*bw* : *stp*^{p}) virgin females. After that, F_1 males were individually backcrossed to 3 virgin *Oster* females from the stock. Translocations were decided if the resulting F_2 progeny consisted of only normal and white eye classes, instead of the four expected phenotypes including orange and brown. Details for translocation design are in the Würgler et al. [27].

RESULTS and DISCUSSION

Sex-linked recessive lethal numbers

Control groups were constructed with only applying one irradiation dose (i.e. 30 Gy). Vitamin applications were described above. Vitamins C and E, and combination of them were applied before and after irradiation. Analysis results of irradiation for vitamin administration experiments are shown in Table 1. For ascorbic acid there was no significant antimutagen effect when it was given in 10 and 15% of the concentrations of the LC_{50} value estimated in this study. The effect profile for the vitamin E was strikingly different from that of vitamin C in both for its single response to irradiation time and its effect dependency on the presence of another vitamin. When vitamin E was applied with ascorbic acid, it did not reduce the lethality significantly from that of the control. Moreover, this effect was independent of the time when the flies were irradiated (Table 1). The administration of vitamin E singly before the irradiation resulted in a remarkably different picture. Both doses of the vitamin E, i.e. 1 and 2.5% of the solution prepared accordingly to the method by Enosco and Verdone Smith [26], reduced significantly the amount of the lethal chromosomes when it was administered before the radiation treatment (for (E1+R) - K and (E2+R) - K comparisons χ^2=5.291 and 7.634, respectively (Table 1). Post irradiation vitamin E treatment produced no significant results.

Translocations

Results from the translocation experiments with a combined or single vitamin treatment are summarized in Table 2. Vitamin C combination with vitamin E administration or singly administration of two vitamins gave nonsignificant profiles in effectively reducing lethal numbers, irrespective of the irradiation time. In this respect the result is similar to that found in recessive lethality test. Vitamin E, as in the recessive lethality, when administered singly before irradiation, significantly reduced the lethal numbers, but only at the highest of the two concentrations applied, i.e. 2.5% of the solution. Reducing of lethals at 1% of solution was not significant, though it was close to the critical χ^2 boundary with 1 df (i.e. 3.84). This would be rather significant when considering the relatively small sample size of the chromosome tested at that concentration (Table 2). No other comparisons in the translocation experiments resulted in significant figures.

Our study aimed in particular at an assessment of the alterations in the gamma irradiations mutagenicity profiles after ascorbic acid and tocopherol administrations. The model system we chose, that is, Drosophila melanogaster for sex-linked and autosomal lethality, is an excellent organism to detect mutagenesis by various putative environmental agents. It is important and necessary to consolidate the results with mammalian organisms that are genetically closer to humans. Once pioneering work has been done in Drosophila, it will need to be studied in mammalian systems, but certain behaviors to be studied and validated will require much smaller numbers of animals and likely only minimal genetic manipulation. Here we present the results which clearly indicate that the effects created by an environmentally effective agent, radiation, and the counter effects responded in a time-dependent manner by the two well known protective compounds, vitamins C and E. Critical inference from our results is that only vitamin E, exclusively before irradiation, could reduce the lethal effects significantly in recessive sex-linked lethality, and almost significantly in the translocation [30]. It has been well established that irradiation disrupts the activities of many vital enzymes including DNA polymerase and dehydrogenases and reduces the efficiency of oxidative phosphorilation [5], causes lethal chromosomal deletions and more importantly for its immediate effect, accelerates lipid peroxidation in cell membrane, which is quite preventory for necessary cell transport dynamics [31,32]. All these radiations mediated harmful effects are assumed stemming from free radicals created by exposure of the cell to radiation. Remarkably, an essential function of the vitamin E is to scavenge the free radicals and hence break the chain of reactions leading to lipid peroxidation [33,34]. Therefore, we suggest the protection in our study was coming from vitamin E's

free radical scavenging function. But why it should be confined to only before- radiation exposure needs further consideration. It is obvious that the cellular processes must be in normal operation for any substance to realize its effect efficiently through the cell machinery. Our conclusion is that a cell machinery having blocked pathways via irradiation might have truncated the putative protective effects of vitamin E when it was administered after exposure. This would have been more pronounced considering the disruption in membrane transport which in turn may prevent the vitamin E entry into the cell. On the contrary, a before-exposure treatment with vitamin E especially at concentrations higher than base levels may mean that the critical levels would have been already in the cell that the protective effects of it could relatively be easy to access. Scavenging radicals effectively before they reach their targets must be the main issue in cases such as presented here in our study.

Table 1. Basic tests and the statistical significancy of the differences (as $\chi 2$ values) between lethal from the controls (R) and the treatments.

Treatments	Number of total chromosomes tested	Number of lethals	Proportion of mutation	Controls	Number of total chromosomes tested	Number of lethal	Proportion of mutation	χ^2
C1 + R	183	13	0.0710	R	234	13	0.0555	0.202
C2 + R								
235	11	0.0468	R	234	13	0.0555	0.057	
E1 + R	349	10	0.0286	R	316	22	0.0696	5.291*
E2 + R	331	7	0.0211	R	316	22	0.0696	7.634*
C1 + E2 →R	226	10	0.0442	R	352	26	0.0738	1.360
R + C1	233	16	0.0687	R	297	21	0.0707	0.010
R + C2	237	17	0.0717	R	297	21	0.0707	0.019
R + E1	326	14	0.0429	R	379	20	0.0527	0.213
R + E2	335	10	0.0299	R	379	20	0.0527	1.795
R → C1 +E2	207	12	0.0580	R	307	21	0.0684	0.098

*P<0.05
R: only radiation
C1: 10% vitamin C concentration
C2: 15% vitamin C concentration
E1: 1% vitamin E concentration
E2: 2,5% vitamin E concentration

Table 2. Translocation tests and the statistical significancy of the differences (as $\chi 2$ values) between lethal from the controls (R) and the treatments.

Treatments	Number of total chromosomes tested	Number of lethals	Proportion of mutation	Controls	Number of total chromosomes tested	Number of lethal	Proportion of mutation	χ^2
C1 + R	430	20	0.0465	R	485	25	0.0515	0.259
C2 + R	393	17	0.0433	R	485	25	0.0515	0.150
E1 + R	269	9	0.0333	R	333	23	0.0691	3.147
E2 + R	351	10	0.0285	R	333	23	0.0691	5.437*
C1 + E2 →R	300	12	0.0400	R	400	22	0.0550	0.674
R + C1	253	13	0.0514	R	290	15	0.0517	0.027
R + C2	220	10	0.0455	R	290	15	0.0517	0.014
R + E1	280	10	0.0357	R	318	16	0.0503	0.547
R + E2	285	11	0.0386	R	318	16	0.0503	0.361
R → C1 +E2	195	8	0.0410	R	284	18	0.0634	0.786
R → C1 +E2	207	12	0.0580	R	307	21	0.0684	0.098

*P<0.05
R: only radiation
C1: 10% vitamin C concentration
C2: 15% vitamin C concentration
E1: 1% vitamin E concentration
E2: 2,5% vitamin E concentration
E2: 2,5% vitamin E concentration

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