THE EFFECT OF "LOSHTAK" PREPARATION ON EXOGENOUS AND ENDOGENOUS OXIDATIVE DNA DAMAGE IN TRANSFORMED HUMAN CELLS

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ВЛИЯНИЕ ПРЕПАРАТА "ЛОШТАК" НА ЭКЗО– И ЭНДОГЕННЫЕ ОКИСЛИТЕЛЬНЫЕ ПОВРЕЖДЕНИЯ ДНК В ТРАНСФОРМИРОВАННЫХ КЛЕТКАХ ЧЕЛОВЕКА

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"Loshtak" preparation (LP) (standardized *Bryonia alba* roots) is used in Armenia as an adaptogenic drug with stress-protective and tonic properties to increase the non-specific resistance of an organism toward harmful stimuli. The effect of aqueous and methanol extracts of LP on exogenous and endogenous oxidative DNA damage was studied on transformed human cells (HeLa and Caco-2) using single cell gel electrophoresis (the comet assay). Extracts of LP lack ability to protect human transformed cells against exogenous DNA oxidative damage induced by H_2O_2. The ability of LP to protect human cells against endogenous oxidative DNA damage adds a new beneficial property to other effects of it.
***Key Words*: "Loshtak" preparation, the comet assay, endonuclease III, formamidopyrimidine DNA glycosylase, oxidative DNA damage, HeLa, Caco-2.**

Ïðåïàðàò "Ëîøòàê" (LP) (ñòàíäàðòèçîâàííûå êîðíåâèùà *Bryonia alba*) èìååò øèðîêîå ïðèìåíåíèå â Àðìåíèè êàê àäàïòîãåííîå ñðåäñòâî, îáëàäàþùåå ïðîòèâîñòðåññîâûì è òîíèçèðóþùèì äåéñòâèåì. Ñ öåëüþ èçó÷åíèÿ âëèÿíèÿ âîäíûõ è ìåòàíîëüíûõ ýêñòðàêòîâ LP (LPÀÅ è LPÌÅ ñîîòâåòñòâåííî) íà ñòåïåíü ýêçî- è ýíäîãåííîãî ïîâðåæäåíèé ÄÍÊ. Áûëî óñòàíîâëåíî, ÷òî ýêñòðàêòû LP íå îáëàäàþò ñïîñîáíîñòüþ çàùèùàòü òðàíñôîðìèðîâàííûå êëåòêè ÷åëîâåêà îò ýêçîãåííûõ îêèñëèòåëüíûõ ïîâðåæäåíèé ÄÍÊ, âûçâàííûõ äåéñòâèåì H_2O_2. Îáíàðóæåíèÿ ñïîñîáíîñòè LP çàùèùàòü êëåòêè ÷åëîâåêà îò ýíäîãåííûõ îêèñëèòåëüíûõ ïîâðåæäåíèé ÄÍÊ äîïîëíÿåò ïðåä÷åíü ïîëîæèòåëüíûõ ëåêàðñòâåííûõ ýôôåêòîâ LP.
***Êëþ÷åâûå ñëîâà:* ïðåïàðàò "Ëîøòàê", ìåòîä ÄÍÊ-êîìåò, ýíäîíóêëåàçà III, ôîðìàìèäîïèðèìèäèí-ÄÍÊ-ãëèêîçèëàçà, îêèñëèòåëüíûå ïîâðåæäåíèÿ ÄÍÊ, HeLa, Caco-2.**

Bryonia, a well–known medicinal plant is used in Armenia beginning from ancient time till now. Tablets prepared from a standardized powder of Bryonia alba roots and called "Loshtak" (LP) are used as an adaptogenic and restorative drug with immunomodulating, stress–protective and tonic properties that increase the non–specific resistance of an organism toward harmful stimuli [1]. LP has also strong antioxidant property [1]. It is well known that antioxidants are also antimutagens and anticarcinogens [2–4]. Possible antimutagenic action of LP was studied on rodents and humans *in vivo*. LP decreased significantly chromosomal aberrations level in bone marrow of rats induced by some clastogenic antitumor drugs [5, 6] and micronuclei level in polychromatic erythrocytes of mice [5]. LP reduced the number of lymphocytes with chromosomal aberrations in Chernobyl clean–up workers and is recommended for treatment of radiation injuries of different degree (against so called clastogenic factor) [7].

Recent investigations have shown that some antioxidants of plant origin (benzyl isothiocyanate, genistein) showing potent anticarcinogenic action on rodents [8, 9] are at the same time potent genotoxins in the comet assay [10, 11]. These compounds can influence endogenous (due to normal metabolic processes [12–14]) and exogenous (induced) oxidative DNA damage in various normal and transformed human cells.

The aim of the present work was to study the possible influence of LP on endogenous and exogenous oxidative DNA damage in human transformed cells (HeLa, Caco–2) by means of the comet assay. This assay is an extremely sensitive method for detecting DNA strand breaks, alkali–labile sites, and (in combination with repair endonucleases) oxidized purines and pyrimidines [12, 13].

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Abbreviations used: FPG — formamidopyrimidine glycosylase; LP — "Loshtak" preparation; LPAE — aqueous extract of LP; LPME — methanol extract of LP.

MATERIALS AND METHODS

A standardized powder of LP (a kind gift of Prof. Dr. A. Panossian) was used in experiments. Because of poor solubility of LP in water and culture medium, aqueous and methanol extracts of LP, LPAE and LPME, respectively), prepared as described earlier [14] were used in experiments. To each flask with cells 100 µl of PBS containing 1µl, 0.1µl and 0.01 µl of extract was added. To control cells only PBS was added at the same volume as extracts – 100 µl.

In experiments transformed human cells — HeLa and Caco–2 (colon carcinoma cells) were used. Caco–2 cells were grown in multilayer culture in Dulbecco's Modification of Eagle's Medium supplemented with 20% (v/v) fetal calf serum and non–essential amino acids. HeLa cells were grown in monolayer culture in Glasgow Minimum Essential Medium supplemented with 5% (v/v) fetal calf serum, 5% (v/v) fetal serum and non–essential amino acids. All culture media routinely contained 100 U/ml penicillin and 100 µg/ml streptomycin. Cell stocks were cultured in 80 ml tissue culture flasks at 37° C in humidified atmosphere of 5% CO_2/95% air. Caco–2 and HeLa cells were passaged at confluence to 5 ml flasks at a density of $1 \cdot 10^6$/flask using 0.25% trypsin and 0.02% EDTA. The cells were allowed to grow in complete medium (containing serum) for 24 h before the start of experiments.

All cell cultures were incubated with various concentrations of extracts for 3 h or 24 h. The cells were then washed twice with PBS, pH 7.4, to remove any residual extract, removed from the flasks using trypsin/EDTA, spun at 200 g for 3 min at 4 °C and resuspended in low melting point agarose for comet analysis. Transformed cells were treated in some experiments with 100 µl of H_2O_2 for 5 min on ice. Treatment on ice minimizes the possibility of cellular processing of damage during the exposure period.

DNA breaks were detected using an adaptation of the single cell gel electrophoresis method (the comet assay) as described previously [12–14]. Briefly, cells were embedded in agarose on a microscope slide, lysed with detergent and high salt. At this point, if required, slides were washed three times with enzyme buffer (0.1 M KCl, 0.5 mM Na_2EDTA, 40 mM HEPES–KOH, 0.2 mg/ml bovine serum albumin, pH 8.0) and incubated with endonuclease III (which converts oxidized pyrimidins to strand breaks) or formamidopyrimidine glycosylase (FPG) which recognizes altered purines, in this buffer (or in buffer alone as control, detecting only strand breaks and alkali–labile sites). Then slides were electrophoresed at pH >13. Under these conditions, the presence of DNA breaks allows DNA to form a "comet tail", observed by fluorescence microscopy after staining with 4',6'–diamidine–2'–phenylindole dihydrochloride. The relative intensity of tail DNA fluorescence – assessed by visual scoring of 100 comets on a scale of 0–400 — reflects the break frequency. The comets were examined with a Zeiss Axioskop fluorescence microscope. One hundred comets on each slide (two slides from any experimental point) were scored visually as belonging to one of five classes according to tail intensity and given a value of 0, 1, 2, 3 or 4 (from undamaged 0 to maximally damaged 4). Thus, the total score for 100 comets range from 0 (all undamaged) to 400 (all maximally damaged). For statistical analysis Student's *t*–test was used.

RESULTS AND DISCUSSION

In a pilot experiment we studied possible effects of LP methanol and aqueous extracts on oxidized DNA bases in HeLa cells. Cells were incubated for 24 h with extract before endonuclease III and FPG treatment. The LPME at dose of 1µl, and LP aqueous extract at doses of 1 µl and 0.1 µl respectively decreased visual scores (in arbitrary units) after endonuclease III and FPG treatment (data are not shown). In all further experiments we used only these doses. It is noteworthy that without endonuclease and FPG treatment both extracts at any dose did not change the visual score. These data confirm the results obtained in our recent experiments [14].

Incubation of HeLa cells with LP aqueous extract (1µl), LP methanol extract (1 µl and 0.1 µl) for 3 h decreased significantly the visual score in endonuclease III treated cells (by 31.9–36.6%). Similar results were obtained in FPG treated cells — significant decrease by 27.5–32.5% (Table 1).

24 h incubation of HeLa cells with mentioned doses of LP extracts decreased significantly the visual score (29.1–42.4%) after endonuclease III treatment. Similar results were obtained with FPG treatment of cells after 24 h incubation with LP (27.6–38.3% significant decrease). The most effective in this experiment was LPME (1µl) decreasing the score by 42.4% (endonuclease III) and 38.3% (FPG) (see Table 1).

Analogous results were obtained with Caco–2 cells treated with both endonuclease III and FPG after preincubation with LP extracts. All doses of LP extracts decreased significantly the visual score by 26.3–31.9% (3 h incubation) and by 20.0–32.8% (24 h incubation) (Table 2). In all experiments LP extracts did not change the total score indicating that they lack the ability to damage DNA in HeLa and Caco–2 cells.

We studied also possible protective effects of LP extracts on DNA damage in HeLa and Caco–2 cells in–

Table 1. Net reduction in oxidized pyrimidines and purines (difference between 0 h and 3 h, 0 h and 24 h in comet score) in HeLa cells treated with "Loshtak" preparation extracts

Dose of LP extract, µl	Enzyme	Reduction in arbitrary units, mean ± SEM
Aqueous[1], 1.0	Endo III	15.5 ± 1.4*
Aqueous[2], 1.0		12.2 ± 0.5*
Aqueous[1], 0.1	Endo III	13.5 ± 1.9*
Aqueous[2], 1.0		10.1 ± 0.7*
Methanol[1], 1.0	Endo III	14.5 ± 1.2*
Methanol[2], 1.0		14.7 ± 1.0*
Aqueous[1], 1.0	FPG	11.2 ± 1.9*
Aqueous[2], 1.0		9.1 ± 0.4**
Aqueous[1], 0.1	FPG	9.5 ± 1.9*
Aqueous[2], 1.0		8.0 ± 0.3**
Methanol[1], 1.0	FPG	10.2 ± 1.7**
Methanol[2], 1.0		11.1 ± 0.5*

[1] Difference between 0 h and 3 h in comet score. Data of 3 experiments; 2 slides per experimental point were studied.
[2] Difference between 0 h and 24 h in comet score. Data of 4 experiments; 2 slides per every experimental point were studied.
In all cases differences are statistically significant at * $p < 0.01$ and ** $p < 0.05$ levels. The differences between negative control — buffer and LP extracts treatment are not significant in all cases (from 0.5 to 4.0).

Table 2. Net reduction in oxidized pyrimidines and purines (difference between 0 h and 3 h in comet score) in Caco–2 cells treated with "Loshtak" preparation extracts

Dose of LP extract, µl	Enzyme	Reduction in arbitrary units, mean ± SEM
Aqueous[1], 1.0	Endo III	10.3 ± 1.1*
Aqueous[2], 1.0		11.8 ± 0.9*
Aqueous[1], 0.1	Endo III	8.5 ± 0.7**
Aqueous[2], 0.1		15.0 ± 0.7*
Methanol[1], 1.0	Endo III	9.5 ± 0.8**
Methanol[2], 1.0		12.8 ± 1.1*
Aqueous[1], 1.0	FPG	5.8 ± 0.6***
Aqueous[2], 1.0		10.0 ± 0.6*
Aqueous[1], 0.1	FPG	7.7 ± 0.7***
Aqueous[2], 0.1		10.0 ± 0.8*
Methanol[1], 1.0	FPG	9.5 ± 0.8**
Methanol[2], 1.0		11.0 ± 0.7*

[1] Difference between 0 h and 3 h in comet score.
[2] Difference between 0 h and 24 h in comet score. In both cases data of 2 experiments; 2 slides per every experimental point were studied.
In all cases differences are statistically significant at * $p < 0.01$, ** $p < 0.02$ and *** $p < 0.05$ levels. The differences between negative control – buffer and extracts treatment are not significant (from –1.2 to 2.5).

Table 3. The effect of "Loshtak" preparation aqueous extracts. (1 µl) on hydrogen peroxide–induced DNA damage (mean ± SEM) in HeLa and Caco–2 cells

Treatment	Treatment time, h	Cell line	Total score, arbitrary units
No treatment	3	HeLa	14.2 ± 3.4
LPAE			17.8 ± 4.5
H_2O_2			166.0 ± 10.7*
LPAE + H_2O_2			142.8 ± 11.8*
No treatment	24	HeLa	12.2 ± 2.8
LPAE			9.8 ± 3.9
H_2O_2			148.8 ± 7.9*
LPAE + H_2O_2			138.0 ± 9.0*
No treatment	3	Caco–2	10.2 ± 2.2
LPAE			13.8 ± 3.4
H_2O_2			151.0 ± 8.4*
LPAE + H_2O_2			139.8 ± 7.3*
No treatment	24	Caco–2	13.2 ± 3.9
LPAE			10.8 ± 2.5
H_2O_2			141.1 ± 6.7*
LPAE + H_2O_2			130.2 ± 9.0*

* The difference between treatment and corresponding controls are significant at $p < 0.001$ level. No difference in score between H_2O_2 and LPAE + H_2O_2 variants.

duced by H_2O_2. The data presented in Table 3 show that no reduced scores were observed in experiments. It means that LP extracts lack ability to protect human transformed cells against exogenous DNA oxidative damage. But at the same time both aqueous and methanol extracts of LP protected significantly human transformed cells against endogenous DNA oxidative damage.

Some compounds of plant origin with antioxidative activity have been investigated for their possible DNA protective properties against endogenous and exogenous DNA strand breaks and oxidized DNA bases with various results. On the bases of contradictory results on genotoxicity, antimutagenic and anticarcinogenic potency of dietary bioflavonoids, it was suggested that the use of mentioned compounds to reduce cancer risk in humans should be carefully reconsidered [16].

In our experiments we have shown that LP extracts do not protect transformed human cells from oxidative DNA damage induced by H_2O_2. But at the same time they are active in prevention of endogenous oxidative DNA damage, like formonetin, dihydrogenistein and enterolactone [11]. It is noteworthy that LP extracts did not induce DNA damage in human cells either after incubation alone or after endonuclease III and FPG treatment. Another difference between LP extracts and other possible chemopreventive compounds is that LP lacks activity in any short–term genotoxic assays [10, 11].

Recent human studies support the experimentally based notion of oxidative DNA damage as an important mutagenic and apparently carcinogenic factor [17, 18]. However, the proof of a causal relationship of oxidative DNA damage and cancer in humans is still lacking [17]. In opinion of other investigators, oxidative modifications of DNA bases may play a role both in carcinogenesis and aging in rats [19], but these findings deserves further studies. LP was studied for possible carcinogenic activity and showed no such activity in mice and rats in long–term experiments [20]. At the same time it was shown that application of LP during lifetime led to decrease spontaneous tumor incidence from 18.2% in control to 8.3% in LP treated mice (subcutaneous injections) and from 25.0% in control to 15.4% in treated mice (oral applications). But in these experiments the difference between the control and experimental groups was not statistically significant because of small number of animals under study (n = 22–26). There were no significant differences in latency period and mean time of first tumor development. In rats there were no differences in any one parameter. The influence of LP on spontaneous tumor development warrants further investigations. We suppose that the mentioned phenomenon in mice may be connected with protection of their cells against endogenous oxidative damage.

LP like other well–known antioxidant compound of plant origin has antimutagenic property but, unlike some other antioxidants of plant origin, has no mutagenic (the Ames assay, rats' and mice bone marrow chromosomal aberrations and micronucleus assay) and genotoxic (the comet assay) property. The ability of LP to protect human cells against endogenous oxidative DNA damage contributes a new very useful property to other ones of LP (for example, lack of genotoxic and carcinogenic activities, antimutagenic effect).

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