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Research Article

Preventive effects of diallyl sulfide on 7,12-dimethylbenz[a]anthracene induced DNA alkylation damage in mouse skin

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Mutations that occur through DNA strand breaks are the prerequisites for the development of tumors, which ultimately leads to various genetic disorders including cancer. A number of naturally occurring compounds including certain dietary constituents play an important role in causation and prevention of a number of genetic diseases. Diallyl sulfide (DAS), a volatile organosulfur compound present in garlic has been shown to possess various pharmacological effects including cancer preventive properties. Now we are reporting the antimutagenic properties of DAS on 7,12- dimethylbenz[a]anthracene (DMBA), a carcinogenic polycyclic aromatic hydrocarbon, induced DNA strand breaks in mouse skin, using an alkaline unwinding assay. DAS (2.5 – 10 mg/kg body-weight) was applied topically, prior and post to DMBA (5 mg/kg body-weight) at the sampling time of 24, 48, 72 and 96 h. DAS application resulted in a significant ($p < 0.001$) protection in DMBA-induced DNA strand breaks. The pre-treatment of DAS (10 mg/kg body-weight) showed 68.35% protection and post-treatment showed 59.49% protection, at an intermittent period of 48 h, against DMBA-induced DNA strand breakage. These findings suggest that DAS can effectively check the mutations induced by environmental toxicants.

Keywords: Antimutagenic / Diallyl sulfide / DNA alkaline unwinding assay / Mouse skin / Prevention

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1 Introduction

Chronic degenerative diseases are the leading causes of death in developing countries. Their control is exceedingly difficult due to their multiplicity and diversity, interconnection with a network of multiple risk factors and protective factors, long latency and multifocal localization. In addition, they share common pathogenetic determinants, such as genotoxic events or oxidative stress [1]. Adducts to nuclear DNA in somatic mutations can not only serve as a useful marker for the early detection of the carcinogenesis process [2, 3] but also plays a role in the pathogenesis of other chronic degenerative diseases [1]. This loss of genetic integrity may occur due to several cumulative factors, including exposure to potent mutagens and carcinogens, like polyaromatic hydrocarbons (PAH), in human and in experimental animal models [4, 5]. Progress in understanding the biological basis of mutation-related disorders revealed that damage to the genome or aberrant DNA methylation resulting in aberrant gene expression (suppression of tumor suppressor genes and inappropriate expression of oncogenes) is fundamental to tumorigenesis. Prevention of cancer and other mutation-related diseases can be pursued both by avoiding exposures to recognized mutagens/carcinogens and by favoring the intake of protective factors or fortifying physiological defense mechanisms. The latter approach, referred to as chemoprevention, is extremely delicate since it involves dietary or pharmacological intervention in the host organism.

Many studies showing a protective role for vegetables and fruits have reported that there is approximately twice the risk of several genetic disorders for low vegetable and fruit intakes as compared with high intakes [6 – 10]. The health-promoting effects of vegetables and fruits may be due to either individual or combined effects of their constituents, including fiber, micronutrients, and phytochemicals that have the potential to modulate the pathways related to the development of various threatening genetic diseases [11]. Among such numerous biologically active phytochemicals

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Abbreviations: DAS, diallyl sulfide; **DAUA,** DNA alkaline unwinding assay; **DMBA,** 7,12- dimethylbenz[a]anthracene; **PAH**, polyaromatic hydrocarbons

present in fruits and vegetables [12], an organosulfur compounds contained in plants, particularly diallyl sulfide (DAS) present in garlic, belonging to the genus *Allium* has received much attention due to its wide spectrum of biological effects [13]. Apart from its beneficial health effects, DAS has been reported to possess tumor inhibitory properties against various types of cancers both in *in vitro* and *in vivo* studies [13 – 17]. Earlier, we have reported the role of DAS as a protective agent against 7,12-dimethylbenz[a]anthracene (DMBA)-induced mouse skin carcinogenesis [17 – 21]. Further extending the mechanistic studies, we earlier demonstrated that DAS administration leads to modulation of the DMBA-induced levels of p21/ras oncoprotein as early as 24 h after the DMBA application [21] and in the present study, the role of DAS on DMBA-induced mutagenesis was studied using DNA alkaline unwinding assay (DAUA).

2 Materials and methods

2.1 Materials

Hydroxylapatite and N, N-dimethylformamide were purchased from Sisco Research Laboratory, India. DAS and DMBA were procured from Sigma (St. Louis, USA). All other chemicals used in the study were of analytical grade purity and procured locally.

2.2 Animal bioassay

Swiss albino mice (female, 20 – 22 g body weight) were obtained from the Industrial Toxicology Research Center Lucknow, animal-breeding colony. The ethical approval for the experiment was obtained from institutional ethical committee. The animals were caged in polypropylene cages and housed ten animals per cage on wood-chip bedding in an air-conditioned (temperature 23 ± 2 C, relative humidity 55 + 5%) animal room. Animals were quarantined for 1 week on a 12/12-h light/dark cycle and were fed solid pellet diet (Ashirwad, Chandigarh, India) and water *ad libitum*. The animals were divided into nine groups consisting of 20 animals each. Topical treatment of DAS was applied everyday; however, DMBA was applied at the single dose (5 mg/kg body-weight) topically, in the shaved 2×2 cm^2 area in interscapular region. The animals of the group I were used as a control group with no treatment. In group II, only DAS (10 mg/kg body-weight) was topically applied to the animals. Animals of the group III served as positive control and only DMBA was given at the dose of 5 mg/kg body-weight. The animals of the group IV, V and VI were given DAS at a dose of 2.5, 5 and 10 mg/kg body-weight, respectively, 1 h prior to DMBA application. The animals of group VII, VIII and IX were also given the same doses of DAS respectively as mentioned above but 1 h post to DMBA treatment.

Five animals from each group were euthanized by cervical dislocation after 24, 48, 72 and 96 h of first treatment, respectively. The skin from the treated area of the interscapular region was excised out, washed in chilled PBS and fat layer was scraped off with the help of sterilized scalpel blade. The skins were stored at −80°C in ultra deep freezer (Revco, USA) until used for further examination.

2.3 DNA alkaline unwinding assay

The DAUA estimates the extent of primary DNA damage based upon the fraction of single- (ss) and double- (ds) stranded DNA. The following types of DNA damage: alkali labile sites, adducts, oxidative damage, dimers, depurinization, depyrimidation, and desamination have been shown to decrease the amounts of ds-DNA following alkaline denaturing (unwinding) [22, 23].

Strand breaks in cellular DNA were quantitated by alkaline unwinding assay using hydroxyapatite batch procedure [24]. In brief, the DNA isolation from the sample skin tissue was conducted by the salting out process. The DNA (50 μg) was subjected to alkaline unwinding by rapid addition of an equal volume of 0.06 N NaOH in 0.01 M Na_2HPO_4, pH 12.5 followed by brief vortexing. Alkaline unwinding was allowed to be completed in dark for 60 min. The pH of the reaction mixture was, then, neutralized to pH 7.0 with the addition of 0.07 N HCl. Subsequently, 20 μM EDTA containing 2% SDS was added and the resultant mixture was transferred to preheated stoppered glass tubes containing 0.5 M potassium phosphate buffer, pH 7.0 and 10% N,N-dimethylformamide. The samples were incubated at 60°C for 2 h with intermittent vortexing. The relative amount of duplex and single stranded DNA present at the end of the alkaline unwinding was quantitated. Single-stranded DNA was selectively eluted from the hydroxyapatite matrix with 0.125 M potassium phosphate buffer, pH 7.0 containing 20% N, N-dimethylformamide. However, duplex DNA was removed with 0.5 M potassium phosphate buffer, pH 7.0 containing 20% N, N-dimethylformamide. The DNA in the eluates was measured [25] and strand breaks were estimated following the equation $\ln F = -(k/M_n)t^{\beta}$ where F is the fraction of remaining double stranded DNA after alkali treatment for the time t, M_n is the number-average molecular weight between two breaks and β is a constant that is less than 1 [26]. The number of unwinding points (P) per alkaline unwinding unit of DNA were calculated according to the equation, $P = \ln F_x / \ln F_0$ [24], where, F_x and F_0 are the fractions of double stranded DNA remaining after alkaline denaturation of treated and untreated samples, respectively. The number of breaks (n) per unit DNA were then determined using the equation $n = P - 1$.

2.4 Statistical analysis

All data are expressed as mean ± SE of three independent experiments. Statistical analyses between groups were performed by Student's t-test ($p < 0.001$).

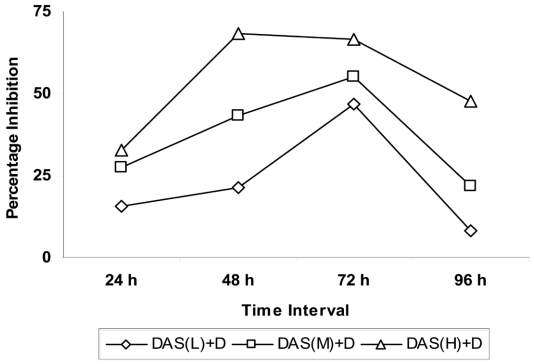

Figure 1. Percentage inhibition by DAS as blocking agent against D: DMBA induced DNA strand breaks. DAS (L) represent topical application of 2.5 mg/kg body-weight of DAS; DAS (M) represent topical application of 5 mg/kg body-weight of DAS; DAS (H) represent topical application of 10 mg/kg body-weight of DAS.

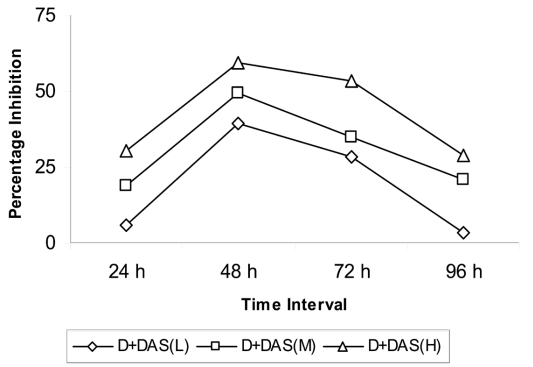

Figure 2. Percentage inhibition by DAS as suppressing agent against D: DMBA induced DNA strand breaks. DAS (L) represent topical application of 2.5 mg/kg body-weight of DAS; DAS (M) represent topical application of 5 mg/kg body-weight of DAS; DAS (H) represents topical application of 10 mg/kg body-weight of DAS.

3 Results

DAS was found to inhibit DMBA induced mouse skin mutagenesis. Based on the amount of duplex DNA remaining after alkali treatment for a specified time, the number of strand breaks formed per unit DNA was determined. A parallel control (Gr. I) does not show any reduction in the amount of duplex DNA. The results revealed that DMBA, when given at a single dose of 5 mg/kg body-weight (Gr. III) causes a significant amount of DNA damage ($p < 0.001$) over control. However, DAS (Gr. II), alone, failed to induce significant DNA strand breaks observed at 24, 48, and 72 h, confirming its non-mutagenicity. However, with repeated doses of DAS at 96 h of exposure, certain level of toxicity was observed over controls. Moreover, when DAS at increasing doses was given prior or after the DMBA application (Gr. IV – IX), increased protection from DNA damage was recorded (Table 1). DAS, as a blocking agent, when given prior to DMBA, offered a significant protection after 24 h to 96 h of treatment (Fig. 1). DAS (2.5 mg/kg body-weight) inhibited the DNA strand breaks from 15.92 to 46.90% in 24 to 72 h over DMBA treated group. Further, inhibition was decreased to 8.50% after 96 h of treatment. At 5 mg/kg body-weight of DAS, the observed inhibition increased from 27.66 to 55.39% in 24 to 72 h, and further inhibition decreased to 21.74% in 96 h. Similarly, at 10 mg/kg body-weight dose of DAS treatment, the percentage inhibition increased from 32.99 to 68.35% in 24 and 48 h, then, decreased to 66.50 and 47.71% in 72 and 96 h, respectively.

DAS administration post-DMBA, offered significant protection against DMBA-induced DNA strand breaks from 24 to 96 h of treatment (Fig. 2). DAS at the dose of 2.5 mg/kg body-weight was found to inhibit DNA strand breaks by 6.15 and 39.30% in 24 and 48 h, respectively, and, then inhibition declined to 28.58 and 3.38% at 72 and 96 h, respectively. At 5 mg/kg body-weight of DAS, the observed protection was 19.13 and 49.50% in 24 and 48 h, respectively, followed by a decline in inhibition to 35.10 and 21.00% in 72 and 96 h.

Table 1. Number of DNA strand breaks inhibited by DAS against DMBA in Swiss albino mice[a)]

Groups	Treatment	Number of DNA strand breaks (*n*)			
		24 h	48 h	72 h	96 h
I	Untreated	–	–	–	–
II	DAS (10 mg/kg body-weight)	0.0002 ± 0.00001	0.0005 ± 0.00008	0.0006 ± 0.00008	0.0014 ± 0.000012
III	DMBA	0.1464 ± 0.0156	0.1464 ± 0.0165	0.1466 ± 0.0146	0.1417 ± 0.0142
IV	DAS (2.5 mg/kg body-weight) +DMBA	0.1231 ± 0.0131	0.1152 ± 0.0124	0.0778 ± 0.0104*	0.1297 ± 0.0112
V	DAS (5 mg/kg body-weight) +DMBA	0.1059 ± 0.0148	0.0829 ± 0.0118*	0.0654 ± 0.0102*	0.1109 ± 0.0115
VI	DAS (10 mg/kg body-weight) +DMBA	0.0981 ± 0.0168	0.0464 ± 0.0086*	0.0491 ± 0.0064*	0.0741 ± 0.0140*
VII	DMBA+DAS (2.5 mg/kg body-weight)	0.1374 ± 0.0165	0.0889 ± 0.0152	0.1047 ± 0.0142	0.1369 ± 0.0161
VIII	DMBA+DAS (5 mg/kg body-weight)	0.1184 ± 0.0126	0.0739 ± 0.0130*	0.0951 ± 0.0146	0.1119 ± 0.0174
IX	DMBA+DAS (10 mg/kg body-weight)	0.1017 ± 0.0124	0.0593 ± 0.0086*	0.0679 ± 0.0018*	0.1005 ± 0.0144

a) DMBA was given topically at a dose of 5 mg/kg body-weight once only. Values are expressed as mean ± SE of five animals in three independent sets of experiments. 'n' represent number of strand breaks of duplex DNA over untreated control group.

* Represents significant prevention over DMBA induced DNA strand breaks ($p < 0.001$).

Similarly, at 10 mg/kg body-weight dose of DAS treatment, the percentage protection increased by 30.53 and 59.49% in 24 and 48 h respectively, and further, decreased to 53.68 and 29.08% in 72 and 96 h, respectively.

4 Discussion

Permanent gene alterations or mutations have been shown to be associated with the cancerous manifestation resulting in dysregulated cell growth and, therefore, tumor development [2, 3]. Occasionally, these changes occur through the formation of carcinogen-DNA adducts, *e.g.* DMBA-DNA. It has been analyzed, both, *in vitro* and *in vivo*, that 99% of the DMBA-DNA adducts are depurinating, formed by one-electron oxidation. Only a fraction of 1% of stable adducts correspond to diol-epoxide products [27]. In present study, the prior and post application of DAS inhibits the DMBA induced DNA strand breaks in mouse skin. This suggests that DAS either inhibits the metabolism of DMBA to its reactive compounds like diol-epoxide products or detoxifies it by inducing phase I and II enzymes. In accordance with the above statement, it has also been reported that DAS prevents mutagenesis by modulating both the phase I and II enzymes [28]. Earlier studies have also demonstrated that DAS induces phase II enzymes to detoxify the xenobiotic compounds [20].

There are substantial evidences from *in vitro* as well as *in vivo* studies that DAS may impart chemopreventive effects against many kinds of genetic disorders [13, 16, 18, 19]. DMBA is known to generate reactive oxygen species that contributes to DNA damage [29]. In the present study, treatment of DAS inhibited the DNA damage because DAS is known to possess reactive oxygen and other free radical species scavenging properties [30]. This is possibly due to the antioxidant action of the compound. The mechanism involved may be the sulfhydryl group present in the compound is able to scavenge the free radicals [31]. In another study, Belloir *et al.* [32] showed that S-allyl cysteine (SAC) and allyl mercaptan (AM) of garlic significantly decreased the DNA breaks in HepG2 cells treated with dimethylnitrosamine. Even if DNA has been damaged, blocking agents can still be effective at limiting further adduct formation [33], which can be a probable mechanism in our study.

A number of studies provide insight into the anticarcinogenic potential of garlic and its constituent compounds like DAS due to its blocking as well as suppressive effects [34]. The pretreatment of DAS effectively reduces the DNA strand breaks up to 68% (Fig. 1). It indicates that DAS inhibits mutagenesis induced by DMBA and its binding to DNA. In rat mammary gland, garlic powder decreased the occurrence of 7,12-dimethylbenz[a]anthracene (DMBA)-DNA adducts *in vivo* and the amounts of total and individual adducts correlated positively with mammary tumor incidence [35]. Garlic powder, garlic water extract, a deodorized garlic powder, a garlic powder with a high sulfur content, and SAC were also effective against mammary DMBA-DNA binding [36]. Furthermore, DAS has been reported to inhibit diethylstilbesterol-induced DNA adducts in the breast of female ACI rats [37]. However, post-treatment with DAS was also found to be effective in reducing the frequency of occurrence of DMBA induced DNA strand breaks, predicting its role as a suppressing agent. These results suggest that DAS enhances the ability of skin tissue to repair DNA damage. DAS induces the expression of nucleotide excision repair genes like p53, Gadd45a, PCNA, and DNA polymerase delta thus preventing the occurrence of cancer [38].

Our results demonstrate that frequency of DNA strand break inhibition increases up to certain time and decreases thereafter which might be due to the excess accumulation of DAS molecules. At higher concentration, garlic compounds such as DAS and DADS (diallyl disulfide) are reported to be toxic in rat models [39, 40]. DAS was also found to have clastogenic activity in *in vitro* short-term tests [41]. However, further analysis suggests that these compounds may alter tumorigenic hazard *in vivo* if consumed as part of a normal diet, which is consistent with our data.

Thus, these findings open the mechanism of action of DAS as a prospective in cancer chemoprevention, although various other factors seem to contribute to it. Taken together, this study has demonstrated that DAS inhibits the DMBA induced DNA strand breaks. The data also imply that DNA nick formation can be used as a target for studies on prevention of different type of cancer.

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