

**EVALUATION OF THE EFFECTS OF EPHEDRINE ON HUMAN LYMPHOCYTES IN THE COMET ASSAY**

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*Ephedrine, a natural alkaloid from plants of the genus Ephedra, has a chemical structure similar to catecholamines. It is well established that catecholamines (adrenalin, noradrenalin and dopamine) cause genotoxic and mutagenic effects. Therefore, the objectives of this investigation were to examine whether ephedrine can exhibit genotoxic effects on isolated human lymphocytes in the Comet assay. Dose-response of human lymphocytes was determined at the concentration range of ephedrine from 0.0005 µM to 500 µM. Three concentrations of ephedrine (1, 50 and 300 µM) which had acceptable cell viability (over 90%) were used for further experiments with inhibitors of DNA reparation (cytosine arabinoside and hydroxyurea). The obtained results showed that ephedrine did not induce DNA damage in isolated human lymphocytes. However, co-treatment of the negative control with DNA repair inhibitors caused a slight but significant increase of DNA damage, due to an endogenous DNA damage. Interestingly, cells treated with ephedrine and DNA repair inhibitors did not express increased DNA damage. On the basis of the obtained results it can be concluded that ephedrine did not exhibit genotoxic effects on isolated human lymphocytes. This result is in accordance with previous investigations showing negative genotoxicological results for ephedrine using bacterial gene mutation test-systems and in vitro cytogenetic analysis.*

*Key words: Comet assay, DNA damage, DNA repair inhibitors, ephedrine*

**INTRODUCTION**

Ephedrine is an alkaloid derived from various plants of the genus *Ephedra*. The herb *Ephedra sinica* contains ephedrine and pseudoephedrine as its principal active compounds. In traditional Chinese medicine, ephedrine has been used in the treatment of asthma, bronchitis, nose and lung congestion and fever

with anhydrosis for centuries (Ford *et al.*, 2001). Both ephedrine and pseudoephedrine act as a bronchodilator and increase blood pressure, but pseudoephedrine has considerably less effect (Drew *et al.*, 1978).

Except for being used as a nasal decongestant (Ma *et al.*, 2007), ephedrine is also used in thermogenic weight loss pills which contain a combination of ephedrine, caffeine and aspirin. This dietary supplement combination is also taken by body builders before workouts (Greenway and Bray, 2008; Zheng *et al.*, 2009).

In veterinary medicine ephedrine may be used to help control urinary incontinence in dogs and cats (Carofiglio *et al.*, 2006). It may also be used to open up the air passages of the lungs and to relieve nasal congestion (Koss *et al.*, 2002).

Ephedrine is an indirectly acting sympathomimetic amine, which acts mainly by stimulating noradrenaline release from presynaptic terminals and thereby stimulating the postsynaptic adrenergic receptors. Ephedrine probably does not interact directly with  $\alpha$  receptors (Ma *et al.*, 2007), but it can stimulate  $\beta$  receptors (Vansal and Feller, 1999).

There are some controversies about the safety of ephedra use. Indiscriminate consumption of ephedrine-type alkaloids has resulted in more than 1000 reported cases of poisoning and other serious effects, some of which were fatal, in the period 1993-2000. in the USA (FDA, 2000). The most serious side effects of ephedrine overdose include hypertension, heart palpitations, psychosis, tremors, myocardial infarction, seizures and stroke. Other signs of ephedrine abuse include nerve damage, muscle injury, memory loss and insomnia.

Despite numerous data concerning toxic effects of ephedrine, there is only limited information about possible genotoxic effects of ephedrine. Ephedrine has not exhibited genotoxic effects in bacterial gene mutation assays and *in vitro* cytogenetic studies (Brambilla and Martelli, 2009). However, to our knowledge, genotoxic effects of ephedrine were not evaluated using single cell gel electrophoresis (Comet) assay. Since the Comet assay is a very sensitive method for detection of DNA damage, the objective of this investigation was to evaluate the effects on isolated human lymphocytes using the *in vitro* Comet assay. For the sake of comparison, we chose human lymphocytes instead of animal lymphocytes because most of the *in vitro* genotoxicological studies on hormones were performed on human lymphocytes.

Moreover, there are some literature data that various catecholamines (adrenaline, noradrenaline and dopamine) may exhibit genotoxic and mutagenic effects (Moldeus *et al.*, 1983; Djelić and Anderson, 2003). Since ephedrine has a similar chemical structure to catecholamines we wanted to examine whether ephedrine can induce DNA damage. Otherwise, the catechol group may be considered necessary for genotoxic effects.

## MATERIALS AND METHODS

### *Blood samples and treatment*

In these experiments we used peripheral venous blood from three healthy volunteers, less than 25 years of age. Heparinized blood samples (sodium heparine, Galenika, Belgrade, Serbia) were immediately processed for isolation of lymphocytes on ficol gradient. The 50  $\mu\text{L}$  of lymphocyte suspension was incubated in PBS solution containing ephedrine hydrochloride (CAS No 50-98-6, Sigma, St. Louis, MO) at concentrations in a range from 0.0005  $\mu\text{M}$  to 500  $\mu\text{M}$ . The negative control was PBS, which is the solvent for ephedrine. The positive control was 100  $\mu\text{M}$  aqueous solution of  $\text{H}_2\text{O}_2$ . Lymphocytes were incubated at 37°C for 1 h. The study was approved by the local Medical Ethics Committee, performed in accordance with the Declaration of Helsinki, and informed donor consent was also obtained.

### *Comet assay*

Before each experiment, microscope slides were precoated with 1% normal agarose (Sigma, St. Louis, MO) in double distilled water and left at room temperature to allow agarose to dry. The alkaline single cell gel electrophoresis (Comet) assay was performed on isolated human peripheral blood lymphocytes. Lymphocytes were isolated using Amersham ficol gradient as described elsewhere (Soltani *et al.*, 2008). The cell suspension in PBS was treated with ephedrine at concentrations 0.0005  $\mu\text{M}$ , 0.001  $\mu\text{M}$ , 0.01  $\mu\text{M}$ , 0.2  $\mu\text{M}$ , 1  $\mu\text{M}$ , 5  $\mu\text{M}$ , 50  $\mu\text{M}$ , 150  $\mu\text{M}$ , 350  $\mu\text{M}$  and 500  $\mu\text{M}$  for one hour at 37°C. After the treatment, cell suspensions were centrifugated at 2000 rpm for 5 min, and the cell pellet was mixed with an equal amount of low melting point agarose (Sigma), rapidly placed on precoated microscopic slides covered with a cover-slip and allowed to solidify for 5 min at 4°C. Then the coverslips were gently removed, and the 1% agarose was placed, covered with a coverslip, left for 5 min at 4°C, then the coverslip was removed and the slides were placed overnight in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X100 and 10% DMSO, pH 10 adjusted with NaOH). After lysis, the slides were placed in electrophoresis buffer (10 M NaOH, 200 mM EDTA, pH 13) for 30 min. at 4°C in the dark to allow DNA unwinding. Electrophoresis was carried out for 30 min. at 25 V and 300 mA. Finally, the slides were gently rinsed with a neutralising solution (0.4 M Tris base, pH 7.5) three times, 5 min each time. Staining of DNA was accomplished with 50  $\mu\text{L}$  of ethidium bromide (20  $\mu\text{g}/\text{mL}$ ) per each slide. The comets were observed and analysed using Olympus X 50 microscope (Olympus Optical Co., GmbH Hamburg, Germany), equipped with a device for fluorescence recording at 100 $\times$  magnification. Evaluation of DNA damage was performed as described by Anderson *et al.* (1994). Namely, cells were graded by eye into five categories corresponding to the following amounts of DNA in the tail: (A) no damage, <5%; (B) low level damage, 5-20%; (C) medium level damage, 20-40%; (D) high level damage, 40-95%; (E) total damage, >95% (Fig. 1).

In order to obtain semi-quantitative analysis of data, the score of DNA damage (the migration of DNA) was calculated as follows:  $2 \times B + 3 \times C + 4 \times D +$

5×E, where B to E represents percentages of cells within the above mentioned categories B to E.

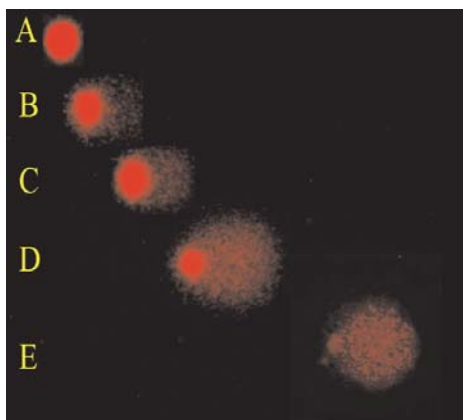


Figure 1. Five classes of comets: (A) no damage, <5%; (B) low level damage, 5-20%; (C) medium level damage, 20-40%; (D) high level damage, 40-95%; (E) total damage, >95%

#### *Statistical analysis*

Data from the Comet assay were evaluated by the non-parametric Kruskal-Wallis ANOVA followed by the Dunn's multiple comparison test. A *P*-value of  $\leq 0.05$  was considered as indicative of statistical significance.

## RESULTS

DNA damage analysis of a wide range of ephedrine concentrations is presented in Fig. 2. In this investigation, we evaluated the effects of a broad spectrum of ephedrine concentrations (range from 0.0005  $\mu\text{M}$  to 500  $\mu\text{M}$ ). However, we did not observe significant changes of the DNA migration in the Comet assay and the cytotoxicity was acceptable (lower than 10%). Thus, percentage of cells without DNA damage was in a range from 72% to 89.5% in vials treated with various concentrations of ephedrine. As for the negative control (PBS), there was 91% of cells without DNA damage (estimated less than 5% of damaged DNA). Only the positive control (100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ) gave a significant rise in DNA damage, so there was only 32% of undamaged cells. Moreover, the distribution of DNA damage in cells treated with 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  clearly showed an increase of the percentage of DNA damage for each of the four categories (B to E).

In order to evaluate the possible changes of DNA damage under the influence of DNA repair inhibitors we carried out two separate experiments with cytosine arabinoside (AraC) and hydroxyurea (HU). Namely, in the first experiment (Fig. 3) we tested three concentrations of ephedrine (1, 50 and 300  $\mu\text{M}$ ) without repair inhibitors, and the same concentrations with

reparation inhibitors (20  $\mu\text{M}$  of AraC + 2000  $\mu\text{M}$  HU). The percentage of undamaged cells was slightly lower in vials with reparation inhibitors, but it did not reach statistical significance. In the second experiment (Fig. 4) we used the same concentrations of ephedrine, but concentrations of reparation inhibitors were higher (40  $\mu\text{M}$  of AraC + 4000  $\mu\text{M}$  HU). The obtained results were similar to the first experiment – lower, but insignificant percentage of undamaged cells. Only the co-treatment of the negative control with higher concentrations of inhibitors of

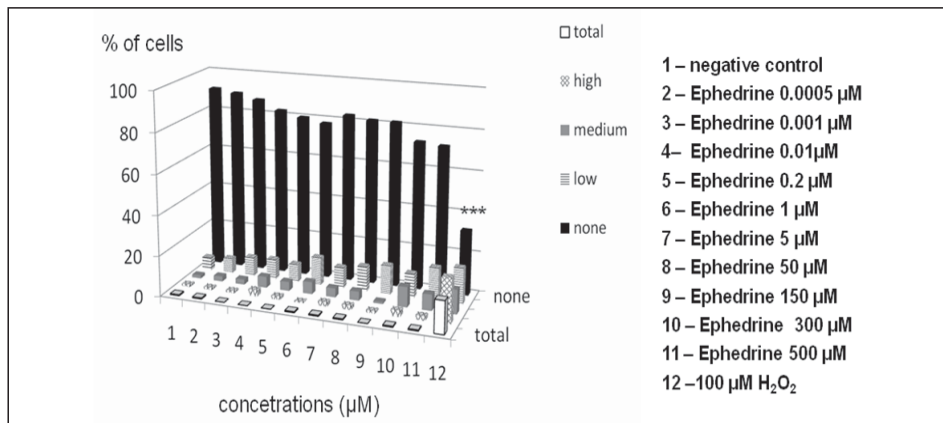


Figure 2. Dose-response analysis of the effects of ephedrine on isolated human lymphocytes. Only the positive control caused significant increase of DNA migration. \*\*\* $p < 0.001$

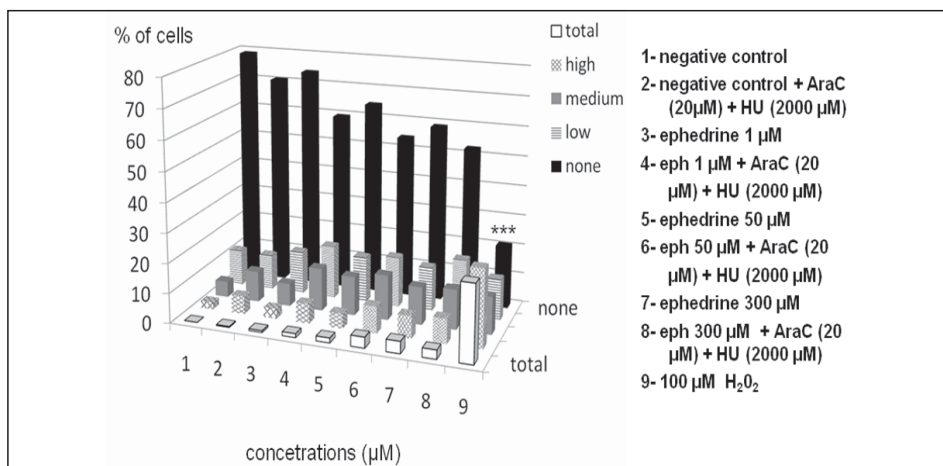


Figure 3. Analysis of DNA damage in isolated human peripheral blood lymphocytes simultaneously treated with ephedrine and DNA repair inhibitors (20  $\mu\text{M}$  cytosine arabinoside and 2000  $\mu\text{M}$  hydroxyurea). \*\*\* $p < 0.001$

reparation produced a slight, but statistically significant rise in DNA damage. Interestingly, DNA damage in the negative control co-treated with reparation inhibitors did not differ significantly from damage in cells treated with ephedrine and reparation inhibitors.

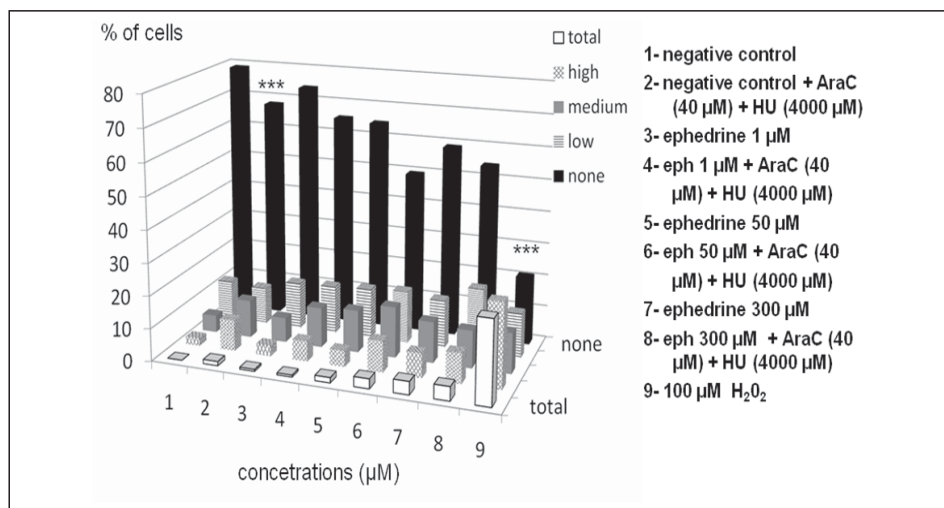


Figure 4. Analysis of DNA damage in isolated human peripheral blood lymphocytes simultaneously treated with ephedrine and DNA repair inhibitors (40 µM cytosine arabinoside and 4000 µM hydroxyurea). Samples treated with AraC and HU only had significantly higher DNA damage compared to the negative control without AraC and HU, due to an endogenous DNA damage. Positive control caused significantly higher DNA damage in comparison to all other experimental points. \*\*\* $p < 0.001$

## DISCUSSION

Ephedrine has a similar chemical structure to catecholamines. There are experimental findings showing that catecholamines (adrenaline, noradrenaline and dopamine) may exhibit genotoxic effects in various test-systems (Moldeus *et al.*, 1983; McGregor *et al.*, 1988; Miura *et al.*, 2000; Djelić and Anderson, 2003; Dobrzynska *et al.*, 2004). Therefore, the main idea of this investigation was to evaluate possible genotoxic effect of ephedrine and compare it with effects of catecholamines.

A broad spectrum of concentrations of ephedrine (range from 0.0005 µM to 500 µM) was investigated in the *in vitro* Comet assay on isolated human lymphocytes. At all applied concentrations, ephedrine has caused less than 10% of cytotoxicity evaluated by Trypan blue exclusion assay, so the conditions in the Comet assay were appropriate for detection of DNA damage. Lymphocytes treated with ephedrine alone, did not express increased DNA damage in comparison to the negative control. However, co-treatment of the negative control with higher concentrations of DNA repair inhibitors (40 µM AraC+ 4000 µM HU)

caused a slight, but significant increase of DNA damage. Possibly, an increase of DNA damage of human lymphocytes co-treated with AraC + HU results from unrepaired endogenous DNA damage. Therefore, it can be concluded that ephedrine does not induce DNA damage in isolated human lymphocytes.

Absence of genotoxic effects of ephedrine obtained in the present study is in accordance with results of several studies on ephedrine genotoxicity in various test systems. Thus, ephedrine has not exhibited genotoxic effects in *Salmonella* mutagenicity tests (Zeiger *et al.*, 1988). In addition, ephedrine did not induce chromosome aberrations *in vitro* (Hilliard *et al.*, 1998).

Bearing in mind that various catecholamines exhibit genotoxic effects, it seems that the catechol group is necessary for these effects. Namely, although ephedrine has a similar chemical structure to catecholamines, it lacks hydroxyl groups in the phenolic ring, so it does not possess the catechol moiety. There is experimental evidence that aromatic catechol groups are necessary for signal transduction after binding of catecholamines to specific membrane receptors (Liapakis *et al.*, 2004). However, catechol moieties can be also involved in redox cycling under the influence of superoxide anion and, therefore, may induce oxidative stress (Genova *et al.*, 2006). Some indirect experimental data corroborate the idea that DNA damage induced by catecholamines mainly results from reactive oxygen species (ROS). Namely, the antioxidant catalase significantly reduces DNA damaging effects of noradrenaline in the Comet assay on human lymphocytes (Djelić and Anderson, 2003) and sperm (Dobrzynska *et al.*, 2004). Therefore, we assume that ephedrine has not expressed genotoxic effects not only in this study, but also in other test-systems probably because it contains the phenolic moiety instead of the catechol moiety which is present in catecholamines.

It should be mentioned, however, that in some test-systems, such as *in vitro* cytogenetic test, adrenaline does not exhibit genotoxic effects (Djelić *et al.*, 2003). Among other hormones which can induce ROS, the contradictory results of genotoxicity evaluation are also observed for oestradiol (Djelić *et al.*, 2006) and thyroxine (Djelić *et al.*, 2007).

In conclusion, although the use of ephedrine can produce serious pharmacological side-effects sometimes including a fatal outcome, it seems that short-term exposure to ephedrine do not induce genotoxic effects. Bearing in mind previous and present studies on ephedrine genotoxicity, probably there is no genetic risk from *Ephedra* use in medicine.

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## EVALUACIJA DEJSTVA EFEDRINA NA LIMFOCITE ČOVEKA U KOMET TESTU

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### SADRŽAJ

Efedrin, prirodni alkaloid iz biljaka roda *Ephedra*, ima sličnu hemijsku strukturu sa kateholaminima. Dobro je poznato da kateholamini (adrenalin, noradrenalin i dopamin) mogu da prouzrokuju genotoksične i mutagene efekte. Stoga su ciljevi ovog istraživanja bili da se ispita da li efedrin može da ispolji genotoksične efekte na izolovanim limfocitima čoveka u Komet testu. Odnos doza-efekat određen je u rasponu koncentracija efedrina od  $0.0005 \mu\text{M}$  do  $500 \mu\text{M}$ . Tri koncentracije efedrina (1, 50 and  $300 \mu\text{M}$ ) koje su imale prihvatljiv nivo ćelijske vijabilnosti (preko 90%) upotrebene su za dalje eksperimente sa inhibitorima reparacije DNK (citozin arabinozid i hidroksiurea). Dobijeni rezultati pokazuju da efedrin nije indukovao oštećenja DNK na izolovanim limfocitima čoveka. Međutim, istovremeni tretman sa inhibitorima reparacije DNK doveo je do malog ali statistički značajnog porasta oštećenja DNK kod negativne kontrole, usled endogenog oštećenja DNK. Interesantno je da ćelije tretirane sa efedrinom i inhibitorima reparacije DNK nisu ispoljile povećan nivo oštećenja DNK. Na osnovu dobijenih rezultata može se zaključiti da efedrin nije ispoljio genotoksične efekte na izolovanim limfocitima čoveka. Ovaj rezultat je u saglasnosti sa prethodnim istraživanjima u kojima je dokazano da efedrin ne dovodi do genotoksičnih efekata u bakterijskim testovima na genske mutacije i u *in vitro* citogenetičkim analizama.

