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Chemopreventive effect of 4′-demethyl epipodophyllotoxin on DMBA/TPA-induced mouse skin carcinogenesis

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The chemopreventive effect of topical application of 4'-demethyl epipodophyllotoxin (DMEP), an antimitotic agent, on a two-stage skin carcinogenesis model in Swiss Albino mice induced by 9,10-dimethylbenz[*a*]anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA) was investigated. Two topical applications with 0.24% DMBA over 1 week, followed later by 5 nmol of TPA twice weekly produced 100% incidence of tumors in these animals by 18 weeks. Treatment of animals with DMEP (until the end of the experiment), 30 min before TPA treatment, significantly reduced the tumor incidence, tumor volume and the conversion efficiency of papillomas to squamous cell carcinomas. The tumor formation and growth was also delayed by DMEP pre-treatment. Application of DMEP protected against the losses provoked in levels of glutathione and activity of catalase and superoxide dismutase in skin and liver of animals by the application of DMBA/TPA. Thus, DMEP might possibly be exerting its chemopreventive activity by acting as an antioxidant.

Introduction

Carcinogenesis is a multi-step process involving the sequential accumulation of mutations that allow cells to gain a selective growth advantage, have the phenotypic attributes of local invasiveness and the ability to form distant metastasis. At the molecular level, progression results from accumulation of genetic lesions. Chemoprevention is the use of one or several agents to prevent the occurrence of cancer (1,2). The mouse skin carcinogenesis model has become very useful in studying the genetic and biological changes involved in tumor promotion (3,4). Some of the genetic changes associated with the chemical initiation of benign papillomas and the transition to squamous cell carcinoma have been characterized in this system (5).

Reactive oxygen species (ROS) have been suggested as causative factors in mutagenesis, carcinogenesis and tumor promotion and have been implicated in the etiology and pathophysiology of many human diseases (6). They induce strand breaks in DNA, and also oxidative modification of DNA bases leading to mutagenic and carcinogenic effects (7). They also modulate gene expression by an epigenetic mechanism (8). Topical application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has been reported to increase release of ROS (9). Many tumor promoters have been shown to exert their action by production of ROS, and many compounds that possess antioxidant activity have been reported to inhibit tumor promotion (10,11). ROS induce chromosomal aberrations, structural genetic changes resulting in alterations in gene expression with high efficiency. Thus, they may play an intermediate role in the induction of promotion-related genes.

4′-Demethyl epipodophyllotoxin (DMEP, Figure 1) is an antimitotic agent which binds to monomeric tubulin, preventing micro-tubule polymerization. The exposure of a dividing cell to DMEP causes the disappearance of the mitotic spindle and blocks the cell in mitosis within a few minutes of exposure to the drug, resulting in cell death (12,13). In many cases, the action of preventing microtubule polymerization is reversible, so that removal of the drug allows the spindle to re-form and mitosis to proceed. The disruption of spindle microtubules, which preferentially kills many rapidly dividing cells, allows DMEP to be classified as a potential chemotherapeutic agent (14,15).

There is constant search for new drugs that can act as chemotherapeutic agents to treat cancer. *In vitro* studies in our laboratory have indicated that DMEP exerts its action via apoptosis in cells of myeloid origin (unpublished data). The two-stage mouse skin carcinogenesis model was used in order to test the efficacy of DMEP to prevent the promotional changes that occur during the process of neoplastic development, under *in vivo* conditions. To the best of our knowledge, this is the first *in vivo* report on the effect of topical application of DMEP. In the present report we describe the effects of topical application of DMEP on TPA-induced tumor promotion in mouse epidermis previously initiated with 9,10-dimethylbenz[*a*]anthracene (DMBA). The effects were studied on histopathological changes in mouse skin. The activity of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) and the alterations in the levels of glutathione and lipid peroxides in mouse liver and skin were investigated to find out whether DMEP mediates its affect by scavenging free radicals.

Materials and methods

Animals

Male Swiss Albino mice, 4–6 weeks old were used in the study. They were kept in well ventilated polypropylene cages. The mice were fed standard laboratory diet and water *ad libitum*.

Treatment of the animals for in vivo studies

The backs of the animals were shaved 2 days prior to the start of the experiment. The animals were divided into three groups (n = 10 for each group). Group I animals served as controls. These animals received acetone (200 µl/mouse) application only. Group II animals received two topical applications of 0.24% DMBA in acetone (200 µl/mouse) over a period of 1 week, followed by 5 nmol TPA in acetone (50 µl/mouse) twice weekly for 24 weeks. The mice of group III were treated as Group II mice, except that they were treated with DMEP (200 µl of the solution in acetone at a concentration of 1 µg/ml), 30 min prior to application of the above dose of TPA twice weekly for 24 weeks.

Body weights of animals were recorded initially and then at 1 week intervals

Abbreviations: CAT, catalase; DMBA, 9,10-dimethylbenz[*a*]anthracene; DMEP, 4′-demethyl epipodophyllotoxin; GSH, glutathione; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

and at the time of death. Papillomas appearing on the skin were recorded every week during the experimental period, with only those having diameter >2 mm considered as positive. At the end of 24 weeks, the animals were killed for histopathological and biochemical studies. The liver and dorsal skin tumors were quickly excised and washed thoroughly with chilled phosphate-buffered saline (pH 7.4). A 10% tissue homogenate (w/v) was prepared from part of the sample (liver/skin or tumors) in 0.15 M Tris–HCl (pH 7.4) and the homogenate was then centrifuged at 12 000 *g* for 15 min. The supernatant thus obtained was taken for estimation of CAT, SOD, glutathione (GSH), malondialdehyde (MDA) and protein.

Hematoxylin and eosin (H&E) staining of the sections

Mouse skin tumors were immediately frozen at –70°C after excision. They were then cut into 5 µ sections using a cryostat and stained with H&E and examined by a pathologist.

Lipid peroxide estimation (MDA)

Levels of lipid peroxides were estimated using the method of Ohkawa *et al.* (16). Briefly, thiobarbituric acid (0.8%), sodium dodecyl sulphate (0.1%) and acetic acid (20%) were added to 100 µl of the tissue homogenate (10%) prepared as described above. This mixture was heated for 30 min, cooled, extracted with *N*-butanol-pyridine, and the OD of MDA recorded at 532 nm. The content of MDA is expressed as nmol/mg protein.

GSH estimation

The level of GSH was estimated by the method of Miron *et al.* (17). The proteins were precipitated in the 12 000 *g* supernatant (obtained as described above) by addition of trichloroacetic acid (TCA) to a final concentration of 5% TCA. This was then centrifuged at 15 000 *g* for 15 min to obtain the protein free supernatant. To 100 µl of this supernatant, 2 ml of 0.6 M 5,5′-dithio-bis(2-nitrobenzoic acid) dissolved in 0.2 M phosphate buffer (pH 8) was added. The absorbance was recorded at 412 nm. Reduced GSH was used as standard. The levels of GSH are expressed as nmol/mg of protein.

SOD assay

This was determined by the method of Misra and Fridovich (18) based upon the ability of SOD to inhibit the auto-oxidation of epinephrine to adrenochrome at alkaline pH. An aliquot of 40 µl of the supernatant of 10% tissue homogenate, obtained as described above, was taken in 0.1 M carbonate buffer (pH 10.2). After addition of epinephrine, the increase in absorbance was measured at 480 nm. The activity of the enzyme is expressed as U/mg of protein, where 1 U of the enzyme is defined as the amount of enzyme required to inhibit the rate of epinephrine auto-oxidation by 50% under the conditions of the assay.

CAT assay

This was assayed by the method of Aebi (19). The change in absorbance was followed spectrophotometrically at 240 nm after the addition of H_2O_2 (30 mM) to 100 µl of the supernatant (of 10% tissue homogenate obtained as described above) in 50 mM phosphate buffer (pH 7). The activity of the enzyme is expressed as U/mg of protein, where 1 U is equivalent to 1 mol of H_2O_2/µg/min/mg protein.

Protein estimation

This was determined by Bradford assay using bovine serum albumin as a standard (20).

Statistical analysis

The significance of the difference in the values of GSH, MDA, CAT and SOD was determined using the Kruskal–Wallis test. *P*-values <0.05 are considered significant.

Results

Effect of DMEP on DMBA/TPA-induced tumors in Swiss Albino mice

Male Swiss Albino mice (4–6 weeks old) had body weights ranging from 29.6 to 31 g at the beginning of the experiment. The average body weights at the end of the experiment, i.e. 24 weeks, were 48.33 ± 1.41 g in the vehicle-treated, 37.5 ± 3.53 g in the DMBA/TPA-treated and 44.66 ± 1.76 g in DMBA/DMEP/TPA-treated animals (Figure 2).

The number of mice bearing tumors in DMBA/TPA-treated and DMBA/DMEP/TPA-treated groups at different weeks is shown in Figure 3. The onset of tumors (>2 mm) commenced at 9 weeks in DMBA/TPA-treated mice and reached 100% at 18 weeks, whereas in DMBA/DMEP/TPA-treated mice the onset was delayed until 12 weeks (i.e. >2 mm). Total tumor incidence was seen from 21 weeks onwards. At the end of the experiment (i.e. at 24 weeks), even the tumor volume in DMBA/DMEP/TPA-treated animals was much lower (29.2%) than tumor volume of DMBA/TPA-treated animals (Figure 4). No tumor formation was seen in the vehicle-treated control animals (Figure 3, Table I).

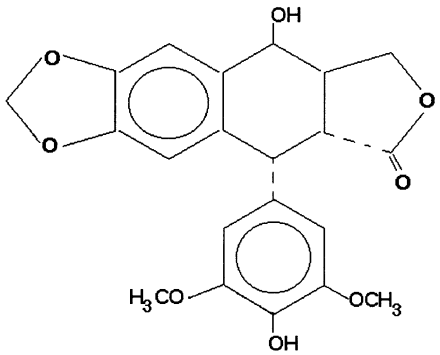

Fig. 1. Structure of DMEP.

Histopathology of mouse skin

The characteristic squamous pearls were clearly visible on examining the H&E-stained sections of the tumors formed on application of DMBA/TPA, i.e. mice belonging to Group II. Islands of dysplastic squamous epithelial cells lying in the dermis distinctly indicated an invasive form of frank squamous cell carcinoma. The necrotic keratinocytes are also visible (Figure 5b and c). Tumors of animals belonging to group III (i.e. DMBA/DMEP/TPA-treated animals) displayed intact basement membrane with hyperplasia of the overlying epidermis. This clearly characterizes the benign nature of these tumors (Figure 5d and e).

Biochemical studies in mouse skin tumor and liver

MDA level. The level of MDA in the skin of animals treated with DMBA/TPA increased by 43.49% ($P < 0.01$), whereas animals treated with DMBA/DMEP/TPA exhibited only a 6.09% ($P < 0.05$) increase with respect to vehicle-treated control animals. In general, a 26% decrease ($P < 0.05$) in the level of MDA was observed in the tumors of animals treated with DMBA/DMEP/TPA in comparison with the MDA levels in the tumors of DMBA/TPA-treated animals (Table II).

The MDA level increased in the liver by 162.93% ($P < 0.05$) in animals treated with DMBA/TPA, whereas animals treated with DMBA/DMEP/TPA exhibited an 84.26% increase ($P < 0.05$) in the MDA level in the liver with respect to the vehicle-treated control animals. In other words, a 29.92% decrease ($P < 0.05$) in the liver MDA level of DMBA/DMEP/TPA-treated animals was observed as compared with the level in DMBA/TPA-treated animals (Table III).

GSH level. A 38.9% decrease ($P < 0.05$) in the levels of GSH in tumors of animals treated with DMBA/TPA, and a 19.2% decrease ($P < 0.05$) were observed in the tumors of animals treated with DMBA/DMEP/TPA as compared with vehicle-treated control animals (Table II). There was an average 32.25% increase ($P < 0.05$) in the GSH levels in the tumors of animals treated with DMBA/DMEP/TPA in relation to the GSH level in the tumors of animals treated with DMBA/TPA.

In the liver, a decrease of 86.1% (in DMBA/TPA-treated animals; $P < 0.01$) and a decrease of 38.2% (in DMBA/DMEP/TPA-treated animals; $P < 0.05$) were observed as

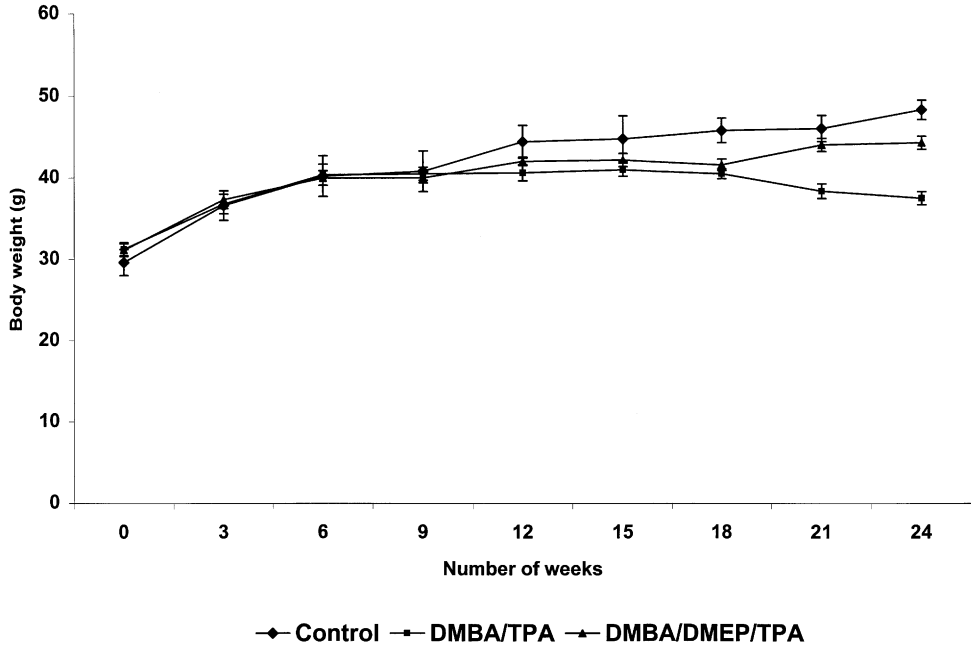

Fig. 2. Body weights (g) of control, DMBA/TPA- and DMBA/DMEP/TPA-treated animals were recorded weekly. Body weights are plotted, showing changes observed every third week until the time of killing of the animals. Data are expressed as means ± SE of 10 animals in each group.

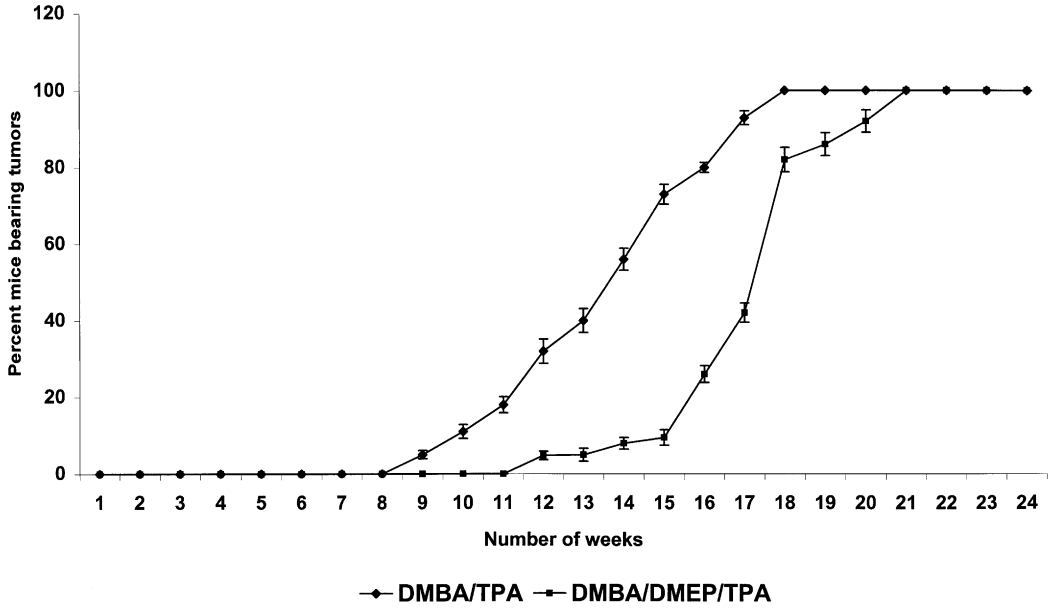

Fig. 3. Effect of topical application of DMEP on DMBA/TPA-induced mouse skin carcinogenesis. Initiation was accomplished with two topical applications of 0.24% DMBA in 1 week. For tumor promotion, 5 nmol TPA in 50 µl acetone was applied twice a week after initiation till 24 weeks, on the dorsal surface of Swiss Albino mice. DMEP was applied 30 min prior to the application of TPA. The number of animals bearing tumors was observed weekly. Data are expressed as the percentages of mice bearing tumors as a function of time in weeks. Controls treated with acetone only did not show any tumor formation.

compared with vehicle-treated controls in the GSH levels. In general, an increase of 34.4% ($P < 0.05$), in the level of GSH was observed in the livers of animals treated with DMBA/DMEP/TPA in comparison with the animals treated with DMBA/TPA (Table III).

SOD activity. A decrease of 72.3% ($P < 0.001$) in the activity of SOD was observed in the tumors of DMBA/TPA-treated animals, whereas animals treated with DMBA/DMEP/TPA exhibited a 59% decrease ($P < 0.001$) as compared with controls. There was an average 48% increase ($P < 0.001$) in SOD activity in the tumors of animals treated with DMBA/DMEP/TPA as compared with the level in DMBA/TPA-treated animals (Table II).

On application of DMBA/TPA, the SOD activity in liver of these animals showed a 53% decrease ($P < 0.01$), whereas an 18.9% decrease ($P < 0.01$) was observed in the DMBA/DMEP/TPA-treated animals as compared with controls. However, the activity of SOD in the liver of animals treated with DMBA/DMEP/TPA showed a 72.58% increase ($P < 0.01$) as compared with the SOD activity in the animals treated with DMBA/TPA (Table III).

CAT activity. As compared with vehicle-treated control animals,

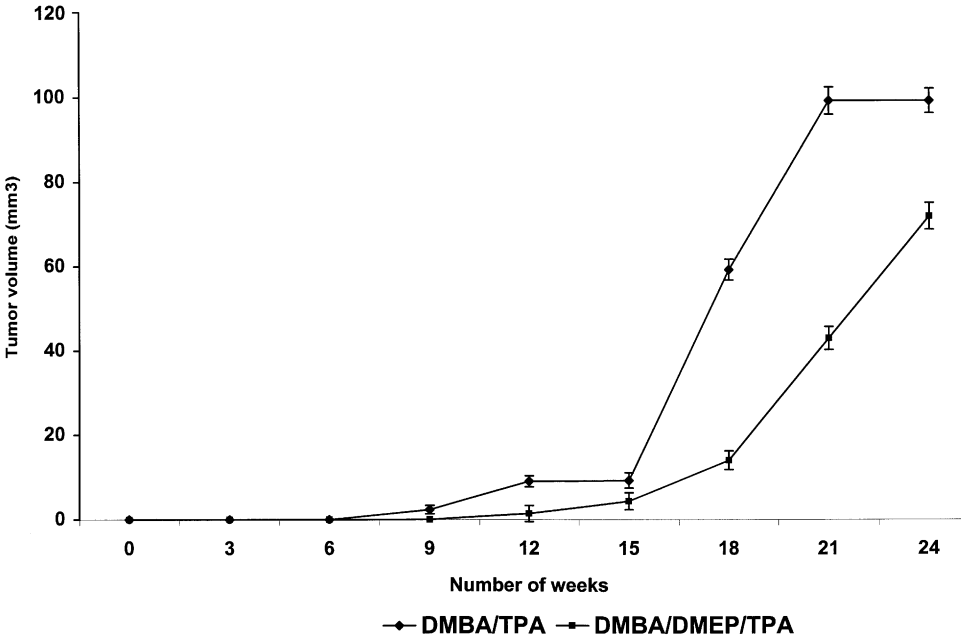

Fig. 4. Effect of application of DMEP on tumor volume. The animals were treated as mentioned in Figure 2 and the changes in tumor volume recorded.

the activity of CAT in tumors of animals treated with DMBA/TPA exhibited a 73.2% decrease ($P < 0.001$), and a 49.8% decrease ($P < 0.001$) in DMBA/DMEP/TPA-treated animals. In general, there was an 87.86% increase ($P < 0.01$) in the activity of CAT in the tumors of animals treated with DMBA/DMEP/TPA as compared with the activity in the DMBA/TPA-treated animals (Table II).

As compared with vehicle-treated control animals, the activity of CAT in the livers of animals treated with DMBA/TPA showed a 31.2% decrease ($P < 0.01$) and a 5.2% decrease ($P < 0.05$) in DMBA/DMEP/TPA-treated animals. In general, a 37.83% ($P < 0.05$) increase was observed in the animals treated with DMBA/DMEP/TPA as compared with the animals treated with DMBA/TPA (Table III).

Discussion

Chemoprevention is an important strategy to control the process of carcinogenesis. Thus, there is a need for exploring drugs/agents which act as chemopreventive agents. In the present study, we have studied the chemopreventive effect of DMEP using a mouse skin carcinogenesis model. Most drugs belonging to the class of podophyllotoxins (e.g. etoposide, teniposide) exert their cytotoxic effect as topoisomerase II inhibitors. DMEP is unique in the class of podophyllotoxins in its mode of action as an inhibitor of microtubule polymerization, thus resembling vinca alkaloids (1). Given the crucial role of microtubules in chromosome segregation, this pathway provides a means to eliminate cells with aberrant chromosome/DNA damage. *In vitro* studies in our laboratory illustrated that DMEP caused apoptotic cell death in CML cells (unpublished data).

This study shows a significant increase in tumor latency by application of DMEP in Swiss Albino mice initiated by DMBA and promoted by TPA. This may be due to the delay in the promotion phase of carcinogenesis. Along with a decrease in tumor volume (29.2%) by the end of the experiment (24 weeks), significant reduction in the number of tumors formed per mouse was observed (27.5% until the end of week 24). Most *in vivo* studies using chemopreventive agents, showing significant reduction in the tumor formation, have been followed only until week 20 (21) whereas we followed it until 24 weeks. However, the percentage inhibition was statistically significant until week 21 (47.4%; $P < 0.05$) but not at week 24. Even in another *in vivo* model studied by us, hamster oral pouch carcinogenesis, DMEP increased tumor latency and decreased tumor volume and efficiency of tumor conversion (unpublished data).

Histopathology of the tumors observed 24 weeks after DMBA/TPA treatment showed necrotic keratinised squamous pearls, suggesting invasive squamous cell carcinoma. On the other hand, intact basal cell layer and dysplastic lesions characterized benign papillomas in DMBA/DMEP/TPA-treated animals. This direct evidence shows that DMEP application inhibits the carcinoma formation and conversion efficiency of papillomas into frank squamous cell carcinoma.

In multistep carcinogenesis, reactive oxygen species have been shown to play a role mostly in the promotion phase. Antioxidants are reported to act as protective agents against cancer (22–25). The liver is a versatile organ involved in drug metabolism and detoxification, hence the antioxidant enzymes and the other parameters were estimated in liver as well as

Table I. Protective effect of DMEP on the number of DMBA/TPA-induced tumors in mice.

Week	DMBA/TPA	DMBA/DMEP/TPA	% inhibition
9	0.33 ± 0.21	–	100
12	1.33 ± 0.54	0.166 ± 0.16	87.5*
15	3.0 ± 1.09	0.5 ± 0.22	83.3*
18	5.56 ± 1.24	1.8 ± 0.58	67.6*
21	11.8 ± 1.65	6.2 ± 1.19	47.45*
24 (killed)	17.4 ± 3.48	12.56 ± 1.69	27.5**

Data represent means ± SE in each group.
*$P < 0.05$; **$P < 0.1$ versus vehicle-treated and DMBA/TPA or DMBA/DMEP/TPA.

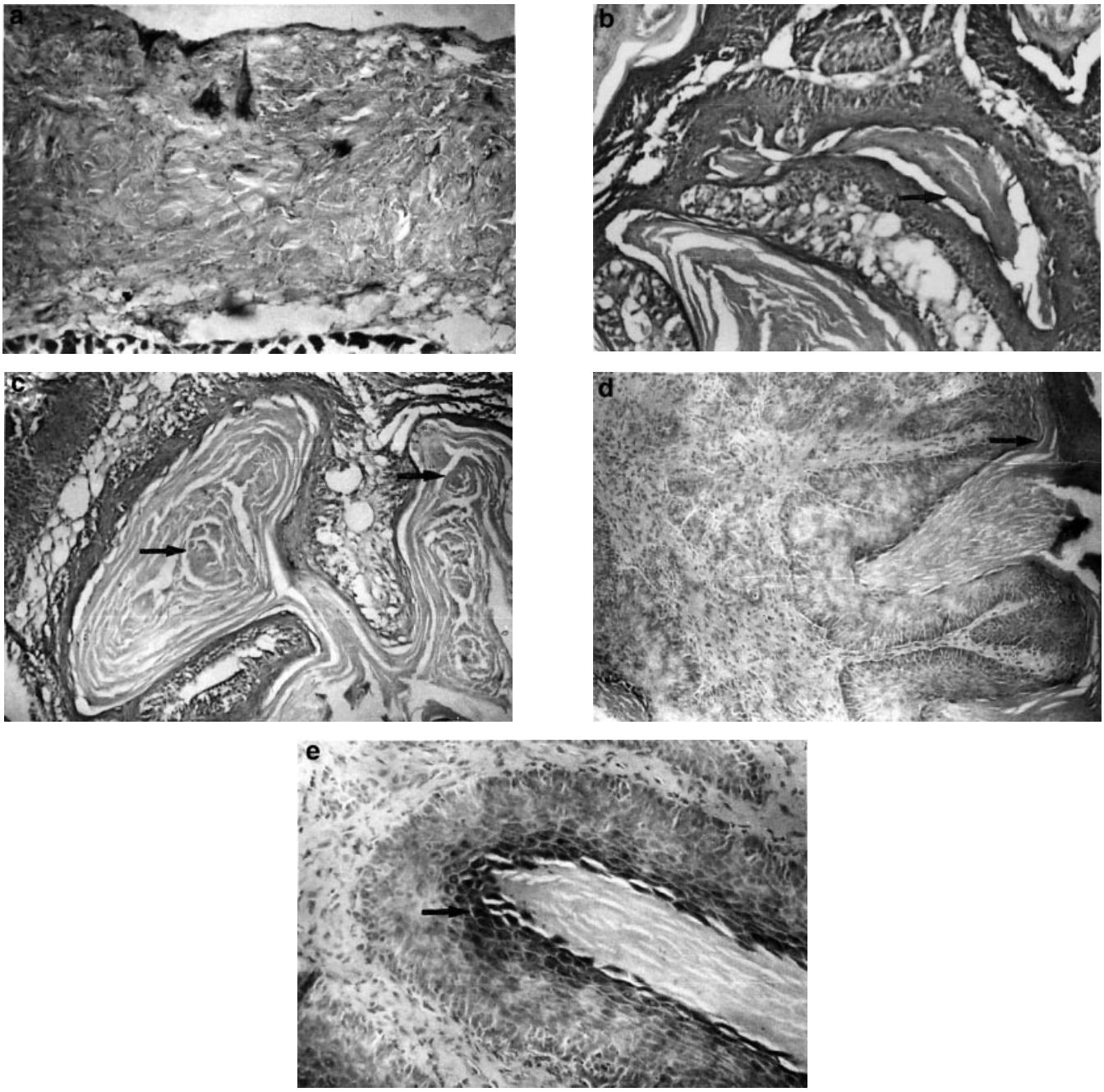

Fig. 5. H&E stained sections of skin tumors of control, DMBA/TPA- and DMBA/DMEP/TPA-treated animals. (**a**) H&E stained section of control skin. (**b**) Squamous epithelial cells lying in the dermis clearly identifying the tumors as invasive in the DMBA/TPA-treated animals. (**c**) Characteristic squamous epithelial pearls. Also identifiable are the necrotic keratinocytes in tumors from DMBA/TPA-treated animals. (**d**) Section showing intact basal layer belonging to the tumors of DMBA/DMEP/TPA-treated animals. (**e**) Hyperplastic lesions indicating the hyperplasia of the overlying epidermis in tumors of DMBA/DMEP/TPA-treated animals.

Table II. Effect of DMEP on lipid peroxides (MDA), GSH level and SOD, CAT activities in mouse skin tumors

Treatment (group)	MDA (nmol/mg protein)	GSH (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
Control (I)	2.46 ± 0.29	35.28 ± 1.30	16.75 ± 1.66	7.71 ± 0.56
DMBA/TPA (II)	3.53 ± 0.23**	21.55 ± 1.4*	4.63 ± 0.19***	2.06 ± 0.5***
DMBA/TPA/ DMEP (III)	2.61 ± 0.23*	28.5 ± 1.3*	6.86 ± 0.26***	3.87 ± 0.21***

Data represent the means ± SE in each group.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus vehicle-treated and DMBA/TPA or DMBA/DMEP/TPA. Comparisons are made in the text between control (Group I) and DMBA/TPA (Group II)-treated animals and between DMBA/TPA- (Group II) and DMBA/DMEP/TPA (Group III)-treated animals.

Table III. Effect of DMBA/TPA and DMBA/DMEP/TPA on MDA, SOD, GSH and CAT in mouse liver.

Treatment (group)	MDA (nmol/mg protein)	GSH (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
Control (I)	2.86 ± 0.32	95.7 ± 10.5	9.24 ± 0.62	5.38 ± 0.33
DMBA/TPA (II)	7.52 ± 0.47*	13.3 ± 0.32**	4.34 ± 0.31**	3.70 ± 1.17**
DMBA/TPA/ DMEP (III)	5.27 ± 0.23*	59.14 ± 2.2*	7.49 ± 0.37**	5.10 ± 0.94*

Data represent the means ± SE in each group.
*$P < 0.05$; **$P < 0.01$ versus vehicle-treated and DMBA/TPA or DMBA/DMEP/TPA. Comparisons are made in the text between control (Group I) and DMBA/TPA (Group II)-treated animals and between DMBA/TPA- (Group II) and DMBA/DMEP/TPA (Group III)-treated animals.

mouse skin tumors. The reduced form of GSH is a biological antioxidant present in high amounts, especially in the liver, and its presence is a pre-requisite for protection against oxidative damage. This is evident even as seen with DMEP treatment, which protects the losses in GSH levels provoked by DMBA/TPA treatment. The low levels of antioxidant enzymes SOD and CAT in DMBA/TPA-treated mice show poor antioxidant status. Oberley and Oberley (26) have also reported decreased activities of SOD and CAT in squamous cell carcinomas. The increase in antioxidant enzymes by DMEP reflects that it inhibits the process of oxidative stress induced carcinogenesis. Several reports suggest that GSH is a more efficient antioxidant agent than SOD or CAT (27). GSH alters the profile of lipoxygenase and cyclooxygenase (28,29), which are involved in tumorigenesis. Oberley and Oberley (26) reported decreased SOD and CAT activity in papillomas and squamous cell carcinoma leading to a pro-oxidant state of cells, facilitating tumorigenesis. But GSH has been found to be highly variable and contradictory, depending on the cell type, nature of the carcinogen and its modulatory pathways (26,30–31). Increase in the level of GSH by the chemopreventive action of flavonoids in mouse skin has also been reported (32). It is thus the combined effect of modulating antioxidant enzyme(s), which may in turn lead to a shift in the intracellular oxidation/reduction balance and to a changed cell and organ sensitivity to induced tumorigenesis.

During oxidative stress, MDA and/or other aldehydes are formed in biological systems. These can react with amino acids and DNA and introduce cross linkages between proteins and nucleic acids, resulting in alterations in replication, transcription (33) and leading to tumor formation. Elevated levels of MDA were observed in skin tumors as well as in livers of animals treated with DMBA/TPA suggesting oxidative stress in DMBA/TPA-induced mouse skin carcinogenesis. Elangovan *et al.* (32) also reported an increase in the level of lipid peroxides in the mouse skin model, which were reduced in response to certain flavonoids. Significant decrease in MDA levels by DMEP treatment indicates reduced oxidative stress, thus indicating its protective potential against skin carcinogenesis. This protective effect of DMEP as indicated by reduced lipid peroxides could be due to increase in GSH and the antioxidant enzymes CAT and SOD. The decrease in lipid peroxides may also explain the reduced invasiveness of the tumor as indicated by the histopathology of tumors of DMBA/DMEP/TPA-treated animals.

DMEP may thus be exerting its chemopreventive effect by acting as an antioxidant. This compound was originally identified as an antitumor agent based on its ability to inhibit microtubule polymerisation. We would like to suggest that other mechanisms may include its capacity to scavenge free radicals, to block or trap ultimate carcinogenic electrophiles by forming innocuous products in a nucleophilic chemical reaction.

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