AMELIORATION EFFECT OF AMLA (*Phyllanthus emblica*) ON DNA DAMAGE INDUCED BY SODIUM FLURIDE

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Abstract : The comet assay (single cell gel electrophoresis assay) is an uncomplicated and sensitive technique for the detection of DNA damage at the level of the indivisdual eukaryotic cell. In the present study DNA damages was assessed by two comet parameters i.e. tail length and olive tail moment and it was found that the amla (*Phyllanthus emblica*) fruit extract showed amelioration effect against sodium fluride induced genetoxicity. It was observed that the tail length and olive tail moment of sodium fluoride induced damage at the significant level.

Keywords : Comet assay, DNA damage, *Phyllanthus emblica*, sodium fluride.

INTRODUCTION

The comet assay **(**single cell gel electrophoresis assay) is an uncomplicated and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell. It was first developed by Ostling and Johansson in 1984 and later modified by Singh et.al. in 1988. Since it has increased in popularity as a standard technique for evaluation of DNA damage/repair, biomonitoring and genotoxicity testing. The term "Comet" refers to the pattern of DNA migration through the electrophoresis gel, which often resembles a comet (Tice, 2000; Kumar et.al., 2011).

Electrophoresis at high pH results in structure resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head effects the number of DNA breaks. The likely basis for this is that loops containing a break lose their super coiling and become free to extend toward the anode. This is followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage. This can be performed by manual scoring or automatically by imaging software (Collins, 2003; Nandha, et.al., 2011). A very wide range of samples including cultured cells, buccal mucosal cells, solid tumor, cancer cells, sperm, yeast cells, bacteria cell etc. can be subjected to SCGE which makes the assay more versatile.

The amount of DNA liberated from the head of the comet depends on effects of the mutagen under evaluation. The brighter and longer the tail have the higher the level of damage. DNA strand breakage is a sensitive marker of genotoxic damage. These strands breaks are potentially pre-mutagenic lesions (Kammann et.al, 2001).

Two principle in the formation of the comet are : (i) DNA migration is a function of both size and the number of broken ends of the DNA, and (ii) Tail length increases with damage initially and then reaches a maximum that is dependent on the electrophoresis conditions, not the size of fragments.

Saleha et.al. (2001) shared a significant increase in comet tail length in blood cells of Arsenic treated mice which clearly gives evidence that arsenic causes DNA damage. The DNA damage potential of sodium arsenite was also confirmed by the *in vivo* comet assay in bone marrow cells of mice (Bandupadhyay, 2013). Therefore, the present work was under taken to study, the DNA damage induced by sodium fluoride and amelioration effect of amla fruit extract if any by the *in vivo* comet assay in bone marrow cells of mice.

MATERIAL AND METHOD

The comet assay was carried out under alkaline condition basically described by Singh et.al. (1988) with slight modification described by Buschini et.al. (2004).

Adult mice of both sexes were separated into four different groups for various treatments as mentioned in table 1.

Table 1 : Treatment Protocol

Sr. No.	Experimental Variant	Symbol	Dose
01	Control	C	No (SF or A)
02	Sodium fluoride	SF	2 ppm (Kumari, 2009)
03	Amla	A	150 mg/kg (Ali et.al., 2013)
04	Sodium fluoride and Amla Concurrently	SF + A	As 2 & 3

After the completion of the treatment the bone marrow cells of mice were extracted from its both femora. The proximal end of femora was cut carefully then about 5 ml of PBS solution (previously incubated at 4°C) was taken in a syringe and its needle was inserted into the proximal part of the femora. By gentle aspiration as well as by flushing the marrow was forced out through the opening around the needle and it was collected in a centrifuge tube as a fine suspension. Comet assay was performed to assess the DNA damage in control, SF, A and SF+A treated of bone marrow cells of mice. 10^5 cells (~ 100 µl sample) were taken and mixed with 200 µl of 0.8% low melting point agarose prepared in 0.9% saline at 39°C. The resulting suspension was layered on the top of fully frosted slides in a zig-zag way and left it in room temperature for 5 minutes. After preparation slides were kept on ice for 15 minutes to allow solidification. After solidification containing box at 4°C wrapped with aluminum foil and kept at 4°C for 1 hour to incubate. The slides were transferred in gel electrophoresis plates/unit with alkaline buffer to incubate for 20 minutes without current. Electrophoresis was then carried out under neutral/highly (pH >13) conditions for 30 minutes at 15 V, 200 mA

i.e. at the rate of 0.6 V/cm using a compact power supply. After electrophoresis, the slides were washed gently with 0.4 M Tris (pH 7.5) to remove the alkali and detergents. Slides were transferred to humid chamber and incubated to prevent the drying of gel. The slides were stained with Propidium iodide (40 µg/ml) and images (70-80 per slide) were grabbed by using fluorescent microscopic (Nikon, Japan) with digital camera (Gupta et.al., 2015). Images were analyzed by using the CASP software for the parameters tail length (TL) and olive tail moment (OTM). Olive tail moment = (tail mean-head mean) × tail % DNA/100; tail moment is = tail length × tail % DNA (tail intensity)/100.

Data were analyzed by ANOVA and the difference between control cells and treated cells were analyzed by post hoc. Dunnett's test or Newman-Keuls Multiple Comparison test. Dunnett's test was performed for comparison between control and Sodium fluoride-treated cells and Newman-Keuls Multiple Comparison were used between SF and SF + A treatment. Values are expressed as the mean ± standard error of the mean. P values less than 0.05 were considered statistically significant.

RESULT

DNA damage was assessed by two comet parameters: tail length and olive tail moment. There is significant increase in both tail length and olive tail moment in SF treated group in comparison to control and amla treated group (table 2 and graph 1 and 2). When tail length and OTM observed in the group fed concurrently with sodium fluoride and amla fruit extract were 14.09 ± 1.57 and 3.02 ± 1.49 respectively. SF + A treated mice significantly ($P < 0.001$) decreased tail length and OTM. Thus, amla fruit extract showed ameliorating effect against sodium fluoride induced genotoxicity.

Table 2 : Mean and standard error Mean of comet tail moment and olive tail moment of cells from bone marrow of mice treated with sodium fluoride and Amla fruit extract.

Treated Group	TL	OTM
	Mean ± SEM	Mean ± SEM
C	13.50 ± 1.26	3.09 ± 1.55
SF	31.46 ± 2.35[a,b]	10.43 ± 1.55[a,b]
A	11.03 ± 1.91	2.76 ± 1.06
A + SF	14.09 ± 1.57	3.02 ± 1.48

Notes : Values expressed as mean ± SEM and were analyzed by using one-way analysis of variance (ANOVA) for multiple comparisons. Newman-Keuls and Dunnett's tests were used to examine the differences between samples. (a = $p < 0.001$ control vs. SF; b = $p < 0.001$ SF vs. SF + A).

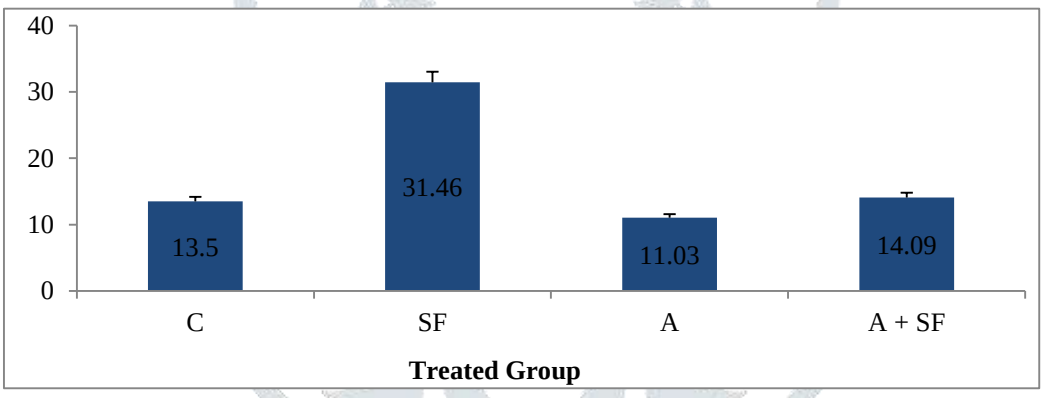


Fig. 1

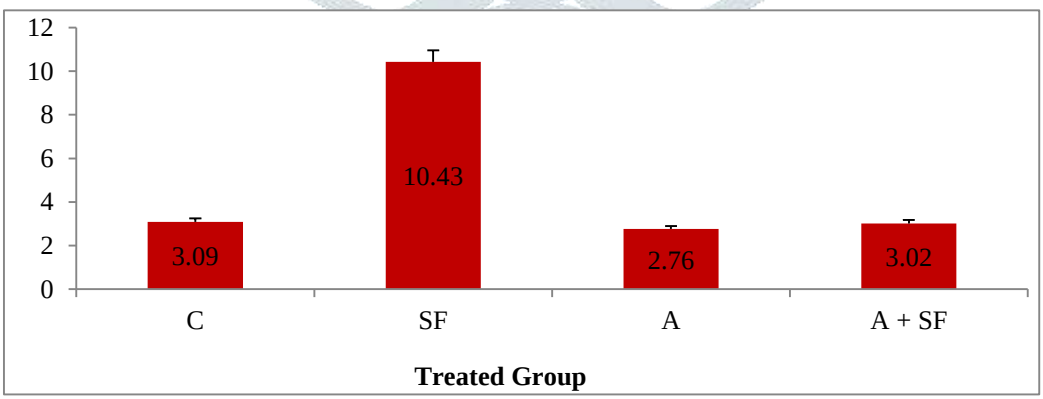


Fig.2

Graphical representation (Fig. 1 & 2) showing the significant increase in DNA damage after SF treatment. Co-treatment with amla attenuated the SF-induced DNA damage at low concentrations, data are expressed as Mean I standard error mean analyzed by ANOVA post hoc. Dunnett's tests and Newman-Keuls Multiple comparison test. ($P < 0.001$, control vs. SF treated; $p < 0.001$, SF vs. SF + A).

DISCUSSION

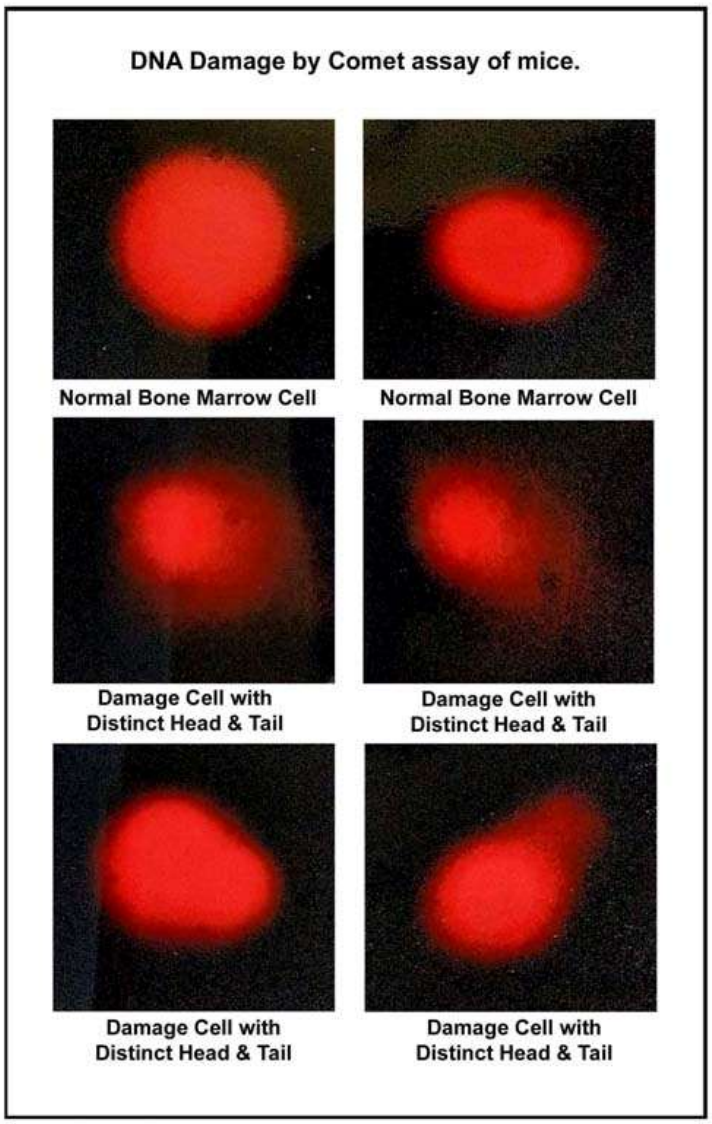
DNA damage induced by fluoride is as following :

1. Fluoride can induce the production of free radicals which can damage DNA strands directly or by lipid peroxidation initiated by free radicals (Wang et.al., 2004; Lobo et.al., 2010).
2. Fluoride may depress enzyme activity such as DNA polymerase which might further affect the process of DNA replication or repair and thereby damage DNA (Aardema and Trutsui, 1995; World, 2006; Department of Environmental Health, 2014).

At the molecular level, free radicals produce breaks in one or both stand of the DNA molecule. Oxyradicals also oxidize bases. Xenobiotics and their metabolite many bind covalently to a base or less frequently another portion of the DNA molecule to form an adduct (Shugart, 1995).

In our study the considered bone marrow cell and germ cell because bone marrow and spermatogenic tissue are very complex not only in compression but also from the kinetic point of view, there are many lines of differentiating cell types, many stages of differentiation and enormous hierarchy of feedback mechanism constantly monitoring the animal status and requirements. Our results showed that sodium fluoride induced significant increase in comet tail length and olive tail moment and amla fruit extract could ameliorate the genetoxicity at significant level. Consequently, the increment in the length of migration observed could be reflecting DNA single strand breaks as a consequence of incomplete excision repair sites. DNA stand breakage is a sensitive marker of genetoxic damage, these stand breaks are potentially pre-mutagenic lesion (Kammann et.al., 2001). Under standard conditions, these comet assay detects the amount of cells with DNA single strand breaks. Review of previous literature showed that DNA effects in the comet assay may be caused by the inhibition of repair of induced DNA damage.

PLATE - A



The result of the present study indicates that the sodium fluoride has definite interaction with DNA in bone marrow cells of mice, resulting in DNA damage, indicating potential carcinogenic effects. Thus, due to ingestion of sodium fluoride through drinking water, cells continuously produce free radicals and ROS as a part of metabolic process. Recently arsenic induced genotoxicity was decreased by papaya fruit extract (Singh and Kumari, 2014, 2015) and arsenic induced genotoxicity was decreased by guava fruit extract (Iqbal et.al., 2016). In present study amla fruit extract could also minimize the sodium fluoride induced genotoxicity. The fruit extract could show minimizing property due to presence of vitamins C that have antioxidant property. Vitamin C is a potent antioxidant (Bendich et.al., 1986), which produces its antimutagenic effect both at the intracellular and extracellular level (Ramel et.al., 1986) through multiple inhibitory mechanisms (De Flora and Ramel, 1988). Vitamin C supplementation (Yu and Hwang, 1985) may help decrease the severity of bone fluorosis.

CONCLUSION

In comet assay, the tail length and olive tail moment of sodium fluoride treated bone morrow cells of mice was much increased in comparison to control, amla and SF+A treated cells. But the concurrent treatment of amla fruit extract and sodium fluoride could also minimize the increased tail length and olive tail moment of sodium fluoride induced damage at the significant level.

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