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REGULAR ARTICLE

In vivo modulation of iNOS pathway in hepatocellular carcinoma by *Nigella sativa*

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Abstract
Objective Nitric oxide (NO) and inducible nitric oxide synthase enzyme (iNOS) have been implicated in various tumors. Hepatocellular carcinoma is a highly aggressive form of solid tumor. The lack of effective therapy necessitates the introduction of novel therapeutic strategies to counter this disease. *Nigella sativa* (NS) has been shown to have specific health benefits. The aim of this study was to investigate the in vivo modulation of the iNOS pathway by NS ethanolic extract (NSEE) and the implications of this effect as an antitumor therapeutic approach against diethylnitrosamine (DENA)-induced hepatocarcinogenesis.
Methods Rats were divided into four groups, normal control, NSEE control, cancer control, and NSEE-DENA groups. The diagnosis of cancer was based on alpha-fetoprotein (AFP) levels and histological variations. Serum NO, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) levels and serum iNOS activity were measured. Liver iNOS expression was investigated by reverse transcriptase (RT)-PCR and western blot assays.
Results Serum AFP, NO, TNF-α, and IL-6 levels and iNOS enzyme activity were significantly increased in rats treated with DENA. Significant up-regulation of liver iNOS mRNA and protein expression was also observed. Subsequent treatment with NSEE significantly reversed these effects and improved the histopathological changes in malignant liver tissue which appeared after treatment with DENA, without any toxic effect when given alone.
Conclusion These results provide evidence that attenuation of the iNOS pathway and suppression of the inflammatory response mediated by TNF-α, and IL-6 could be implicated in the antitumor effect of NSEE. As such, our findings hold great promise for the utilization of NS as an effective natural therapeutic agent in the treatment of hepatocarcinogenesis.

Keywords *Nigella sativa* ethanolic extract · Hepatocellular carcinoma · Inducible nitric oxide synthase · Nitric oxide · Tumor necrosis factor-alpha

Introduction

Nitric oxide (NO), a membrane permeable gaseous radical and an important cellular second messenger that is produced by three NO synthase (NOS) isoforms [inducible (iNOS), endothelial (eNOS) and neuronal (nNOS)], has been postulated to mediate numerous physiological and pathophysiological processes [1–3]. It plays an important role as a redox-based signaling mediator, modulating enzyme activities, signal transductions, and cytokine networks and also produces reactive nitrogen species (RNS). RNS cause the aberrant *S*-nitrosylation of proteins, DNA damage, as well as lipid peroxidation, apoptosis, and necrosis [4, 5].

NO, which is produced by iNOS during inflammation and overexpressed in neoplastic lesions, is also an endogenous candidate molecule involved in the predisposition to cancer [6–9]. Induction of gene alterations by NO through DNA damage, mutations, inhibition of DNA repair, and effects on p53, together with activation of DNA methyltransferase might thereby be important in studies on cancer.

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NO can also inhibit caspases and lymphocyte proliferation, activate telomerase and matrix metalloproteinases, and induce vascular permeability and pro-angiogenic factors, thus impacting on apoptosis, angiogenesis, cell migration, and invasion [6, 8, 10, 11].

Hepatocellular carcinoma (HCC) is one of the most frequently occurring forms of a solid tumor, with a high prevalence of 620,000 cases per year reported worldwide [12]. It is highly aggressive, as shown by the mortality of 595,000 cases per year, a mortality rate that nearly matches the incidence of this tumor type [13]. At the present time, there is a limited number of therapeutic options for HCC patients. Hence, a thorough understanding of the biological bases of this malignancy might be the first step in developing new strategies for effective treatment [14]. iNOS is known to be overexpressed in HCC [15, 16], and in the livers of patients with hepatitis, fibrosis, and cirrhosis [17–21]. Epidemiological studies have shown that inflammation predisposes to certain cancers and that most cancerous tissues show signs of inflammation, in association with specific inflammatory signaling molecules (i.e., cytokines), remodeling, and angiogenesis. It has also been shown that cancer-associated inflammation actually promotes tumor growth and progression [22, 23]. Several pro-inflammatory gene products [i.e., tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)] play a critical role in apoptosis, proliferation, angiogenesis, invasion, and metastasis. Their expression is mainly regulated by nuclear factor kappa-B, which is constitutively active in most tumors and induced by chemotherapeutic agents and carcinogens [24]. Although improper up-regulation of iNOS and pro-inflammatory cytokines has been linked to the pathophysiology of certain cancers [25–32], the involvement of iNOS in hepatocarcinogenesis remains largely unclear.

On account of the high morbidity and mortality of cancer diseases, the problems encountered in their treatment, and the toxic effects of cancer chemotherapy, various attempts have been made to investigate the anticancer effects of relatively safe edible herbs [33]. One of these herbs is *Nigella sativa* (NS). NS seed has long been an important natural remedy for many ailments in ancient systems of medicine (e.g. Unani, Ayurveda, Chinese, and Arabic medicines), and many active components have been isolated from this herb. The pharmacological effects of NS seed, its oil, various extracts, and active components have been identified to include stimulation of the immune system and anti-inflammation, hypoglycemic, antihypertensive, antiasthmatic, antimicrobial, antiparasitic, antioxidant, and anticancer effects [34, 35]. However, only a few authors have reviewed the medicinal properties of NS and reported on its anticancer effects. Toxicity studies have recently confirmed the safety of NS oil and its most abundant active component, thymoquinone [34, 36–38].

The molecular effect of thymoquinone and NS oil on the iNOS pathway in lung and testicular injuries has recently been shown [39–43], but to date the molecular effect of NS seed extracts on that pathway has not been investigated. Here we report our study on the in vivo molecular mechanism underlying the antitumor properties of NS ethanolic extract (NSEE) through the modulation of the iNOS pathway against diethylnitrosamine (DENA)-induced hepatocarcinogenesis. To this end we examined the effects of NSEE on DENA-stimulated increased alpha-fetoprotein (AFP), NO, TNF-α, and IL-6 levels, as well as the effects of NSEE on iNOS activity and expression.

Materials and methods

Animals

Animal care and experimental protocols were performed in accordance with the guidelines established by the Research Ethics Committee of the Experimental Animal Center, University of Toyama. In total, we used 32 male Wistar rats (weight 160 ± 5 g) which were maintained on a standard rodent diet and water ad libitum. The rats were housed in polyethylene cages in a humid room under a controlled light regime of 12 h light and 12 h dark for 2 weeks before the experiment.

Chemicals

All chemicals were of analytical grade and came from local suppliers.

NS ethanolic extract

Nigella sativa seeds (Kahira Pharmaceuticals and Chemical Industries Co., Cairo, Egypt) were washed, dried, and then ground to powder using an electric micronizer (Jiangyin Hongke Shredder Machinery Co., Jiangsu, China). The powder was extracted three times with 80 % ethanol and the solvent evaporated at 40 °C under reduced pressure. This procedure resulted in a two-phased extract. The oily and solid phases were recombined in proportion to their yield (typically 70 and 30 %, respectively). The NSEE was stored at 4 °C and protected from light and humidity.

Experimental design

The rats were divided into four equally sized groups (8 rats/group). Rats in the normal control group were given normal saline 0.5 ml by gavage daily for 5 consecutive days and received a single intraperitoneal (i.p.) injection of normal

saline (2.5 ml/kg) on day 3. Rats in the NSEE control group were given NSEE 250 mg/kg orally by gavage daily for 5 consecutive days [44]. Rats in the cancer control group were given the same doses of normal saline as the normal control group (0.5 ml) for 3 consecutive days before and 2 days after a single i.p. dose of DENA (Sigma Chemical Co., St. Louis, MO) 200 mg/kg in saline [45]. Rats in the NSEE-DENA group received the same doses of NSEE as the NSEE control group (250 mg/kg) for 3 consecutive days before and 2 days after a single i.p. dose of DENA (200 mg/kg). Fourteen weeks after the administration of DENA, the animals were fasted overnight, then weighed and killed by exposure to an increasing concentration of CO_2. Blood samples were obtained by cardiac puncture, left to clot for 1 h at 37 °C, then centrifuged at 10,000 rpm at 4 °C to separate out the serum samples which were kept at −80 °C for further use.

Liver samples were quickly removed, washed in ice-cold phosphate buffered saline (PBS) and desiccated. Individual liver weights were recorded. The liver was cut into portions for subsequent studies. The portion intended for histological examination was kept in 10 % buffered formalin. A second portion was either frozen in liquid nitrogen and stored at −80 °C for further use or homogenized in 0.25 mM sucrose, 10 mM Tris, 1 mM ethylene diamine tetraacetic acid (EDTA) (pH 7.4), and 1 % protease inhibitor cocktail (Boehringer Mannheim, Indianopolis, IN). The liver homogenates were centrifuged at 4,000 rpm for 15 min at 4 °C and the supernatant collected; total protein in the supernatant was determined. For biochemical investigations, samples were distributed into aliquots in Eppendorf tubes and kept at −80 °C.

Serum AFP, TNF-α, and IL-6 measurements

Samples were analyzed in triplicate. AFP serum levels were determined using the UBI MAGIWEL™ AFP CM-101 assay, a highly sensitive enzyme-linked immunosorbent assay (ELISA) kit (United Bioresearch, Sunnyvale, CA) following the recommendations of the manufacturer. TNF-α and IL-6 serum levels were determined using highly sensitive ELISA kits from R&S Systems (Minneapolis, MN) according to the manufacturer's recommendations.

Serum NO measurement

The NO stable end products nitrite + nitrate (NO_x) were measured in serum using the Total Nitric Oxide Assay kit (Thermo Scientific, Rock Ford, IL). In brief, after nitrate had been reduced by 0.25 % vanadium(III) chloride in 0.5 M HCl, a 100-µl sample was mixed with 100 µl of Griess reagent [mixture of equal amounts of A (1 % sulphanilamide) and B (0.1 % naphthylethylene diamine dihydrochloride in 5 % H_3PO_4)] followed by incubation for 10 min at room temperature. Absorbance at 540 nm was measured, and the NO levels were calculated by using a standard calibration curve prepared from different concentrations of sodium nitrite. Standards were analyzed in triplicate.

Serum iNOS enzymatic activity assay

Serum iNOS enzymatic activities were measured using the Mouse iNOS ELISA kit from ElAab (Wuhan ElAab Science Co., Wuhan, China) according to the manufacturer's instructions. In this kit, a monoclonal antibody specific for iNOS has been pre-coated onto a microplate. Standards and samples are pipetted into the wells, and any iNOS present is bound by the immobilized antibody. An enzyme-linked polyclonal antibody specific for iNOS is then added to the wells. Following a wash to remove any unbound antibody–enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of iNOS bound in the initial step. The color development is then stopped and absorbance at 450 nm measured. In our study we used an HTS Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT) to measure absorbance, and enzymatic activities of the different groups were expressed relative to those of the normal control group.

RNA isolation, cDNA synthesis, and reverse transcription-PCR for iNOS mRNA

Total RNA was extracted from frozen mouse livers using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Aliquots of 1 µg of total RNA were treated with 0.1 U/µl deoxyribonuclease I (DNase I; Sigma-Aldrich) at room temperature for 15 min. cDNAs were synthesized using 0.5 µg DNase I-treated RNA by using a Rever Tra Ace qPCR RT kit (TOYOBO Co., Osaka, Japan) according to the manufacturer's instructions. The resulting cDNA was subjected to PCR using a Taq DNA polymerase kit (QIAGEN, Hilden, Germany). PCR cycling was performed in a 25-µl reaction mixture and consisted of one cycle of 70 s at 95 °C, 25 cycles of 55 s at 93 °C, 45 s at 61 °C, and 40 s at 72 °C, and one cycle of 100 s at 72 °C, according to the manufacturer's instructions for the thermal cycler (Gene Amp PCR System 2700; Applied Biosystems, Foster City, CA). The primers (Invitrogen, Carlsbad, CA) used were: mouse *iNOS* (835 bp; forward 5′-CAACCAGTATTATGGCTCCT-3′, reverse 5′-GTGACAGCCCGGTCTTTCCA-3′) and mouse *β-actin* (840 bp; forward 5′-GGAGAAGATCTGGCACCA CACC-3′, reverse 5′-CCTGCTTGCTGATCCACATCTGC TGG-3′). The *β-actin* primer was used as an internal control. PCR products were separated by electrophoresis in

2 % agarose gels containing 0.1 μg/ml ethidium bromide. Digital images were captured by LAS-3000 (Fujifilm, Tokyo, Japan) and Multi-Gauge v3.0 software (Fujifilm). Bands corresponding to iNOS of the different groups were analyzed densitometrically relative to the normal control group using Image J software. Data were normalized to the corresponding internal control *β-actin* mRNA bands.

Western blot assay of iNOS protein expression

Samples containing 50 μg of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12–14 % acrylamide) and transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). Nonspecific binding was blocked by preincubation of the membranes in PBS containing 3–5 % nonfat dried milk for 30 min at 37 °C. The membranes were then incubated overnight at 4 °C with rabbit polyclonal anti-iNOS antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). Bound primary antibody was detected using a horseradish peroxidase-conjugated antirabbit secondary antibody (Dako, Glostrup, Denmark) by chemiluminescence using an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. Immunoreactive proteins were visualized using a luminescent image analyzer (LAS-4000; Fujifilm Co.). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology) was used (1:1000) as a loading control to ensure equal loading and even transfer from the gel to the membrane across the whole gel. Bands corresponding to iNOS protein of the different groups were analyzed densitometrically, using Image J Software, relative to the normal control group. Data were normalized to GAPDH levels. Electrophoresis and electroblotting were carried out in a Bio-Rad Trans-Blot SD Cell apparatus (Bio-Rad, Hercules, CA) using a discontinuous buffer system.

Histological examination

For histological examination, a piece of the liver was fixed by immersion in 10 % buffered formalin for 24 h. The blocks were dehydrated in a graded series of ethanol and embedded in Paraffin wax. Serial 4-μm-thick sections were stained with hematoxylin and eosin (H&E) stain.

Statistical analysis

All results were expressed as mean ± standard deviation of $n = 8$ rats. Statistical comparison was conducted using Student's t test after one-way analysis of variance (ANOVA) using GraphPad Prism 5 statistical software (GraphPad, La Jolla, CA) and Excel software (Microsoft, Redwood, WA). The results were considered to be significant when the probability values (P) were <0.05.

Results

Serum AFP level

Serum AFP levels were measured as an indicator of cancer incidence. As shown in Table 1, animals treated with NSEE showed non-significant changes in serum AFP levels, while animals treated with DENA (cancer group) showed significantly ($P < 0.001$) higher serum levels of AFP when compared to their counterparts in the normal control group. The treatment of rats which had developed cancer with NSEE (NSEE-DENA group) significantly ($P < 0.001$) reduced serum AFP levels compared to rats treated only with DENA.

Serum levels of the pro-inflammatory cytokines (TNF-α and IL-6)

The level of the pro-inflammatory cytokines TNF-α and IL-6 in the serum was determined by appropriate ELISA assay according to the manufacturer's instructions. As shown in Table 1, the administration of DENA significantly ($P < 0.001$) increased the serum levels of both pro-inflammatory cytokines in rats of the cancer control group relative to those of the normal control group; this increase was significantly ($P < 0.001$) reversed in the rats of the

Table 1 Serum alpha-fetoprotein, tumor necrosis factor-alpha, and interleukin-6 levels in all rat groups

Group[a]	Serum alpha-fetoprotein (ng/ml)	Serum tumor necrosis factor-alpha (pg/ml)	Serum interleukin-6 (pg/ml)
Normal control	6.3 ± 0.56	18.5 ± 2.30	40.8 ± 4.63
NSEE control	7.2 ± 0.63 ns	19.2 ± 3.17 ns	43.1 ± 4.81 ns
Cancer control	61.5 ± 4.63*	75.6 ± 8.43*	120.4 ± 11.12*
NSEE-DENA	20.7 ± 4.1[§]	34.3 ± 5.85[§]	71.3 ± 11.73[§]

Data are presented as the mean ± standard deviation ($n = 8$)

Significant difference between groups was analyzed by one-way analysis of variance, where: * $P < 0.001$ compared to normal control group, [§] $P < 0.001$ compared to cancer control group; ns, non significant ($P > 0.05$) compared to normal control group

[a] See Materials and methods subsection Experimental design for a detailed description of the animal groups

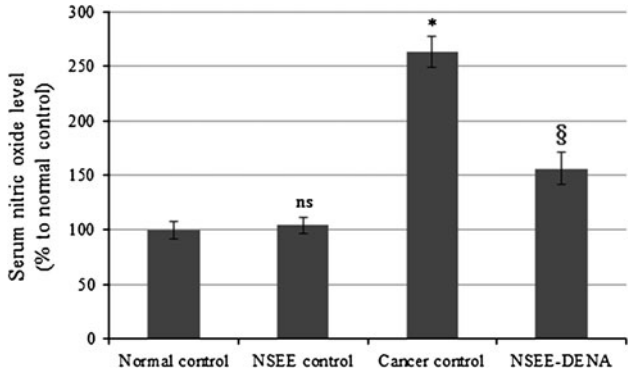

Fig. 1 Serum levels of the pro-inflammatory mediator nitric oxide. Serum levels are expressed relative to those of the normal control group. *Bars* Mean ± standard deviation (SD), $n = 8$. Significant differences between groups are analyzed by one-way analysis of variance (ANOVA). $^{*}P < 0.001$ compared to normal control group, $^{\S}P < 0.001$ compared to cancer control group, *ns* non-significant ($P > 0.05$) compared to normal control group. *NSEE Nigella sativa* ethanolic extract, *DENA* diethylnitrosamine. See Materials and methods subsection Experimental design for a detailed description of the animal groups

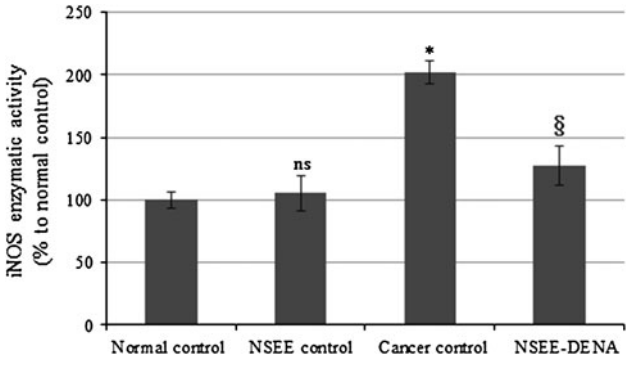

Fig. 2 Serum inducible nitric oxide synthase (*iNOS*) enzymatic activity. Enzymatic activities of the different groups are expressed relative to that of the normal control group. *Bars* Mean ± SD, $n = 8$. Significant differences between groups are analyzed by one-way ANOVA test. $^{*}P < 0.001$ compared to normal control group, $^{\S}P < 0.001$ compared to cancer control group, *ns* non-significant ($P > 0.05$) compared to normal control group

NSEE-DENA group. Rats treated with NSEE alone did not show any significant changes in their serum levels compared to the normal control group rats.

Serum levels of the pro-inflammatory mediator NO

The serum levels of the NO stable end products were estimated, and the results are shown in Fig. 1. NO serum levels were significantly ($P < 0.001$) increased in rats treated with DENA, but did not significantly change in those treated with NSEE when compared to those in the normal control group animals. The serum level of this pro-inflammatory mediator was significantly reduced in rats in the NSEE-DENA group ($P < 0.001$) compared the cancer control group rats.

iNOS enzymatic activity

The inhibitory effect of NSEE on the activity of serum iNOS enzyme was also examined. Figure 2 shows that DENA significantly ($P < 0.001$) elevated the enzymatic activity of iNOS in the cancer control rats while NSEE did not elevate iNOS enzymatic activity, when compared to iNOS activity in normal control rats. More interestingly, iNOS enzyme activity in rats in the NSEE-DENA group was significantly ($P < 0.001$) reduced when compared that in the cancer control group rats.

Liver iNOS mRNA expression

Liver tissues from rats of different groups were investigated for their iNOS mRNA expression by reverse transcription (RT)-PCR (Fig. 3a). When the iNOS bands of the different groups were densitometrically analyzed relative to the normal control group and normalized to β-actin levels, iNOS mRNA expression in the cancer control group was found to be significantly ($P < 0.001$) elevated compared to that of normal control group. Surprisingly, iNOS mRNA expression in NSEE control rats and NSEE-DENA

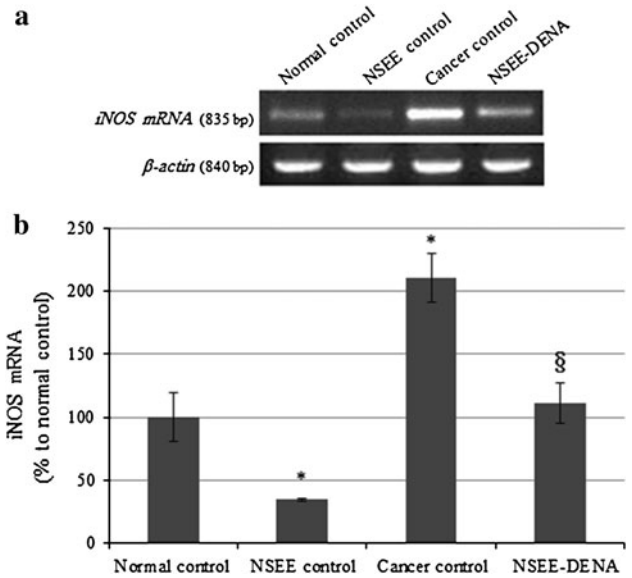

Fig. 3 Liver *iNOS* mRNA expression. **a** Representative reverse transcription (RT)-PCR photographs for the different groups. **b** Relative expression of iNOS mRNA of the different groups is expressed densitometrically as a percentage of that of the normal control group using the *bands* in **a**. Expression was normalized to that of the corresponding internal control *β-actin* mRNA. *Bars* Mean ± SD, $n = 8$. Significant differences between groups are analyzed by one-way ANOVA, where $^{*}P < 0.001$ compared to the normal control group, $^{\S}P < 0.001$ compared to the cancer control group

rats were significantly ($P < 0.001$) reduced compared to that of normal control and cancer control rats, respectively, as shown in Fig. 3b.

Liver iNOS protein expression

Nigella sativa ethanolic extract is known to down-regulate NO production induced by DENA. Because NO overproduction is always associated with the up-regulation of iNOS protein expression, we examined iNOS protein expression by the liver by western blot (Fig. 4a). The densities of bands corresponding to the iNOS protein were normalized on the basis of GAPDH and analyzed relative to that of the normal control group, as shown in Fig. 4b. The administration of DENA significantly ($P < 0.01$) up-regulated liver iNOS protein expression in the cancer group rats, while NSEE significantly ($P < 0.001$) down-regulated its expression, when compared to the normal control group rats. Also, iNOS protein expression in the liver of NSEE-DENA rats was significantly ($P < 0.001$) reduced when compared to that of cancer control rats.

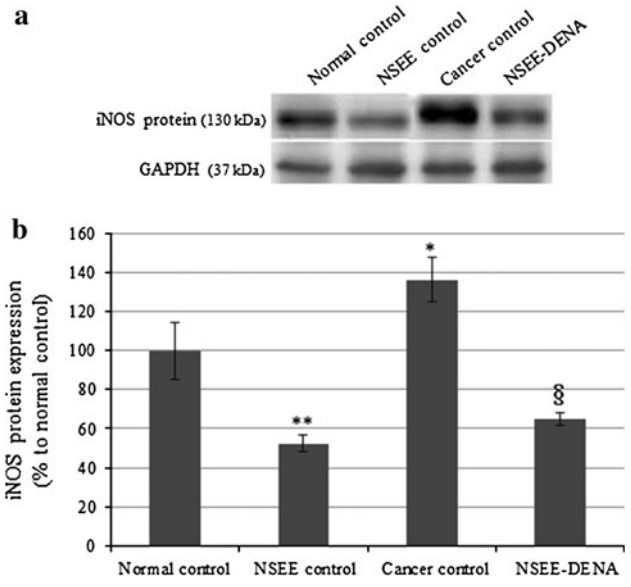

Fig. 4 Liver iNOS protein expression. **a** Representative immunoprecipitation blots of iNOS protein expressed in the liver of rats in the different groups. **b** Relative expression of iNOS protein by the different groups expressed densitometrically as a percentage of that of the normal control group using the *bands* in **a**. Expression was normalized to the expression of the corresponding internal control glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). *Bars* Mean ± SD, $n = 8$. Significant differences between groups are analyzed by one-way ANOVA, where: $^{*}P < 0.01$ compared to normal control group, $^{**}P < 0.001$ compared to normal control group, $^{\S}P < 0.001$ compared to cancer control group

Liver histological examination

The liver tissue was examined histologically using H&E staining, and serum AFP levels were used as an indicator of cancer incidence. Histological examination of the liver normal control animals (Fig. 5a) revealed normal cells with granulated cytoplasm and small, uniform nuclei, as well as normal sinusoids and normal architecture, while no obvious changes were observed in livers of NSEE animals (Fig. 5b). Liver sections of cancer control rats (Fig. 5c) showed a loss of normal architecture, enlargement of sinusoids, focal areas of necrosis, and irregularly shaped neoplastic hepatocytes with polymorphic and hyperchromatic nuclei. These changes were observed to be reduced in liver sections of NSEE-DENA rats, indicating a certain degree of improvement (Fig. 5d).

Discussion

The precise role of NO in tumor pathophysiology has been the focus of numerous studies, with particular emphasis on its involvement in a multitude of inter- and intra-cellular signaling pathways that are crucial to the malignant character of cancer. It is not surprising that in heterogeneous tumors, a variety of responses have been observed following modification of NO levels [46]. iNOS has been implicated in different stages of cellular changes that lead to malignancy, growth of transformed cells, angiogenesis, and metastasis of malignant cells. Up-regulation of iNOS expression can be observed in a variety of human malignant tumors, such as breast, lung, prostate, bladder, colon, gastric, and malignant melanoma [10, 25–28, 30–32, 47–49]. Further studies are required to determine the role of the iNOS pathway in tumorigenesis and to establish the utility of iNOS inhibitors as chemoprevention agents. In fact, specific iNOS inhibitors and iNOS gene ablation have been demonstrated to prevent colon and lung carcinogenesis in rats [50–52]. Nevertheless, an enhancement of colon lesion development with iNOS gene ablation was reported in one case [53], and thus the involvement of iNOS in this and other organs remains controversial [6, 7].

Hepatocarcinogenesis induced by DENA is an ideal animal model to investigate liver tumor formation because it proceeds in stages similar to that of human liver cancer—i.e., formation of preneoplastic foci, neoplastic nodules, and HCC nodules [54, 55]. In our study, liver carcinogenesis was induced chemically by DENA, a well-known hepatic cancer model in which serum AFP level is highly elevated and hepatocarcinogenesis can be histopathologically identified [45, 56, 57]. Serum AFP levels and liver histological examination were used as indicators of cancer incidence. Our data show that animals treated with DENA

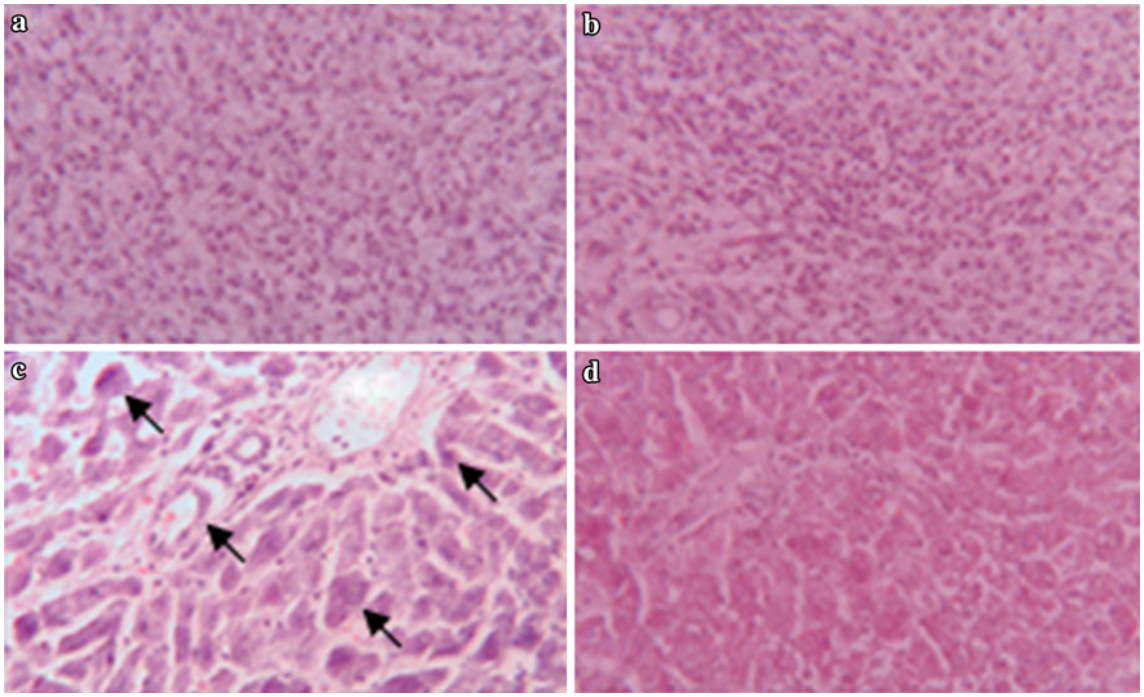

Fig. 5 Histological examination of the livers of animals in all groups. **a**–**d** Representative photomicrographs of livers of normal control rats (**a**), NSEE control rats (**b**), cancer control rats (**c**), and NSEE-DENA rats (**d**). Hematoxylin and eosin staining revealed normal architecture and normal cells with granulated cytoplasm and uniform nuclei in liver sections of normal control and NSEE control rats. Loss of normal architecture, enlargement of sinusoids, and irregularly shaped neoplastic hepatocytes with polymorphic and hyperchromatic nuclei were observed in liver sections of cancer control rats (*arrows*), while these changes were reduced in NSEE-DENA rats with few neoplastic hepatocytes. Magnification ×200

alone had elevated serum AFP levels and definite histological changes reflecting severe hepatocellular damage. AFP is always used to assess and follow up both HCC diagnostically and prognostically [24]. In our study we investigated the in vivo modulation of the iNOS pathway by NSEE and the implication of this effect as an antitumor pathway against hepatocarcinogenesis induced by DENA.

Our data reveal that the administration of NSEE alone did not significantly affect the biochemical parameters studied (AFP, TNF-α, IL-6, and NO) or change the liver histological examination. Furthermore, NSEE significantly down-regulated the expression of iNOS. More interestingly, we found no significant changes in the body weights or hepatosomatic indices of rats treated with NSEE alone (data not shown), suggesting the great safety of this extract. These results are in accordance with those reported earlier [58, 59]. NSEE also significantly down-regulated the elevation of serum AFP levels and reduced the histopathological changes caused by DENA in the livers of the rats. These anti-neoplastic effects have been confirmed by others in different models [60–64].

The inhibitory effect of NS oil and thymoquinone on pro-inflammatory cytokines release and iNOS activation has been proved in lung and testicular injuries, but there is nothing about the effect of NSEE [39–43].

Overproduction of the inflammatory mediators (NO, TNF-α, and IL-6) and stimulation of iNOS enzyme activity and expression have been reported in DENA-induced hepatocarcinogenesis [55, 64, 65]. In our study, NSEE blocked DENA-induced various responses in rats, including NO overproduction, the release of pro-inflammatory cytokines (TNF-α and IL-6), and the stimulation of both iNOS enzymatic activity and expression. Overall, our results suggest that NSEE (which contains natural constituents, such as thymoquinone, alpha-hederin, quercetin, and kaempferol) prevented DENA-induced hepatocarcinogenesis in rats through the inhibition of the iNOS pathway. Various other studies have reported that iNOS pathway inhibition may result in an antineoplastic effect [28, 31, 47, 55, 64].

In conclusion, the results of our investigation provide evidence that attenuation of iNOS pathway and suppression of the inflammatory response mediated by TNF-α and IL-6 could be implicated, in part, in the antitumor effect of NS. Consequently, our findings hold great promise for the utilization of NS as an effective natural therapeutic agent in the treatment of liver cancer.

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Conflict of interest None.

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