IN VITRO EVALUATION OF MUTAGENIC EFFECT OF VITAGNUS AND SHIRAFZA IN HUMAN LEUKOCYTES BY SINGLE CELL GEL ELECTROPHORESIS

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Abstract

The use of herbal drug in Iran has a long history. In spite of the place and efficacy of medicinal plants it is worthy to evaluate their potential mutagenicity and genotoxicity. Vitagnus and Shirafza are herbal medicines, which are widely used in Iran. The purpose of this investigation was to find out the potential mutagenic effect of these herbal drugs by performing Single Cell Gel Electrophoresis (SCGE) or Comet assay in human Leukocytes. In this method the positive control group received H_2O_2 at dose of 100µl as a standard mutagenic agent. The test groups received Vitagnus and Shirafza at doses of 10, 50, 100, 500 and 1000 µl. The following parameters were used as indicators for mutagenicity measurement. No migration, short migration and long migration and the results of the test groups were compared with the mutagenic agent. After analysis of the data the following result were obtained: The results of Shirafza indicated that the drug is safe as did not show any DNA damage or migration. Similarly Vitagnus at low doses did not show any significant mutagenic effect. However, at high dose (500 µl) had considerable effect as pointed in long migration. In Conclusion: from the above results and data obtained it is clear that Vitagonus at dose of 500 µl seems to have DNA damaging effect but to prove this statement it is better to perform in vivo studies on different organ / tissue for evaluation of genotoxicity of this herbal drug.

Keywords:

Mutagenicity, Vitagnus, Shirafza, Comet assay

Introduction

There has been an increase used of herbal drugs due to their less side effects as compared with synthetic drugs and greater acceptance especially by medical; practitioners. Acceleration in the use of herbal medicine in Iran lead to perform toxicity and safety tests in this kind of medicines. Darly et al., introduced Single Cell Gel Electrophoresis or comet assay in 1984 as microelectrophoretic technique for the direct visualizations of DNA damage (1). The comet assay is a sensitive, fast and reliable technique which is widely applied for evaluation of pharmaceutical, chemical and herbal products for genotoxicity. Also it provides an excellent approach to detect small number of DNA single strand break in individual mammalian cells (2). The sensitivity of comet assay depends on the accurate and the reproducible measurement of DNA in the comet head and tail regions (3). Single Cell Gel Electrophoresis can produce DNA fragments observed in the form of a comet (head and tail) as the fragment is separated between the cathode (head) and anode (tail) (4,5). The aim of this study

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was to assess mutagenicity potential of Vitagonus and Shirafza by this method.

Materials and Methods

The following materials were used: Hanks Balance Salt Solution (HBSS) calcium and magnesium free was obtained from GIB company New York and prepared according to Bio whitaker Company formula in our laboratory. Low melting point agarose was purchased from Merck, Germany, Disodium salt (Na_2-EDTA), Trice base ethidium bromide and Triton X100 were purchased from Sigma Chemical Company. Hydrogen peroxide (H_2O_2) 30% solution obtained from Fisher Scientific. Co. Calcium and magnesium free Phosphate Buffer Saline (PBS) was purchased from GIB Company. Electrophorese instrument, centrifuge, fully frosted slides, centrifuge tubes, fluorescent microscope, glass wears and other materials were obtained from our toxicology laboratory. From Vitagnus and Shirafza which are available in the form of drops in Pharmaceutical market in Iran doses of 10, 50, 100, 500l and 1000 µl/ml, were prepared. In Single Cell Gel Electrophoresis method about 10000 cells are needed since each microliter of human blood contains about 7000 leukocytes; therefore for each concentration of drug less than 1 µl whole blood is required. With lancet blood was taken in microtubes and 1µl of Hanks Balance Salt Solution (HBSS) was added and centrifuged for 10 minutes at 3500 rpm. After centrifugation the supernatant was discarded and the lower layer, which contained lymphocytes, was diluted with 1ml HBSS and each 100 µl of this mixture contains about 3500 leukocytes (5,6). For each drug seven micro tubes were used and to each tube doses of 10, 50, 100, 500 and 1000 µl of test herbal extract were added. Tube six (negative control) contained distilled water and tube seven (positive control) contained H_2O_2. All the above tubes were incubated for 40 minutes at 37^{o}C to show the effect of tested material on lymphocytes cells and by this time the lysine solution was prepared and kept at 4^{o}C. All content of tubes were centrifuged for 10 minutes at 3500 rpm. The upper layer was discarded and 125 mg/25ml of 0.5% solution from low melting point agarose in phosphate buffer solution was prepared and mixed with lymphocytes cell suspension (7). Form this suspension 100 µl was applied smoothly on each microscopic slide. All slides were kept in lysine solution for one hour. To prevent probable damages by light, thus it is better to perform the rest of procedure in dark or under red light or rapped in aluminum foil (8). Then slides were removed from lysine solution and kept in electrophorese set for an hour at 25 V and 300 mA for electrophoresis (by providing salty bridges to prepare a better conductivity). Slides were washed three times with neutral buffer and stained with ethidium bromide for 15 minutes. After staining, all the slides were washed and cover slip was placed on each slide. These slides can maintain their properties for at least 72 hours at 4^{o}C (9,10). Fluorescence microscope (with mercury filter) at 560 nm was used to analyze slides for DNA migration. In each slide 25 cells were randomly selected and studied. The migration pattern or length of comet measured for no migration, short migration, medium migration and long migration.

Results

Effect of different doses of Vitagnus and Shirafza in comparison with H_2O_2 on human leukocytes cells are given in Table 1. The migration pattern of DNA in positive control group (H_2O_2) is shown in Fig. 1. The results obtained in this study indicate Vitagnus had some

mutagenic effect, which is dose dependant. For example at dose of 500 µl the migration pattern was long and it was comparable to positive group (Fig. 2). However, in case of Shirafza it seems that the drug is safe because the migration pattern was not significant as compeered with positive group (Fig. 3).

Table 1: Effect of different doses of Vitagnus and Shirafza and comparison with H_2O_2 on leukocytes cell migration

	-ve C	+ve C (H_2O_2)	V 10µl	V 50µl	V 100µl	V 500 ml	V 1000µl	S 10 µl	S 50 µl	S 100µl	S 500µl	S 1000µl
Nomigration	24	1	20	19	19	11	7	23	23	21	20	17
Medium Migration	1	1	4	5	4	7	7	1	0	2	2	5
Short Mirgation	0	9	1	1	2	6	8	1	2	2	2	2
Long Migration	0	14	0	0	0	1	3	0	0	0	1	1
Calculated Index	0.007	0.407	0.040	0.047	0.053	0.147	0.213	0.020	0.027	0.040	0.060	0.08

-ve C= Negative control +ve C= Positive control V= Vitagnus S= Shirafza

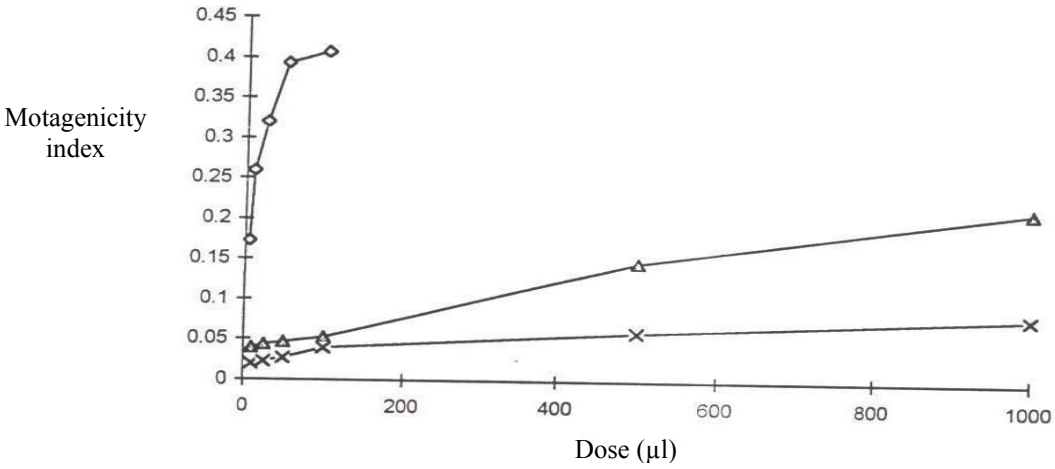

Fig. 1: Human leukocytes exposed to different concentrations of Vitagnus, Shirafza and H_2O_2.
Note: ◊= Positive control (H_2O_2); Δ= Vitagnus; x = Shirafza

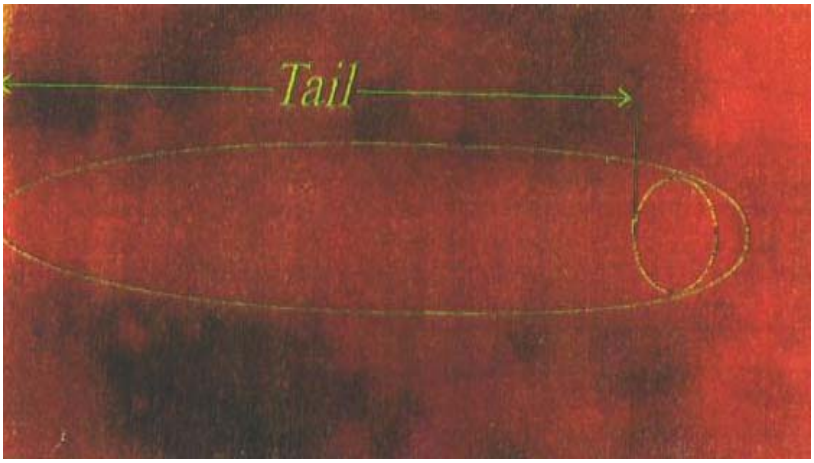

Fig. 2: Human leukocytes exposed to H_2O_2 (Long migration)

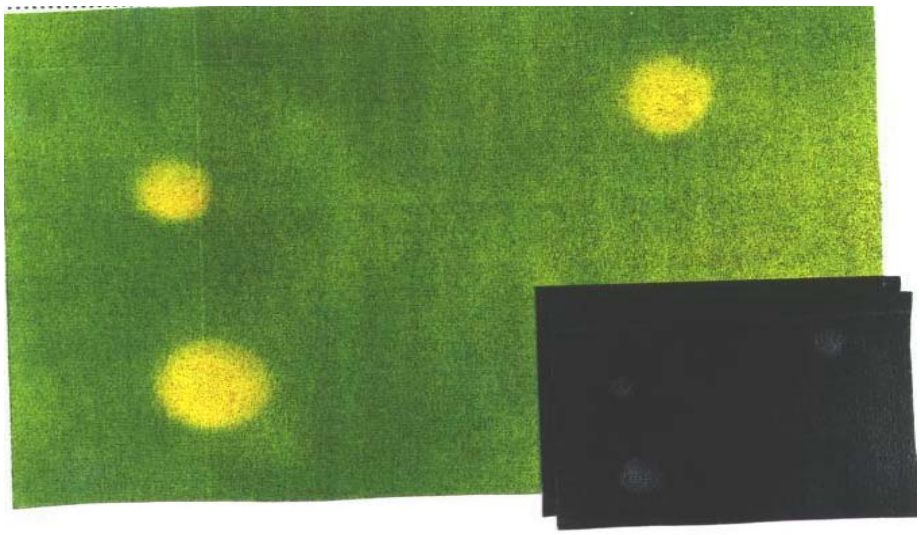

Fig. 3: Human leukocytes exposed to Shirafza (500 µl)

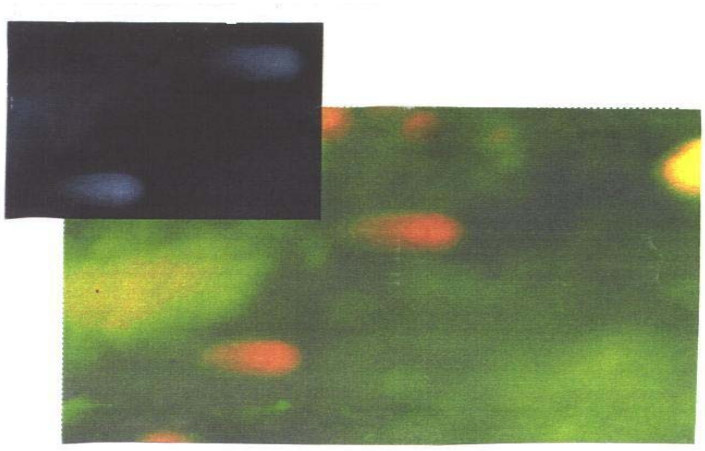

Fig. 4: Human leukocytes exposed to Vitagnus (500 µl)

Discussion

Neil and Kim in 1995 reported that DNA damage could be measured by applying SCGE, which is a rapid, most sensitive and reliable technique (8). A great advantage of the comet assay is the possibility to analyze single cells for DNA damage, thus offering the opportunity to study human lymphocytes.

The comet assay detects DNA strand breaks induced directly by genotoxic agents as well as DNA degradation due to cell death. (11). Thus it is essential to perform toxicity test for these mentioned herbal medicines. For these reasons we tested that whether these drugs are safe or not. The DNA damage was detected after exposure of Vitagnus and Shirafza as apart of DNA migration. In experiments conducted for two herbal drug drops indicated that Vitagnus was capable of increasing DNA migration in dose dependant manner.

The use of head and tail (Comet) as an indication of DNA damage was originally based on the observation that both, the amount of DNA in the comet tail and the length of tail were increased. These results wee compared with H_2O_2 (mutagenic agent) as positive control and (no mutagen) as negative control. It has been reported that (H_2O_2) produces free radicals which causes damage to the DNA of the lymphocyte cells (12). This study revealed that Vitagonus showed a significant DNA damage at high dose as compared to Shirafza. However, one should find out how this change in DNA takes place. For example, is DNA fragmentation in SCGE move towards anode and after staining they become as a comet. According to Narendra et al., 1998 the damage of DNA is directly proportional to the length of comet (13). The microscopic analysis and resultant calculation of a number of affected damaged cells were comparable to the results of other observations of previous studies using SCGE (13-16).

Conclusion

This method of detection provides an efficient technique to determine the genotoxicity of herbal products. Due to its simple design, it is very easy to use and runs very fast , although, it is based on a rather slow interpreted. We concluded that the increase of DNA migration induced by drug.

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