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Modification by Compositional Antimutagens of the Key Stages of a Mutation Process

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Abstract: In experiments with Escherichia coli B/r WP2(trpE-), mice marrow cells and human lymphocytes the antimutagenic activity of an extract from Zizyphus mill was performed. The extract was tested separately and in mixture with Triticum aestivum. In experiments with different treatment schemes it has been established that the extract from wheat sprouts added to an antimutagenic preparation from Zizyphus mill intensifies the genoprotective properties of the Zizyphus mill extract.

Key Words: mutation, antimutagens, plant extracts, chromosome aberrations, gene mutations

Introduction

As a result of intensive research in the field of genome protection, associated with the possibility for most antimutagens (AMs) to be used not only against the processes of induced mutagensis but also against carcinogenesis, more than 100 genoprotective agents are already known and their numbers are growing every year. Among these compounds, plant AMs are the most promising ones (1-7). The mechanisms by which AMs decrease mutation rates are different due to the specific characters of these compounds to influence particular stages of the mutation formation process. It has been shown that these compounds may inactivate genotoxicants or inhibit the metabolic activation of pro-mutagens as well as correct the mutation process through influencing the process of DNA replication and repair (7-10). The efficiency of AMs is largely determined by the genoprotective agent's ability to participate in the correction, at least, of some key stages of multistage mutation processes. The solution to the problem of how to achieve increases in the inhibitory effects of genoprotective compounds was found in the concept of compositional AMs developed by Alekperov (11). According to the concept, this goal can be achieved through the creation of antimutagenic compositions, individual compounds that are capable of inhibiting different stages of the mutation production process. The idea of compositional antimutagens is one of the most feasible ways for human gene-pool protection and biodiversity conservation (7, 11).

The object of the present study is to investigate the antimutagenic properties of a compositional preparation from extracts of *Zizyphus mill (ZM)* and *Triticum aestivum (TA)* by tracing its role in the correction of the initial and final stages of a mutation formation process.

Materials and Methods

The objects

The experiments were performed with the cells of *Escherichia coli B/r* WP_2 *(trp*$^-$*)* wild type, white mice in vivo, and a culture of human peripheral blood lymphocytes.

Chemicals and reagents

As inducers of mutations, the following chemicals were used: N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Serva), N-methyl-N-nitrosourea (MNU) (Institute of Chemical Physics, RAS, Moscow, Russia), DimethylNitrosamine (DMNA) (Serva), Mitomycin C (Sigma), Vincristine (Merck) and Sodium fluoride (SF) (C.P. Producer Factory, Nizhnii Novgorod, Russia).

Mutagens of direct action used in experiments with *E. coli* such as Mytomicyn C, MNNG and Vincristine form initial damage of the following types: crosslinks DNA-DNA and DNA-protein, as well as monoadducts; one- and two-

thread ruptures of DNA, crosslinks DNA-DNA and DNA-protein; and one-thread DNA ruptures, respectively. In experiments with mice and human lymphocytes MNU, DMNA and SF, which are inducers of direct, pro-mutagenic and co-mutagenic effects, respectively, were used (12).

Reagents for the cultivation of bacterial cells: beef-extract agar (BEA) and beef-extract broth (BEB). As a minimal full-bodied medium, M-9 was used (per 1 l of distilled water: 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl and 1 g of NH_4Cl). As a buffer solution for minimal agar, the phosphate buffer concentrated (PhBC) was used (per 1 l of distilled water 2 g of $C_6H_6O_7Na_3$, 42 g of K_2HPO_4, 18 g of KH_2PO_4 and 4 g of $(NH_4)_2SO_4$) was added. For resuspending the cells, PhBC, 4-fold diluted in distilled water (pH 7.0), was used. For the preparation of minimal agar, to 900 ml of aqueous agar were added 300 ml of phosphate buffer, 1.2 ml of $MgSO_4$ 20% solution, 12 ml of glucose 20% solution, 0.6 ml of thiamine (B_1) 1% solution and the necessary amino acids until a final concentration of 1 µg/ml was achieved (13).

Reagents for lymphocyte culture: Heparin (Reanal), Phytohemagglutinin (Wellcome), Colchicine (Fluka AG), Giemsa (Merck), Trypsin (Varion, Russia), bovine serum (Moscow, Russia), Medium IGLA 199, Henx solution and cattle serum (Nizhnii Novgorod, Russia).

Test substances

As genoprotective agents the extracts from *ZM* fruit pulp and 3-day *TA* sprouts were used. Both extracts had a 35-37% content of dry substances and were prepared carefully to keep the biologically active substances in a relatively native state. The process includes the following: 3-time extraction with 70% ethanol, filtration, primary distillation of eluent, precipitation in a cold chamber from ballast substances, centrifugation and secondary distillation in a vacuum at 50-55 ºC.

As the antimutagenic action of the extract from *TA* sprouts has been investigated earlier (14,15), the present study explores the antimutagenic effect of *ZM* fruits and a compositional preparation created on their base.

The extracts were tested in the most efficient doses (16). Thus, the extract from *ZM* fruits was tested at a dose of 0.01 µg/ml on bacterial cells and on cultures of human peripheral blood lymphocytes, and at a dose of 0.3 mg/100 g of animal weight on mammals. The compositional preparation made up from an extract of *ZM* fruits and *TA* sprouts was tested in proportional ratios of each constituent on bacterial cells and on cultures of human peripheral blood lymphocytes at 0.001 µg/ml, and on mammals at 0.2 mg/100 g of animal weight.

The experiments were performed in 4 different variants:

- prior to mutagen: when AM was administrated prior to mutagen (M) treatment
- simultaneously: when AM was administrated simultaneously with M
- in mixture: when AM and M were left in mixture for 1 h before treatment
- after mutagen: when AM was administrated after treatment with M.

Microorganisms: Treatment. Gene mutations

In the first variant, *E. coli* cells grown to the stationary phase in M-9 medium with additives, were treated by extracts by adding them to the same medium and incubating them on a water bath for 30 min at 37 ºC. Then the cells were precipitated by centrifugation for 10 min at 5,000 revs/min, resuspended in buffer and each chemical mutagen (MNNG–5 µg/ml, Mitomycin C–0.01 µg/ml, Vincrestin-0.5 µg/ml) was added separately. After a 30-min incubation, the cells were washed to remove mutagen, resuspended in phosphate buffer (pH 7.0) and inoculated with BEA for the calculation of surviving cells, and placed on a minimal agar with an appropriate addition of amino acid to calculate revertants from auxotrophicity by triptophane to prototrophicity.

In other variations of the treatment, the method of the experiment remained the same, only the sequence of treatment by AM and M was changed.

Animals: Treatment. Chromosome Preparations

In experiments 1.5-2 month-old pubescent mice weighting 18-20 g were used. Both the experimental and control groups of animals were housed 6 per cage (97 x 97 with wood bedding), in an environment maintained at 23 ± 2 ºC, relative humidity 50 ± 5% and with a 12 h light/dark cycle. The animals were fed a standard diet (chemical content in percentages: raw protein 22.8%, raw fat 4.9%, carbohydrates 51.2%, cellulose 1.96%; the cellulose deficit was compensated for by the addition of 20 g grassy flour per 1 kg of feed, and tap water *ad libitum*).

The first 3 experimental groups of animals for 24 h were orally administrated by means of a special tube

directly into stomach the extract from *ZM* fruits (0.3 mg/100 g of weight) or the compositional preparation (0.2 mg/100 g of the extract from *ZM* fruits + 0.2 mg/100 g of extract from *TA* sprouts) and then separately Ms: MNU (3.75 mg/100 g), DMNA (3 mg/100 g) and SF (2 mg of fluoride/100 g of weight). The second and third experimental groups of animals were given for 24 h the extracts and Ms simultaneously or a mixture of components in equal ratios, and the fourth groups; at first for 24 h Ms and then the AMs. In each variant of the experiment 24 h after the last M or AM action the animals were decapitated humanely. The thigh-bone was isolated and after the cutting of the distal and proximal ends it was fixed in absolute alcohol and acetic acid (3:1). As a control, the intact animals as well as those subjected to the action of each of the Ms separately were used. For chromosome preparations, isolated marrow was stained with acetoarsein (Merck). The frequency of chromosome aberrations was determined in squash preparations. This technique allowed us to analyze single and double fragments of chromosomes, chromatid and chromosome dicentrics.

Humans: Treatment. Chromosome aberrations

The experiments were performed on a culture of peripheral blood lymphocytes of practically healthy donors (each group consisted of 5 men and 5 women, aged 30-35 years). The analysis of chromosome aberrations was done in the metaphase cells according to standard methods (17). Before cultivation, lymphocyte mitogenesis was stimulated with phytohemagglutinin at a dose of 20 mg. After cultivation of the mixture for 5 h at 37 ºC, in the first variant the test preparations (extract from *ZM* fruit 0.01 µg/ml, compositional preparation – in proportional ratio of the constituents 0.001 µg/ml) were added to the mixture for 17 h and then the cultivated medium was changed by adding mutagens – MNU (0.02%), DMNA (0.1 mg/ml of activating medium, of which S9 mix fraction donors were males of white breedless rats, and as an inducer of microsomal enzymes, sodium phenobarbital was used) or SF (0.1 µg/ml). In the fourth variant, the treatment scheme was changed to the reverse, and in the second and third variants the test agents were administrated simultaneously or as a mixture of the components. Three hours before fixation colchicine at a final concentration 0.5 mg/ml were added to the cultivation medium. Aberrations in the human cells were scored as breaks if the fragments were clearly separated and disoriented from the main chromatid.

Statistical Analysis

In the mice experiment 6 animals for each experimental variant were used, and a minimum of 150 cells from each animal were examined (in total a minimum of 900 cells per experimental variant) for anaphase analysis. A similar method was used for metaphase analysis of human blood lymphocytes: a minimum of 200 cells per human were analyzed (in total around 2000 cells per experimental variant). The chromosome aberration level was scored as a percentage of the number of abnormal anaphases to the number of observed ones. In experiments with *E. coli* cells gene mutation level was scored as a ratio of revertant colonies to survived ones.

All experiments were performed with 4 repeats. The data were analyzed using Student's t-test criteria and presented with standard error (18,19).

In experiments with E.coli Student's t criteria was scored by following formula:

$t = \frac{k - 1}{\delta k}$, where $k = \frac{M}{C}$, M – mutagen level of gene mutations , C – control intact level,

$\delta k = k \cdot \sqrt{\left(\frac{\delta \bar{x}}{x}\right)^2 + \left(\frac{\delta x_1}{x_1}\right)^2}$, $\bar{x}$ average frequency of gene mutations of the variant,

$\bar{x} = \frac{\sqrt{\sum x_i}}{n}$, x_i -frequency of gene mutations in the variant, and n– number of experiments.

Antimutagenic efficiency (AE) was determined as a proportion of the difference between initial and modified levels of mutability to the initial one (in relative units),

$AE = \frac{M - AM}{M}$, where M – mutagen level of gene mutations, and AM – antimutagenic level of gene mutations (20).

Results

In experiments with microorganisms, it has been shown that irrespectively of inducer of mutations used (e.g., MNNG, Mitomycin C and Vincrestin) and specificity of DNA initial damage types, the extract from *ZM* fruit, though with different efficiency, decreases chemically induced rates of gene mutations (Table 1).

The antimutagenic efficiency is highest when AM is administrated prior to M administration. With less efficiency, but at equal values of confidence ($P < 0.001$), it exhibits antimutagenic properties when it is preliminarily left in mixture with chemical mutagens. The efficiency of AM decreases though remains within the ranges of confidence values in those variants when extract is added to bacterial medium simultaneously with M or after mutagenic treatment.

The comparative analysis of AM efficiencies in both *ZM* fruit and compositional preparation in experiments with *E. coli* cells (Figure) showed that the compositional preparation significantly increased the antimutagenic efficiency of *ZM* fruit extract in all experimental variants. Thus, we conclude that it exhibits a similar effect at the removal stage of initial DNA damage as well as transports, the mutagen products to the hereditary substrate. Moreover, it was determined that this effect is independent of DNA initial damage types induced by chemical mutagens of a different nature.

The trend established on the model of the microorganisms and expressed in correlation between antimutagenic efficiency and the treatment protocol, was confirmed in the experiments with mice marrow cells (Table 2) and human peripheral blood lymphocytes (Table 3).

Discussion

Different approaches have been employed in studies of AM mechanisms, of which many are mainly based on experimental results carried out using inducers of mutation processes that initiate specific damage to both DNA and the systems supporting its structural and functional integrality (1,11). In some cases different mechanisms of antimutagenic activity may occur simultaneously. In research carried out with various native compounds it has been established that they simultaneously affect genotoxicant inactivation, free-radical suppression as well as DNA repair correction processes (8,9,21,22). Such influence is more typical for native compounds, especially for the sum of extractive substances, the components of which may be effective in different processes supporting genome integrality. This is one of the reasons to transfer from investigations of separate AM substances to complex of native compounds. The findings of investigations carried out indicate the higher antimutagenic efficiency of the sum of extractive substances because of the synergy of their actions (11).

Table 1. Antimutagenic efficiency (AE) of *ZM* fruits in experiments with *E.coli*.

Mutagen, gene mut.	Variants of the experiment	Extract from *ZM* fruits	
		Gene mut.	AE
MNNG 192 ± 16.07***	Prior to mutagen	145.74 ± 12.71 ***	0.24
	Simultaneously	163.5 ± 14.05 **	0.15
	In mixture	154.5 ± 13.63 ***	0.20
	After mutagen	167.3 ± 14.85 *	0.13
Mitomycin C 147.63 ± 12.38***	Prior to mutagen	97.54 ± 8.39 ***	0.34
	Simultaneously	121.58 ± 10.62 **	0.18
	In mixture	106.86 ± 9.13 ***	0.28
	After mutagen	125.31 ± 11.25 *	0.15
Vincrestin 161.19 ± 13.53***	Prior to mutagen	119.21 ± 10.84 ***	0.26
	Simultaneously	135.18 ± 11.09 **	0.16
	In mixture	124.36 ± 10.93 ***	0.23
	After mutagen	139.48 ± 12.39 *	0.13

Control level of gene mutations (gene.mut.) in E.coli – 28.5 ± 2.36
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

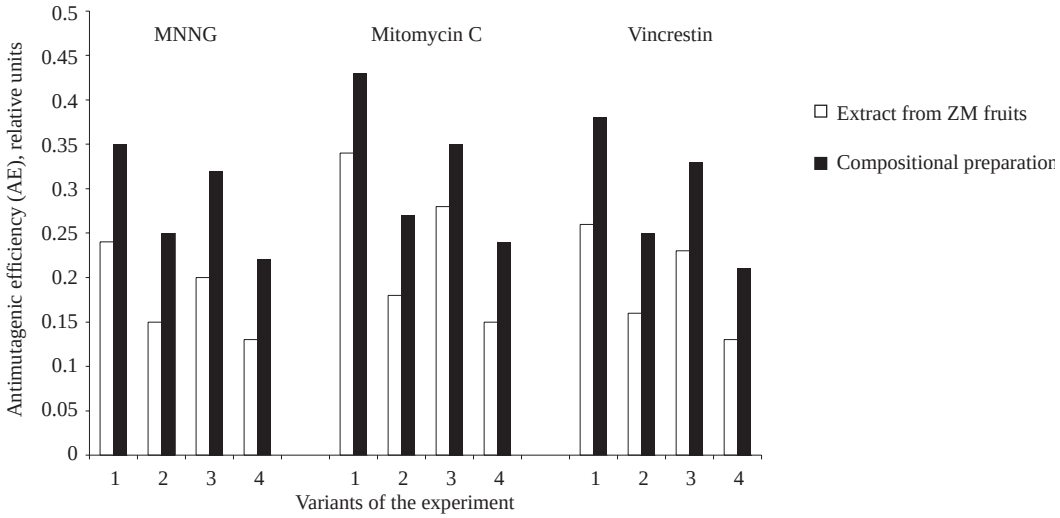

Fig. Histogram comparing antimutagenic efficiency of ZM fruit and compositional preparation in *E. coli* cells
□ Extract from ZM fruits
■ Compositional preparation
1. Prior to mutagen
2. Simultaneously
3. In mixture
4. After mutagen

Table 2. Antimutagenic efficiency of *ZM* fruit and its compositional preparation in mice marrow cells.

Mutagen, Chr.A.,%	Variants of the experiment	Extract from *ZM* fruit		Compositional preparation	
		Chr.A.,%	AE	Chr.A.,%	AE
	Prior to mutagen	4.86 ± 0.71 ***	0.47	3.26 ± 0.59 ***	0.64
MNU	Simultaneously	5.73 ± 0.77 **	0.34	4.75 ± 0.71 ***	0.48
9.15 ± 0.95***	In mixture	5.13 ± 0.72 ***	0.44	4.08 ± 0.67 ***	0.55
	After mutagen	6.19 ± 0.80 *	0.32	5.07 ± 0.73 ***	0.44
	Prior to mutagen	4.25 ± 0.67 ***	0.49	3.18 ± 0.58 ***	0.62
DMNA	Simultaneously	4.99 ± 0.72 **	0.40	3.79 ± 0.63 ***	0.54
8.30 ± 0.90***	In mixture	4.47 ± 0.69 ***	0.46	3.42 ± 0.59 ***	0.59
	After mutagen	5.44 ± 0.76 *	0.34	4.36 ± 0.67 ***	0.47
	Prior to mutagen	4.06 ± 0.66 ***	0.48	3.13 ± 0.58 ***	0.60
SF	Simultaneously	4.64 ± 0.70 **	0.41	3.89 ± 0.64 ***	0.50
7.81 ± 0.88***	In mixture	4.12 ± 0.66 ***	0.47	3.44 ± 0.60 ***	0.56
	After mutagen	5.05 ± 0.73 *	0.35	4.16 ± 0.66 ***	0.47

Control level of chromosome aberrations (Chr.A.) in mice marrow cells – 1.92 ± 0.42%
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3. Antimutagenic efficiency of *ZM* fruit and its compositional preparation in human blood lymphocytes.

Mutagen, Chr.A.,%	Variants of the experiment	Extract from *ZM* fruit		Compositional preparation	
		Chr.A.,%	AE	Chr.A.,%	AE
	Prior to mutagen	5.41 ± 1.12 ***	0.54	3.25 ± 0.85 ***	0.72
MNU	Simultaneously	6.29 ± 1.19 **	0.46	4.74 ± 1.03 ***	0.60
11.71 ± 1.56***	In mixture	5.42 ± 1.10 ***	0.53	4.07 ± 0.97 ***	0.65
	After mutagen	6.85 ± 1.30 *	0.42	5.33 ± 1.11 ***	0.54
	Prior to mutagen	4.41 ± 0.99 ***	0.57	3.06 ± 0.80 ***	0.70
DMNA	Simultaneously	5.16 ± 1.09 **	0.50	4.07 ± 0.97 ***	0.61
10.37 ± 1.51***	In mixture	4.89 ± 1.07 **	0.55	3.75 ± 0.92 ***	0.64
	After mutagen	5.70 ± 1.13 *	0.45	4.53 ± 1.02 ***	0.56
	Prior to mutagen	3.77 ± 0.95 ***	0.61	2.84 ± 0.81 ***	0.71
SF	Simultaneously	4.58 ± 1.03 **	0.53	3.84 ± 0.88 ***	0.61
9.79 ± 1.51***	In mixture	4.17 ± 0.99 **	0.57	3.29 ± 0.88 ***	0.66
	After mutagen	5.38 ± 1.12 *	0.45	3.83 ± 0.94 ***	0.61

Control level of chromosome aberrations (Chr.A.) in lymphocytes-1.71 ± 0.69%

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

In previous experiments (23) it has been established that ZM fruit extract decreases the frequency of spontaneous and MNNG-induced mutations. In these experiments a direct treatment of bacterial cells was used, when extract from *ZM* fruits was introduced to a medium of *E. coli* cells prior to mutagenic action. In such treatment the antimutagenic effect of the modifier reflects its influence practically at all stages of the multistage process of the occurrence of initial DNA damage and its realization in the final mutation events (8).

In our experiments, differences in the efficiency of genome protection by the tested preparations within variants, specifying the different sequence of the combined use of extracts with mutagens, allowed us to judge the peculiarity of their antimutagenic mechanism. Thus, the highest antimutagenic efficiency in variants when the extract of *ZM* fruits is introduced prior to mutagenic treatment in comparison to that after mutagenic treatment reveals that the genoprotective effect is largely associated with the correction of induced mutagenesis even before the initial damages of DNA molecules have occurred. This conclusion is also confirmed by the results obtained in the other variations of treatment. The sufficiently high indices of genome protection when the extract and mutagen are mixed before introduction to the test object provide a basis for the assumption that one of the ways of antimutagenic action is associated with the mechanism of adsorption as well as chemical or enzymatic inactivation of chemical mutagen molecules at a pre-cell level as well as after their penetration inside the cell that provides the evacuation of DNA-tropic agents and restricts their access to the cell.

At the same time the results of experiments showed that *ZM* fruit extract with relatively less efficiency corrects the mutation process on the basis of already occurred premutations. These data have been obtained in a variant of the experiment, when extract from *ZM* fruit was introduced after mutagenic treatment, i.e. after the formation of potential damages to DNA molecules. The efficiency of this removal in a great part depends on the activity of repair systems.

The antimutagenic efficiency increases synergistically in all experiment variants with the introduction of combined extract from *ZM* fruit and *TA* sprouts. Thus, the extract from *TA* sprouts in the compositional

preparation participates in the correction of the mutation process not only at the early stages but also on the basis of already present initial damage to DNA molecules, thereby strengthening the antimutagenic activity of *ZM* fruit extract at many key stages of the process of the occurrence of initial damages to DNA molecules and their realization in the final mutation events.

The genoprotective effect of the *ZM* fruit extract is associated with the presence in its composition of biologically active metabolites, such as ascorbic acid, carotenoids, cumarine derivatives, flavonoids, phenols and polyphenols (24), which take part in correcting possible stages of mutation formation (22).

When using the compositional preparation its effect is intensified by these or other metabolites (e.g., a -tocopherol) in the extract from wheat sprouts (25). The metabolites realize their potential both at the transport stages of the mutagen products to the DNA molecule and on the basis of already occurred initial damage to this molecule (1-3,8).

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