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RESEARCH

Chemopreventive Efficacy of Naringenin-Loaded Nanoparticles in 7,12-dimethylbenz(a)anthracene Induced Experimental Oral Carcinogenesis

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Abstract Nanochemoprevention has been introduced recently as a novel approach for improving phytochemicals bioavailability and anti-tumor effect. The present study is designed to evaluate the chemopreventive efficacy of prepared naringenin-loaded nanoparticles (NARNPs) relative to efficacy of free naringenin (NAR) against 7,12-dimethyl benz(a)anthracene (DMBA)-induced oral carcinogenesis by evaluating the status of lipid peroxidation, antioxidants and immunoexpression patterns of proliferating cell nuclear antigen (PCNA) and p53 proteins. Transmission electron microscope (TEM) and dynamic light scattering (DLS) investigations have confirmed a narrow size distribution of the prepared nanoparticles (40–90 nm) with ~88 % encapsulation efficiency. Oral squamous cell carcinoma (OSCC) was developed in the buccal pouch of golden Syrian hamsters by painting with 0.5 % DMBA in liquid paraffin three times a week for 14 weeks. DMBA painted animals revealed the morphological changes, hyperplasia, dysplasia and well-differentiated squamous cell carcinoma. Moreover, the status of lipid peroxidation, antioxidants and immunoexpression of PCNA and p53 were significantly altered during DMBA-induced oral carcinogenesis. Oral administration of NARNPs (50 mg NAR/kg body weight/day) to DMBA-treated animals completely prevented the tumor formation as compared to the free NAR and significantly reduced the degree of histological lesions, in addition to restoration of the status of biochemical and molecular markers during oral carcinogenesis. In addition, NARNPs have more potent anti-lipid peroxidative, antiproliferative effect and antioxidant potentials compared to free NAR in DMBA-induced oral carcinogenesis. In conclusion, the present study suggests that NARNPs could be a potentially useful drug carrier system for targeted delivery of naringenin for cancer chemoprevention.

Keywords Nanochemoprevention · DMBA · Hamster buccal pouch carcinogenesis · Naringenin

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Introduction

Oral cancer is one of the most common malignancies and is a major cause of cancer morbidity and mortality worldwide. Globally, about 500,000 new cases of oral cancer are diagnosed annually and approximately 75 % of these occur in developing countries. Oral cancer accounts for 40–50 % of all cancers in India [1]. The incidence rate of oral cancer in India is rapidly increasing due to the habit of betel nut chewing with tobacco and heavy alcoholic consumption. Oral squamous cell carcinoma (OSCC) constitutes 90 % of oral cancer. Despite significant advance in various forms of treatment, the 5-year survival rate of this disease has not improved over the last 4–5 decades and remains around 50 %. Recent efforts to control the incidence of OSCC have focussed on developing chemopreventive strategies. Therefore, it is important to establish chemoprevention in an experimental animal tumor model that mimics specific characteristics of human oral OSCC before embarking on clinical trials.

The hamster buccal pouch (HBP) model is the well-characterized animal system for the investigation of oral cancer development and the efficacy of chemopreventive agents [2]. The 7,12-dimethylbenz(a)anthracene (DMBA) is one of the widely used carcinogens in experimental oral carcinogenesis. In mammalian cells, DMBA can be bioactivated via formation of a reactive diol-epoxide metabolite, which subsequently adducts to adenine and guanine residues in DNA [3]. The formation of these adducts has been suggested as the ultimate step in the carcinogenic mechanism of DMBA. DMBA-induced tumors in the hamster's buccal mucosa closely mimic the human oral cavity tumors, morphologically, histologically, biochemically and at molecular level [4, 5]. The development of both human OSCC and HBP carcinoma is associated with sustained genetic mutation that leads to excessive cell proliferation, prolonged cell survival and evasion of apoptosis [5, 6].

Chemoprevention serves as an attractive alternative to control malignancy by a pharmacological intervention aiming to arrest the process of carcinogenesis. The major focus of research in cancer chemoprevention includes identification, characterisation and development of new and safer cancer chemopreventive agents. The search for new compounds in foods or in plant medicines showing anticancer effects is one realistic and promising approach to prevention. Flavonoids are a class of phenolic compounds widely distributed in the plant kingdom. These agents display diverse biological activities including prevention of cancer initiation and inhibition of cancer progression. Naringenin (4′, 5, 7-trihydroxy flavonone) is one of the naturally occurring flavonoids found in grape fruits, oranges, tomatoes and cherries. It exerts a wide variety of activities such as anti-inflammatory, anti-mutagenic, anti-atherogenic and anti-cancer effects [7–10]. Naringenin possesses three hydroxyl groups in its aromatic rings which are responsible for its potent antioxidant activity and widespread pharmacological property.

Inspite of the wide spectrum of pharmacological properties, the use of naringenin in pharmaceutical field is limited due to its aqueous solubility and instability in physiological medium [11]. These properties of naringenin result in poor bioavailability, poor permeability, instability and extensive first pass metabolism before reaching the systemic circulation. In order to improve the efficacy and bioavailability of naringenin, many approaches such as β-cyclodextrin inclusion complexes, phospholipids complexes and polymeric nanoparticles mediated naringenin delivery have been developed [12–14]. Among these, polymeric nanoparticles offer promising enhanced therapeutic performance of anticancer drugs by increasing their bioavailability. Due to their small size, NPs penetrate into even small capillaries and are taken up within cells allowing an efficient drug accumulation at the targeted sites in the body.

Nanoparticles are well-known to transport and deliver drugs which are unstable in the biological fluids and cannot readily diffuse across the mucosal barriers [15]. Oral nanoparticles are promising drug delivery system due to improved bioavailabilty, targetability and controlled release of drugs in gastrointestinal tract (GIT). Hence, nanochemoprevention by encapsulation of naturally occurring phytochemicals in polymeric-loaded nanoparticles has been introduced recently as a novel approach for improving phytochemicals bioavailability and anti-tumor effect [16]. In the preparation of drug nanoparticles system, polymers are required for the immediate entrapment of the free compound and are used as carriers. The Eudragit® E cationic copolymer has been widely used to improve the solubility of poorly water-soluble drugs [11]. It has basic site containing tertiary amine groups which are ionized in gastric fluid. This makes Eudragit® E readily easy to dissolve in the gastric environment. In the previous work, we reported the enhanced anticancer effect of the NARNPs in HeLa cells [14]. Yen, et al. have also observed significant hepatoprotective effects of NARNPs in CCl_4 administered rats [11]. To the best of our knowledge, there were no scientific studies on chemopreventive efficacy of NARNPs in DMBA-induced hamster buccal pouch carcinogenesis. Hence, the present study was designed to elucidate the protective role of free NAR and NARNPs on DMBA-induced oral cancer by assessing lipid peroxidation (LPO), enzymatic and non-enzymatic anti-oxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH) and vitamin E. Histopathological and immunohistochemical localisation of PCNA and p53 in hamster buccal mucosa tissue was also done to validate the chemopreventive efficacy of NARNPs in comparison with free NAR against DMBA induced HBP carcinogenesis.

Materials and Methods

Chemicals

DMBA, naringenin and polyvinyl alcohol (PVA, Molecular weight=30,000–70,000) were obtained from Sigma-Aldrich Chemical Pvt. Ltd, Bangalore, India. Aminoalkyl methacrylate copolymers (Eudragit® E) were supplied as gift samples by Evonik Industries (Mumbai, India). All other chemicals used were of analytical grade, purchased from Hi-media Laboratories, Mumbai, India.

Preparation of Naringenin-loaded Nanoparticles (NARNPs)

Naringenin-loaded nanoparticles (NARNPs) system was prepared in the ratio of NAR: EE: PVA (1: 10: 10 w/w/w)

using nanoprecipitation method [11]. Precisely, 50 mg of naringenin and 500 mg of Eudragit® E were dissolved in 25 ml of ethanol. The internal organic phase solutions were quickly injected into the 75 ml external aqueous solution containing 500 mg of PVA, and then the solutions were homogenized at 18,000 rpm for 40 min. The ethanol was completely removed by rotary vacuum evaporation at 40 °C water bath and the remaining fraction was then lyophilized with a freeze dryer.

Characterization of NARNPs

Particle Size Analysis

The mean particle size of NARNPs was determined by using Nanotrac (Microtrac, Inc, USA) based on the principle of dynamic light scattering (DLS). Briefly, 1 mg of NARNPs was suspended in 3 ml of double distilled water and sonicated for 30 s. The measurement was performed in triplicate.

Morphological characteristics of the NARNPs were observed by JEOL JSM-100CX transmission electron microscope (TEM). The lyophilized NARNPs (0.5 mg/ml) were suspended in water and sonicated for 30 s. A drop of this suspension was placed over a carbon coated copper TEM grid and allowed to air dry. The sample was then negatively stained with 1 % (W/V) uranyl acetate solution for 10 min; any excess acetate solution was removed with filter paper before viewing on the TEM machine and the images were visualized at 100 kV under microscope.

Determination of %Encapsulation Efficiency (% EE)

Weighed amount of dried naringenin-loaded nanoparticles were dissolved in distilled water and then centrifuged, followed by the collection of the supernatant. The absorbance of the supernatant solution was measured by UV spectrometer (UV-2450 Shimadzu) at the wavelength of 266 nm and the weight of the drug loaded was calculated using calibration curve. The EE was calculated from the following formula:

$$\%EE = \frac{\text{Total Weight of NAR in NARNPs} - \text{Free NAR released from NARNPS}}{\text{Total Weight of NAR in NARNPs}} \times 100$$

Animals and Diet

Male golden Syrian hamsters, 8–10 weeks old, weighing 80–120 g, were purchased from Kerala Agricultural University, Mannuthy, Kerala, India and were maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University, India. Five animals were housed in a polypropylene cage and provided with a standard pellet diet (Agro Corporation Pvt. Ltd., Bangalore, India) and water ad libitum. The animals were maintained under controlled condition of temperature (27±2 °C) and humidity (55±5 %) with an alternating light/dark cycle in accordance with the guidelines of the Indian Council of Medical Research, and approved by the ethical committee, Annamalai University, India.

Experimental Design

The experimental animals were randomized into control and experimental groups and divided into 6 groups of 10 animals each. Group 1 animals served as the control and were treated with liquid paraffin (vehicle) alone three times a week for 14 weeks on their left buccal pouches. Group 2 animals were treated with 0.5 % solution of DMBA in liquid paraffin using a No. 4 sable brush, three times a week for 14 weeks on their left buccal pouches [4]. Group 2 animals received no other treatment. Groups 3 and 4 animals were treated with DMBA as in Group 2, in addition to oral administration of free naringenin (50 mg NAR/kg body weight/day), dissolved in 1 ml of 5 % dimethyl sulphoxide (DMSO) and NARNPs (50 mg NAR/kg body weight/day), dissolved in distilled water, respectively, starting 1 week before exposure to the carcinogen and continued on days alternate to DMBA painting until the end of the experiment. Groups 5 and 6 animals received oral administration of free NAR (50 mg NAR/kg body weight) and NARNPs (50 mg NAR/kg body weight) alone, as in Groups 3 and 4, throughout the experimental period. The experiment was terminated at the end of 16 weeks and all animals were sacrificed by cervical dislocation after an overnight fast. The buccal pouch tissues were variously processed for each experiment. Biochemical studies were conducted on one portion of the buccal mucosa tissue. The remaining tissues were fixed in 10 % formalin, embedded in paraffin, sectioned and mounted on polylysine-coated glass slides. One section from each specimen was stained with haematoxylin and eosin. The other sections were used for immunohistochemical staining.

Induction of Oral Squamous Cell Carcinogenesis

The total number of tumors in the hamster's buccal pouch was determined macroscopically at the time of sacrifice of animals. The diameter of each tumor was measured with a Vernier calliper. The tumor volume was calculated by the formula $V=(4/3)\pi(D_1/2)\,(D_2/2)\,(D_3/2)$, where D_1 D_2 and D_3 are the three diameters (mm^3) of the tumor. Tumor burden was calculated by multiplying tumor volume and the number of tumors/hamster.

Biochemical Analysis

Buccal mucosa tissue samples were washed with ice cold saline and homogenized using an appropriate buffer in an

all-glass homogenizer with a teflon pestle and used for biochemical estimations.

TBARS in buccal mucosa were assayed by the method described by Ohkawa et al. [17]. The GSH levels were determined with the method of Beutler and Kelly [18]. The activity of GPx, SOD and CAT in buccal mucosa was assayed using the method of Rotruck et al. [19], Kakkar et al. [20] and Sinha [21], respectevely. Vitamin E was measured using the fluorimetric method of Palan, et al. [22].

Immunohistochemical Staining

Immunohistochemical analysis was performed as described earlier [23]. Each slide was microscopically analyzed and the percentage of the positively stained cells was estimated semi-quantitatively. The percentage of positive cells was scored according to the method of Lyzogubov et al., [24]: 3+=strong staining, more than 50 % of cells was stained; 2+=moderate staining, between 20 and 50 % of cells was stained; 1+=weak staining between 1 and 20 % of cells was stained; 0=negative, less than 1 % of cell staining.

Statistical Analysis

The data were expressed as mean ± S.D. Statistical comparisons for biochemical parameters were performed by one-way analysis of variance followed by Duncan's multiple range test (DMRT). Statistical evaluation for PCNA and p53 expression was analyzed using Chi-square (χ^2) test. The results were considered significant if the p values were less than 0.05.

Results

Characterisation of NARNPs

Figure 1a shows the particle size distribution of the prepared naringenin-loaded nanoparticles (NARNPs). The size distribution for the nanoparticles was obtained using DLS measurements. The average particle size of the prepared NARNPs was found to be around 90 nm (Fig. 1a). In TEM analysis, NPs are found to be spherical and well-separated, and most of them are smaller than 50 nm (Fig. 1b). It is noteworthy that the diameter of the particles measured by particle size analyzer is higher than the size estimated from TEM predominantly because of high swelling capacity of NARNPs. Interestingly, these nanoparticles have not showed any aggregation or adhesion. The encapsulation efficiency of NARNPs was 88±2.7 %.

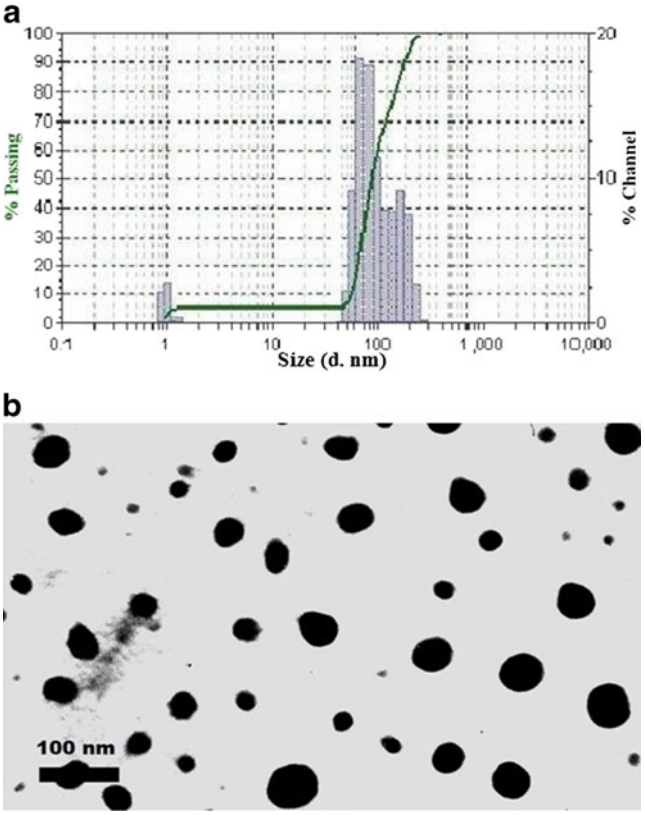

Fig. 1 **a** Mean particle size of NARNPs measured by dynamic light scattering. **b** Transmission electron microscopic (TEM) images of NARNPs

Tumor Incidence

Table 1 shows the incidence of oral neoplasm in the control and experimental animals in each group. In DMBA painted animals (Group 2), the tumor incidence was 100 % and these tumors were large, exophytic and well-defined, with a mean tumor burden of 1,318 mm^3. Histologically, DMBA-induced HBP tumors were found to be well-differentiated squamous cell carcinoma with evidence of keratin formation. In Group 3 (free NAR), three of ten animals developed SCC, while others displayed mild to moderate dysplasia. Oral administration of NARNPs (Group 4), on alternate days to DMBA painting, to DMBA-treated hamsters for 14 weeks completely prevented the tumor incidence as well as the formation of oral squamous cell carcinoma. No tumor was observed in the control animals painted with liquid-paraffin alone (Group 1), as well as free NAR (Group 5) and NARNPs alone (Group 6) administered hamsters.

Histopathology

The histopathological features observed in the buccal mucosa of hamsters in the control and experimental animals in each group are shown in Fig. 2 and Table 2. Severe hyperkeratosis,

Table 1 Incidence of oral squamous cell carcinoma in the control and experimental groups (n=10)

Parameter	Group 1 (Control)	Group 2 (DMBA)	Group 3 (DMBA + NAR)	Group 4 (DMBA + NARNPs)	Group 5 (NAR alone)	Group 6 (NARNPs alone)
Tumor incidence	0	100 %	30 %	0	0	0
Total number of tumors/animals	0	35/10	6/3	0	0	0
Tumor volume (mm^3/animals)	0^a	376 ± 42^b	115 ± 9^c	0^a	0^a	0^a
Tumor burden (mm^3/animals)	0^a	1318 ± 128^b	230 ± 23^c	0^a	0^a	0^a

Values are expressed as mean ± Standard deviation (S.D.) for ten hamsters in each group. Values that do not share a common superscript in the same column differ significantly at $p<0.05$ (DMRT)

Tumor volume was measured using the formula, $\upsilon=(4/3)\,л\,[D_1/2][D_2/2][D_3/2]$, where D_1, D_2 and D_3 are the three diameters (mm) of the tumor. Tumor burden was calculated by multiplying tumor volume and the number of tumors per animal

hyperplasia, dysplasia and well-differentiated squamous cell carcinoma were observed in the buccal pouches of DMBA-alone treated hamsters (Fig. 2b). A mild to moderate preneoplastic lesions [hyperplasia, keratosis and dysplastic] were noticed in Group 3 animals (Fig. 2c), while Group 4 animals exhibited mild dysplasia (Fig. 2d). Hamsters administered with free NAR and NARNPs alone showed well-defined and intact epithelial layers similar to that of the control hamsters (Fig. 2a, e and f).

Status of Buccal Mucosa TBARS and Antioxidants

The status of TBARS and antioxidants levels in the buccal mucosa of the control and experimental animals in each group is shown in Fig. 3. As seen from the figure, topical application of DMBA (Group 2) for 14 weeks decreased TBARS level in the buccal pouch when compared to the control animals (Group 1). The concentration of GSH, GPx, and vitamin E increased and SOD and CAT decreased in the tumor-bearing animals (Group 2) as compared to the control animals. Oral administration of free NAR (Group 3) and NARNPs (Group 4) to DMBA-treated animals restored the concentration of TBARS and antioxidants to a near normal range. Overall, the treatment of NARNPs was found to be more effective than free naringenin in restoring the status of lipid peroxidation and antioxidants. Hamsters treated with free NAR and NARNPs alone (Groups 5 and 6, respectively) showed no significant difference in TBARS and antioxidants status as compared to the control hamsters (Group 1).

Immunohistochemical Analysis

Figure 4 and Table 3 depict the immunohistochemical analysis of PCNA and p53 expression in the hamster buccal

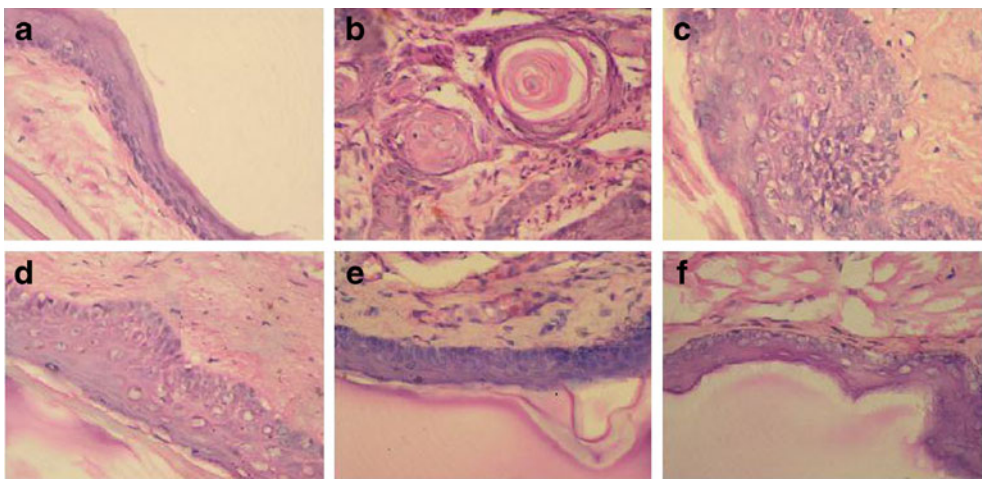

Fig. 2 Histopathological features observed in the buccal mucosa of the control and experimental animals in each group. **a**, **e** and **f** Photomicrographs showing well defined buccal pouch epithelium from control, NAR-alone and NARNPs alone treated hamsters respectively (H&E, 40×). **b** Photomicrographs showing well differentiated squamous cell carcinoma with keratin pearls in hamsters treated with DMBA-alone (H&E, 40×), **c** Photomicrographs showing moderate dysplastic epithelium in hamsters treated with DMBA + NAR (H&E, 40×) and (**d**) Photomicrographs showing mild dysplastic epithelium in hamsters treated with DMBA + NARNPs (H&E, 40×)

Table 2 Histopathological changes in the buccal mucosa of the control and experimental groups (n=10)

Parameter	Group 1 (Control)	Group 2 (DMBA)	Group 3 (DMBA + NAR)	Group 4 (DMBA + NARNPs)	Group 5 (NAR alone)	Group 6 (NARNPs alone)
Hyperkeratosis	–	+++	++	+	–	–
Hyperplasia	–	+++	++	+	–	–
Dysplasia	–	+++	+/++	+	–	–
Squamous cell carcinoma	–	Well differentiated (10)	Well differentiated (3)	–	–	–

−=no change; +=mild; ++=moderate; +++=severe

pouch mucosa of the control and experimental animals in each group. Tumors were considered positive when more than 10 % of the tumor cells were stained. A significant increase in the expression of PCNA and p53 was found in DMBA painted hamsters when compared to the control. Administration of free NAR and NARNPs at a dose of 50 mg NAR/kg body weight to DMBA painted hamsters significantly decreased the PCNA and p53 expression compared to group 2 animals. However, NARNPs (Group 4) were more effective when compared to free NAR (Group 3).

Discussion

Drug development from natural products is currently emerging as a highly promising strategy to identify novel anticancer agents. During the past decade, a large number of phytochemicals from the diet and medicinal plants have been evaluated for anticancer activity owing to their ability to interfere with multiple pathways controlling growth and apoptosis of cancer cells. Naringenin is one of the potent dietary phytochemicals and possesses various biological and pharmacological properties. Despite its promising pharmacological activity, naringenin's low oral bioavailability has remained a major hurdle. Therefore, to achieve maximum response of a chemopreventive agent, novel strategies are required to enhance the bioavailability of potentially useful agents and reduce perceived toxicity. Considering the potential of nanoparticles as oral drug delivery system, the present investigation involved development and characterisation of NARNPs with a view to improve its oral bioavailability.

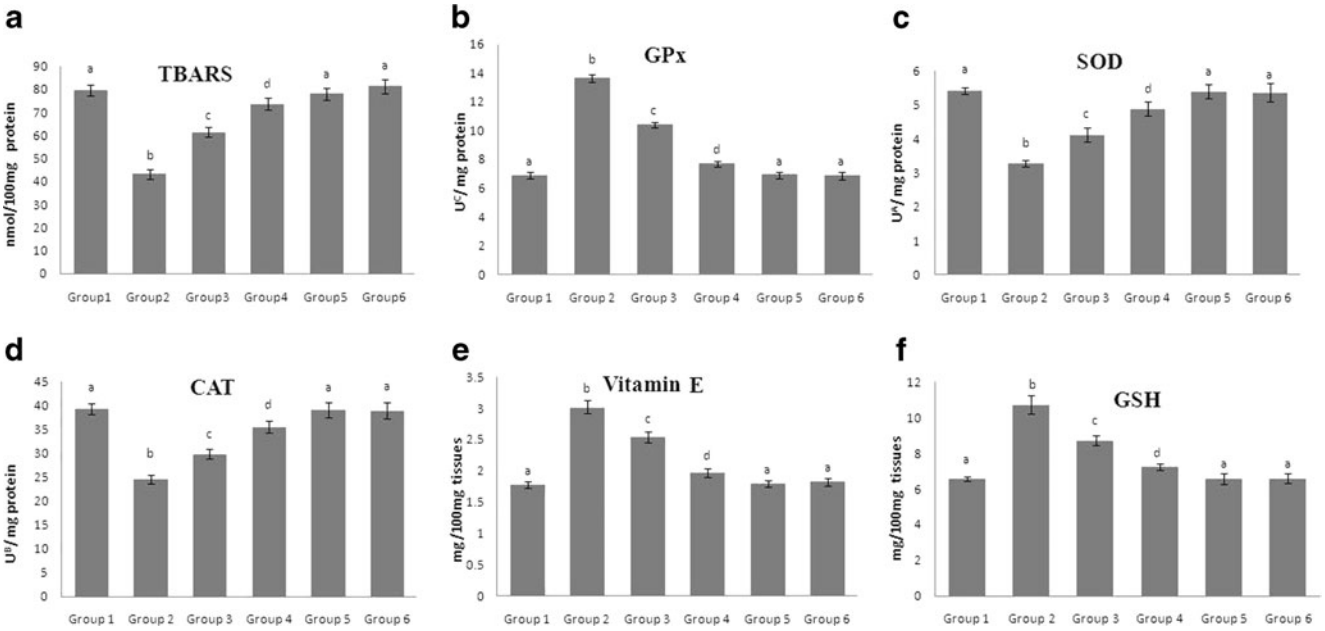

Fig. 3 The status of lipid peroxidation (TBARS), enzymatic antioxidants (GPx, SOD and CAT) and non-enzymatic antioxidants (Vitamin E and GSH) in the buccal mucosa of the control and experimental groups. Bars are mean ± SD for 10 animals in each group. $^{a–d}$ values that do not share a common superscript letter (**a**,**b**,**c** and **d**) in the same column differ significantly at $p<0.05$ (DMRT). U^A-micromoles of glutathione utilized/minute; U^B- amount of enzymes required to inhibit 50 % nitroblue-tetrazolium (NBT) reduction; U^C-micromoles of H_2O_2 utilized/second

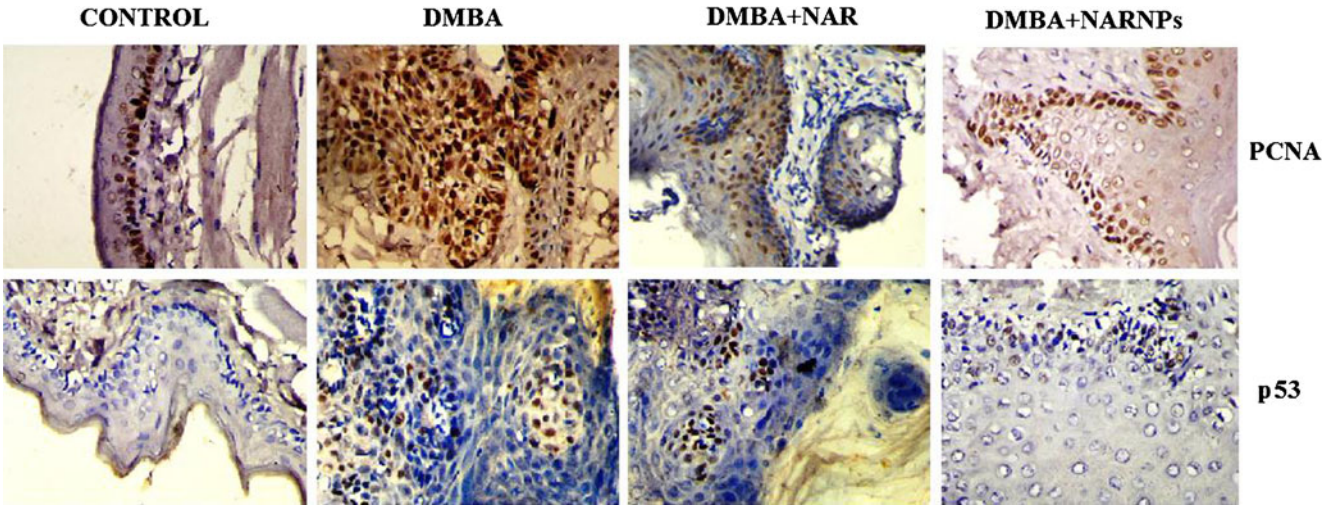

Fig. 4 Representative photographs of immunohistochemical staining of PCNA and p53 expression in the control and experimental animals (n=10)

In the present study, the naringenin-loaded nanoparticles of around 90 nm were synthesized and developed for the treatment of oral cancer. The particle size measured by DLS was found to be higher than that of TEM measurements because DLS measures the apparent size, including the hydrodynamics layers that form around hydrophilic particles, thus leading to overestimation of the size of the NARNPs [25]. Moreover, it was observed that naringenin was efficiently loaded in NPs, reaching a high encapsulation efficiency of 88±2.7 %. This implies that ~88 % of the drug was entrapped inside the nanoparticles. The amount of entrapped drug in the NP is an important factor for determining the therapeutic efficacy of drug delivery system. Furthermore, our previous results have confirmed that the encapsulated naringenin retained its properties even inside the nanoparticles [14].

In DMBA induced HBP tumors, the extent of lipid peroxidation was low compared with the control group. Lipid peroxides are recognized to prolong the G1 phase of the cell cycle and decrease the rate of cell proliferation. Increased cell proliferation was thought to be involved in the pathogenesis of carcinogenesis. An inverse relationship has been observed between lipid peroxidation and the rate of cell proliferation, with highly proliferating tumors showing low levels of lipid peroxidation [26]. Rapidly proliferating tumor cells show resistance to lipid peroxidation and overexpress GSH and GSH-dependent enzymes. Reduction in lipid peroxidation observed in the present study could be related to lack of peroxidisable substrate and increased rate of removal of lipid peroxidation products. The low availability of polyunsaturated fatty acids (PUFA) has been suggested to be a limiting factor for lipid peroxidation in tumor tissues [26,

Table 3 Intensity of staining for PCNA and mutant p53 expression in the control and experimental groups (n=10)

Parameter		Group 1 (Control)	Group 2 (DMBA)	Group 3 (DMBA + NAR)	Group 4 (DMBA + NARNPs)	Group 5 (NAR alone)	Group 6 (NARNPs alone)
PCNA	0	6	0	0	0	5	6
	1+	4	0	3	4	5	4
	2+	0	2	5	6	0	0
	3+	0	8	2	0	0	0
p53	0	10	0	1	2	10	10
	1+	0	1	2	4	0	0
	2+	0	3	6	4	0	0
	3+	0	6	1	0	0	0

Values are given as number of animals (n=10). The percentage positive cells were scored as: 3+=strong staining, more than 50 % of cells were stained, 2+=moderate staining between 20 and 50 % of cells were stained, 1+=weak staining between 1and 20 % of cells were stained, 0=negative, less than 1 % of cells staining

27]. Lowered TBARS levels observed in the buccal mucosa of hamsters treated with DMBA alone might be due to the decreased PUFA content in tumor tissues or abnormal cell proliferation occurring in oral carcinogenesis. Our previous studies have found diminished lipid peroxidation associated with a loss of = CH groups of olefinic bands (unsaturated lipids) in DMBA-induced HBP tumors [27]. Decreased lipid peroxidation in HBP tumors may be attributed to an increase in GSH and GPx that are involved in scavenging reactive oxygen species (ROS), maintenance of thiol status, detoxification and regulation of cell proliferation [28].

Glutathione peroxidase and its co-substrate reduced glutathione are essential for the maintenance of cellular integrity due to their regulatory effects on cell proliferation. The present results showed increased levels of GSH and enhanced activities of GSH dependent enzyme GPx in HBP tumors, which might be an adaptive mechanism by which tumor cells gain selective growth advantage over their surrounding cells. Higher levels of GSH and overexpression of GSH-dependent enzymes GPx have been reported in several malignant tumors including oral tumors [26, 29]. Moreover, these results are consistent with the hypothesis that decline in lipid peroxidation is associated with increased enhanced GSH-dependent antioxidant activity.

SOD and CAT are primary antioxidant enzymes involved in the inactivation of environmental carcinogen and direct elimination of toxin-free radicals and electrophiles, which might result in amelioration of oxidative damage. In the present study, activities of SOD and CAT were found to be decreased in hamsters treated with DMBA. Lowered activities of SOD and CAT were also reported in several malignancies including oral cancer [26, 29]. An increase in vitamin E in tumor tissues indicates that the malignant tumor can sequester these antioxidants for their growth and can also maintain the low levels of lipid peroxidation by-products. The decrease in TBARS levels and functional compromise of antioxidant defense mechanisms observed in the tumor tissues of DMBA-treated hamsters reflects a decreased susceptibility of oral tumor tissue to lipid peroxide. Several experimental studies supported our present findings [26, 29]. Oral administration of free NAR and NARNPs restored the status of lipid peroxidation and antioxidants in the buccal mucosa tissues of DMBA-painted animals. The chemopreventive potential of NAR and its nanoparticles may also be attributed to its antioxidant properties. The antioxidant potential of NAR might be probably due to the presence of three phenolic groups and keto groups, which can react with hydroxyl radicals, hydrogen peroxide and peroxynitrite and thereby inhibit both oxidative and nitrosative reactions. Comparing NAR and its nanoparticles, NARNPs have potent antioxidant potential and free radical scavenging property during oral carcinogenesis. NARNPs offer many active sites for free radical scavenging because of their large surface/volume ratio and also nanoparticulate drug delivery systems can enhance the oral bioavailability of NAR by NARNPs. Moreover, prevention of degradation of NAR in the GIT along with the enhanced intestinal uptake and protection from first pass metabolism by nanoparticulate carriers could contribute to its improved oral bioavailability. The present results thus suggest that naringenin-loaded nanoparticles improved the antioxidant defense mechanism by scavenging excessively generated reactive oxygen species during DMBA-induced hamster buccal pouch carcinogenesis.

Cell proliferation plays an important role in multi-stage carcinogenesis and is believed to be involved in the pathogenesis of OSCC. Increased cell proliferation has been proposed to be a biomarker of increased susceptibility to oral cancer. Thus, over expression of PCNA and p53 observed in the current study reflects increased cell proliferation as confirmed by immunohistochemical analysis in HBP tumors. The content and expression of PCNA, a 36-kDa non-histone nuclear acidic protein, have been linked to the late G1 as well as early S-phase of the cell cycle. Moreover, PCNA is one of the helper proteins of DNA polymerase O', which is a key protein in cell cycle regulation [30]. Besides proliferation, PCNA also exhibits anti-apoptotic functions. PCNA interacts with Gadd 45, a growth arrest and DNA damage protein, as well as MyD118, a myeloid differentiation primary response protein. These interactions inhibit apoptosis and differentiation and promote cell growth. Therefore, PCNA expression has been considered to reflect the proliferation rate of tumor cells. Shin, et al. found a gradual increase in PCNA expression during progression of normal epithelium from hyperplasia through dysplasia to oral squamous cell carcinoma [31]. Over expression of PCNA has been reported in a wide range of human tumors as well as in DMBA-induced HBP tumors [5, 6, 23].

Immunohistochemical localisation of p53 protein in HBP carcinomas was similar to that of PCNA with increased expression in areas of high proliferative activity. Mutations of p53, the most frequent alterations in human OSCCs and HBP carcinomas induce conformational changes that prolong the half-life of the p53 protein enabling immunolocalisation in the nuclei of malignant cells [32]. Overexpression of mutant p53 may enhance genetic instability by facilitating cell proliferation and inhibiting DNA repair and apoptosis. Thus, overexpression of PCNA and p53 observed in HBP carcinomas may confer a selective growth advantage on tumor cells.

Oral administration of NARNPs to DMBA painted animals not only prevented the formation of squamous cell carcinoma, but also maintained the expression of PCNA and p53 in the buccal mucosa of hamsters during DMBA induced oral carcinogenesis. Down regulation of PCNA and p53 by free NAR and NARNPs suggest regulatory effects

on cell cycle progression. Moreover, NARNPs was more active than free NAR in suppressing the expression of PCNA and p53 proteins. The enhanced efficacy of NARNPs could be due to difference in bioavailability of NAR by NARNPs which might be the reason for better antiproliferative activity of nanoparticulate naringenin. Greater bioavailability and stability of nanoparticulate naringenin results in greater accumulation of delivered naringenin inside tumor cell and consequently showed more pronounced down regulation of PCNA and p53 compared to free naringenin treated animals. These findings suggest that nanoparticulate naringenin might have inhibited abnormal cell proliferation either by inhibiting DMBA metabolic activation or by suppressing DMBA-induced mutations in p53 and PCNA during DMBA-induced oral carcinogenesis.

The results of the present study showed that the NARNPs exhibited more efficient anti-tumor efficacy than free NAR by significantly preventing the tumor formation during DMBA-induced oral carcinogenesis. Numerous investigations have shown that nanoparticulate drug delivery systems can increase anti-tumor efficacy while reducing systemic side effects [33, 34]. It is well known that nanoparticles could escape from the vasculature through the leaky endothelial tissue that surrounds the tumor and then accumulates in certain solid tumors by the process called "enhanced permeation and retention" (EPR) effect [35]. This EPR is mainly due to the differences in the vasculature between tumor tissue and normal tissue. Normal tissue vasculatures are lined by tight endothelial cells which prevent the nanoparticles from escaping into the tissue, whereas tumor tissue vasculatures are porous with leaky endothelium, which easily allows the nanoparticles to permeate in the tissue. Hence, more drugs can enter the interior of the cells with the help of nanocarriers, which is crucial to exert the pharmacological activity of drug more efficiently. The possible mechanism underlying the superiority of NARNPs against free NAR may include the continous exposure of tumor mass to released NAR from the nanoparticles. In our previous study, we demonstrated that NARNPs showed a more sustained release of the drug than free NAR, which might produce a prolonged exposure of tumor cells to the anticancer drugs [14]. Therefore, for the NARNPs, drug nanoparticulates may arrive at the tumor through EPR effects and then sustain a high concentration over time, all of which would lead to higher antitumor efficacy compared to free NAR.

In conclusion, oral administration of free NAR and NARNPs restored the status of lipid peroxidation and antioxidants in the buccal mucosa tissues of DMBA-painted animals. In addition, the free NAR and NARNPs exhibited superior antiproliferative effect by down regulated the expression of PCNA and p53. On a comparative basis, NARNPs was found to have more potent anti-lipid peroxidative, antiproliferative and antioxidant potentials when compared to free NAR in DMBA-induced HBP carcinogenesis. Thus, the present study confirms that NARNPs could be a potentially useful drug carrier system for targeted delivery of naringenin for cancer chemoprevention.

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