Recovering the angiogenic/angiostatic balance in NNK-induced lung carcinoma via 12 weeks of submaximal swimming and *Nigella sativa* nanocapsule

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**A R T I C L E   I N F O**

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**A B S T R A C T**

**Background:** Carcinogen nitrosamine 4-(methyl-trosamino)–1-(3-pyridyl)–1-butaneone (NNK) remarkably affects the actions of growth factors: EGFR, VEGFR-2, as well as the natural tumor suppressors: TGF-1 and TIMP-1. We propose that utilizing non-chemical interventions such as swimming and *Nigella sativa* nanocapsule play role in controlling cancer progression through direct effects on tumor-inherent factors.

**Material and methods:** Male rats were randomly placed into seven groups: Control (C), Solvent (S), (NNK), NNK + N.sativa (NNK + NS), NNK + Exercise (NNK + E), *N. sativa* + Exercise (NS + E), NNK + N.sativa + Exercise (NNK + NS + E). The exercise program consisted of 12 weeks of submaximal swimming. NNK and NS groups received weekly doses of 12/5 mg/kg and 125 μg/kg of NNK and *N. sativa*, respectively. By the end of the protocol, the levels of VEGFR-2 and TIMP-1 were determined using immunohistochemistry method and EGFR, and TGF-1 levels were measured by RT-PCR assay.

**Results:** In comparison with control group, there was a significant increase in the levels of VEGFR-2 in NNK, NNK + E, NNK + NS, NS + E, and NNK + NS + E groups (*P* ≤ 0.001), also TGF-1 levels of NNK + E and NS + E groups significantly increased (*P* > 0.001). While EGFR levels did not change remarkably (*P* > 0.05), except in NNK group (*P* ≤ 0.001). TIMP-1 in NNK, NNK + E, NS + E, NNK + NS + E groups significantly decreased (*P* ≤ 0.001).

**Conclusion:** We recommend 12 weeks of submaximal swimming and 125 μg/kg *N. sativa* nanocapsule are safe interventions to recover the balance of selected angiogenic/angiostatic markers and to control tumor initiation, growth, and metastasis in lung carcinoma induced by 12/5 mg/kg of NNK injection.

1. **Introduction**

Lung cancer phenomena, a multifactorial and multi process disease, includes several genetic and epigenetic deregulations that still remain with several challenges. In the United States, non-small cell lung cancer continues to be the leading cause of cancer-related deaths among men (90%) and women (75–80%) [1]. Based on dose calculations the total amounts of NNK taken up by people who used tobacco products for a period of 30 years or more, approximate the total amounts that induce tumors in rats. The metabolic activation process of genotoxic compound nitrosamine 4-(methyl-trosamino)–1-(3-pyridyl)–1-butaneone (NNK) has been extensively documented in rodents. In addition to the classical mechanisms of carcinogenesis that proceed through the formation of DNA adducts, NNK also binds to nicotinic and other receptors, which leads to downstream effects contributing to the cancer development. Injected NNK has reported to contain the highest levels of tobacco-specific N-nitrosamines. The degree of exposure to tobacco-specific N-nitrosamines depends not only on the levels of these compounds in smoke, but also on the manner in which the products are used (in water, oral swabbing, injection, second hand smoke) [2].

Imbalance of angiogenic and angiostatic markers that forms pathologic angiogenesis supplies the vital need of any growing malignant tissue. Compensatory mechanisms within non-small-cell lung carcinoma (NSCLC) overexpress endothelial growth factor (VEGF) and its receptor...
(VEGFR-2) that are the major responsible for pathologic angiogenic surge within the tumor [3]. EGF (Epidermal growth factor) which stabilizes hypoxia-inducible factor (HIF) leads to more angiogenesis transcription, cancer cell division and progression [4]. Dysregulated angiogenesis and natural cell growth influence the actions of multifunctional cytokines such as potential angiogenesis stimulator and proliferation: transforming growth factor β (TGFβ-1). In cancer progression, the over-expressed TGFβ-1 signaling pathway suppresses immune cells and increases angiogenesis ending in metastasis [5]. The action of endogenous protein regulator of the matrix metalloproteinase (TIMP-1) has been examined in different metastatic behaviors of small-cell lung cancer. This natural inhibitor modulates cell proliferation, cell migration and invasion, anti-angiogenesis process, and pro-apoptosis pathways. In stage I lung cancer, the clinical benefits of angiogenesis-inhibitory therapy through TIMPs has been considered [6].

Applying safe adjuvant therapeutic interventions help maintaining the balance of angiogenic/angiostatic markers. The molecular mechanisms by which submaximal exercise, especially swimming, influence lung cancer outcomes remain elusive. Based on literature, exercise may counteract some hallmarks of cancer such as: aberrant angiogenesis, besides moderating chemotherapy-related adverse effects. Epidemiological studies repeatedly evidence for applying exercise interventions to reduce tumor incidence, tumor growth, and metastasis across a wide range of genetic, transplantable, and chemical-induced tumor in rodent models [7].

Repetitive bouts of exercise results in widespread adaptations, i.e., increase in blood flow, shear stress on the vascular bed, pH regulation, sympathetic activation, and endocrine effects with the potential to regulate cancer progression and biology [8]. Additionally, adaptive response to hypoxia induced by submaximal exercise under normal and pathological conditions, activates HIF-1α pathway to promote angiogenesis through proangiogenic factors, mainly VEGF-α and VEGFR-2 [9]. Nevertheless, how submaximal exercise training in different cancers modulates angiogenesis in an oncological setting is not fully clear. Under normal circumstances treadmill exercise for a period of 4 weeks, five times per week and 60 min each session, significantly increased VEGF serum levels in mice inoculated with lung cancer cells [10]. Recently, it has been reported that in cancer cells, submaximal intensities of exercise deactivated Hippo/YAP (molecular signaling events involved in tumor formation) signaling through an epinephrine-dependent mechanism, blockade of adrenergic signaling [11], reducing the phosphoinositide 3-kinase–AKT pathway, downregulating the RAS-MAP kinase cascade, and deactivating Akt/mTOR (the central for control of growth and protein synthesis) pathway [12]. Other proved antitumorigenic properties of exercise training are related to immunomodulation in which exercise leaves affects through increasing proinflammatory cytokine levels, natural killer (NK) cell infiltration, and upregulation of proinflammatory cytokines (IL-1a and inducible nitric oxide synthase [iNOS]) in the tumor microenvironment [13]. Intratumoral metabolism is unquestionably regulated during exercise, but how this affects tumor growth and metastatic rate is currently not mechanistically understood.

N. sativa (NS) with its similar effect to dexamethasone, down regulates inflammatory factors and increases white blood cells in cancer patients. This annual flowering plant, in the family Ranunculaceae, native to Eastern Europe and Western Asia consists of 32–40% of oils also linoelic acid, oleic acid, palmitic acid, and other minor constituents, such as nigelemic, and nigellimines N-oxide. Aromatics include active ingredient: thymoquinone. Protein and alkaloids are present in the seeds. Today, focuses are on the biochemical mechanisms of N. sativa in protection against oxidative stress, cellular damage, atherosclerosis, cardiovascular disease and cancer [14]. Here, by utilizing immunehistochemical and RT-PCR analysis on VEGFR-2, TGFβ-1, EGF, and TIMP-1, we have investigated whether 12 weeks of endurance swimming and treatment with N. sativa nanocapsule, separately and in cooperation with one another, modulate the lung carcinoma progression through both activating or suppressing some intracellular signaling.

2. Material and methods

Ethical approval: All procedures in this study were following the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). This study was approved by HRI Ethics Committee of the Babol University of Medical Sciences (Code No: MUBABOL.HRI.REC.1395.109).

Animals, NNK and N. sativa injection: Male Wistar rats aged 6–8 weeks and 105.8 ± 27.93 g weight range (digital laboratory scale: Samurai Techno weight India Pvt. Ltd) were purchased from the Pasteur Institute of Iran. The animals were adapted to the laboratory environment of the Sport Sciences Faculty at the University of Mazandaran for two weeks. Then, rats were randomly assigned in 7 groups (n = 8): Control (C), Solvent (S), (NNK), NNK + N. sativa (NNK + NS), NNK + Exercise (NNK + E), N. sativa + Exercise (NS + E), NNK + N. sativa + Exercise (NNK + NS + E).

NNK chemical compound and injection: 4-(Methyl-nitrosamino)–1-(3-pyridyl)–1-butane, a pale yellow crystalline solid (molecular weight: 207.23) that is easily soluble in water and any organic solvent (10–50 mg/mL at 65.3 °C) was ordered from TRC, North York, Ontario, Canada. NNK was IP injected as single weekly dose of 12.5 mg/kg body weight to induce lung cancer in rats during a 12-week period [15].

Preparation of herbal extract: N. sativa used in this study was commercial seeds obtained from a local market (Babolsar, Iran). After confirming the variety of seeds by the herbarium official expert of University of Mazandaran, voucher specimens of seeds were transferred and kept in exercise physiology laboratory for the next steps.

N. sativa extraction: Soaking method was used to prepare the primary N. sativa extract. This method is introduced to preserve and to extract the major active components of N. sativa such as thymoquinone. To extract N. sativa, seeds were manually crushed with a mortar and pestle and stored in a voucher specimen. 55 g of N. sativa powder (weighted with scale of 0.0001) was soaked in a solution of 70% ethanol and 30% distilled water for 72 h. During this time the lid of the container was well covered with paraffin and maintained in the temperature of 20–25. The mixture was passed through a filter paper (Whatman 42, Germany) in a mild temperature (below 60 °C). Finally, Rotary model C-V RV8 by IKA Germany was used to eliminate the solution. 10gr prepared powder was mixed with 20 mL of PBS (phosphate-buffered saline), and then whipped consistently until the color of the solution was stabilized. The obtained solution was kept in sterile tubes overnight at 4 °C, and then, centrifuged at 3000 rpm, for 15 min. To purify the brownish-orange color supernatant a Whatman paper filter was used, and then it was restored in disinfected microtubes at 4 °C until consumption. In closing, the total proteins were quantified by using the Bradford reagent, and a DU-70 spectrophotometer used for spectrophotometry [16]. The chemical compositions of the essential oil and the oils extracted from N. sativa seeds grown in Iran were previously determined by gas chromatography-mass spectroscopy (GCMS) (Shimadzu A17 instrument with flame ionization detection (FID)) and high performance liquid chromatography (HPLC) (Hewlett Packard 6890-5972). Oils and essential oils of Persian N. sativa seeds respectively contained 8 and 32 components. The highest amount of oil was consisted of %55/6 linoleic acid, %23/4 oleic acid, and %12/5 palmitic. Additionally, Trans-anethole (%38/3), p-cymene (%14/8), and carvacrol (%94) were the major components of Persian N. sativa essential oil [17].

Preparation of N. sativa nanocapsule: After preparation of the hydroalcoholic extract of N. sativa, human albumin was used to nanocapsule the herbal components. Human serum albumin with its synthesized hollow spherical capsules traps compounds such as N. sativa extract to be sent into targeted points. Thus, to prepare nanocapsules 50 mg of human albumin serum dissolved in 1 mL of water pH: 7.4, Tween 80 (0.5% v/v) was added to the samples, then stirred 30 min at 500 rpm
using a magnet. 4 mL Ethanol was added to the stirring solution. Glutaraldehyde (117 microliters) was surcharged to the samples to produce particle cross-likings, then stirred continuously at 500 rpm for 24 h. Fig. 1-A indicates the image of the synthetic nanocapsule under scanning electron microscope. After adding *N. sativa* extract to the nanocapsule solution, the shape remained spherical as the indicator of the synthesized nanocapsules containing *N. sativa* extract (Fig. 1-B). Finally, 0.029 gr of *N. sativa* nanocapsule was obtained from 600 μl of *N. sativa* extract weighted 0.522 gr. Albumin electron microscopy was used to evaluate the synthesis of albumin capsules.

The stability and durability of synthetic nanocapsules in transferring biological materials was evaluated by Dynamic light scattering (DLS). This evaluation method was accomplished through synthesized capsule sampling during 2 weeks. We observed that in over a period of 14 days, nanocapsules remained approximately unchanged in the manner of size and shape. The average size of nanoparticle capsule was 253/8 nm with−20.5 surface charge while, bonding between extract and amine groups after adding the extract reduced the size of nanocapsules to 230.7 nm (Table 1).

This study was the first in examining the dose of 125 μg/kg body weight of *N. sativa* nanocapsules as weekly IP injection in rats. Solvent group received the same amount of normal saline (0.9% NaCl) on the same schedule [16].

**Training protocol:** The rats got familiarized with the swimming program in a 5 days/week and 20 min/section protocol, in a 50 × 50 × 100 cm dimensioned pool, and temperature of 30–32 °C [18]. Then, blood lactate threshold was measured in 4, 7, 10 and 13 liter/min intensities (Lactate Scout 4; EKF Diagnostics Company, Germany), and based on lactate levels, the power of water arranged in submaximal levels (4–13 liter/min intensities) during each swimming section. The animals were trained for 12 weeks (25–60 min, 5 days/week) (Fig. 2) [19].

**Tissue sample preparation:** 48 h after the last training session and after weighting, the animals were anesthetized by 50 mg/kg of ketamine and 40 mg/kg of xylazine [20]. By thoracic surgery, the lung tissues were removed and weighted ([Control 71/0 ± 14 gr], (Solvent: 59/1 ± 11 gr), (NNK: 62/1 ± 26 gr), (NS+ E: 68/1 ± 25 gr), (NNK+E: 72/1 ± 42 gr), (NNK+NS75/1 ± 28 gr), (NNK+NS+E: 59/1 ± 28 gr)), rinsed with physiological serum, isolated and then kept in the micro tube, placed in a LN2, freezer at -80 °C in liquid nitrogen for following gene expression analysis. Left lobe of the lungs were immobilized by 5% formaldehyde for subsequent immunohistochemistry (IHC) analysis.

**Immunohistochemistry (IHC) evaluation of VEGFR-2 and TIMP-1:** Immunohistochemical analysis was assigned based on the manufacturer’s instruction. After dewaxing paraffin-embedded blocks, endogenous peroxidases were deactivated through 3% H2O2. Samples were pre-incubated with 10% goat serum, after thermal remediation with citrate buffer, then, diluted primary antibodies (1: 100) (VEGFR-2 antibody: abcam 39256, and TIMP-1 antibody: AB61224) were added to the samples. The samples were incubated at 4 °C overnight and then washed three times with PBS buffer. A peroxidase-labeled polymer (secondary antibody; ab97050) was used to coat the samples 30 min and then washed with PBS three times. In the end, DAPI staining solution (1:100) (ab228549) was added to samples and then washed with PBS. Finally, pictures were taken under Olympus fluorescent microscope with x400 lenses to confirm VEGFR-2 and TIMP-1 expressions. In this regard, 10–15 high fields in each section was observed [21,22]. The numbers of VEGFR-2 and TIMP-1 positive cells were quantified using AxioVision software.

**Gene expression (TGFβ-1, and EGFR):** To validate EGFR and TGFβ-1 gene expression, the quantitative RT-PCR method was performed. In order to isolate the lung RNA, TRIzol Reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was performed as indicated by the manufacturer’s protocol. First-standard cDNA synthesis (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used, according to the manufacturer’s procedure. Expression levels of EGFR, and TGF-β1 were detected by RT-PCR assay using GAPDH as an internal control. The following primers were used for real-time PCR for EGFR: forward 5’-GTCGTTCTAGTCTCTGGAG-3’, reverse 5’-CATCTGACGACTCATCAGC-3’, for GAPDH control, forward 5’-AAGTTCAACGGCACAGTCAC-3’, and reverse 5’-CAGTGGCTGTCAGTACGAGG-3’, reverse 5’ GTCCGTTCACTGACTGTTGTTG3’, for GAPDH control, forward 5’-AAGTTCAACGGCACAGTCAC-3’, and reverse 5’-CAGTGGCTGTCAGTACGAGG-3’.

**Fig. 1.** Scanning Electron Microscopy (SEM). Image of nanocapsules: A: Before loading *N. sativa* extract; B: After loading *N. sativa* extract. Surfaces and spaces among nanocapsules are coated with *N. sativa* extract. The photomicrographs were taken with 10,000 x magnification.

**Fig. 2.** Blood lactate levels at different intensities of swimming exercise.

**Table 1**

| Distribution | Surface load | Nanoparticle size | Sample              |
|-------------|-------------|------------------|-------------------|
| Monomodal   | -20.5       | 253.8            | Unloaded nanocapsule |
| Monomodal   | -8.38       | 230.7            | *N. sativa*-loaded nanocapsule |

**Table 1** The data of size and surface load of synthetized nanocapsules and nanocapsules containing *N. sativa* extract.
Quantitative values of EGFR, and TGFβ-1 were obtained from the threshold cycle value (Ct), and the relative expression of EGFR and TGFβ-1 genes: were calculated using the ΔΔCT method [23].

Statistical analysis: Data analysis was done in SPSS version 26.0. One-way ANOVA and Tukey test were performed to show the difference between control and other groups. Mean ± SD was used to report the values of measured variables. The significance level was adjusted to 5% (P ≤ 0.05). The graphs were drawn with Sigma Plot version 14 and excel version 2016. RGB profile plots were drawn by ImageJ 2021.

3. Results

The results of One-Way ANOVA test showed that VEGFR-2 levels in NNK, NNK+E, NNK+NS, NS+E, NNK+NS+E groups, significantly increased compared with control groups (P ≤ 0.001) (Fig. 3-A). According to the statistical analysis of EGFR levels shown in Fig. 3-B, NNK group had a meaningful increase versus control group (P ≤ 0.001) while, EGFR levels in NNK+E, NNK+NS, and NNK+NS+E significantly decreased compared with NNK group (P<0.01). The amounts of EGFR of NS+E group did not have any remarkable change versus control and NNK groups (P>0.05). Based on observations (Fig. 3-C), TGFβ-1 levels in NNK+E and NS+E groups showed a remarkable increase compared to NNK group (P ≤ 0.001) (Fig. 3-D). The results of statistical analysis (mean±SEM). A: VEGFR-2, B: EGFR, C: TGFβ-1, and D: TIMP-1 levels in lung tissue of experimental groups (n = 8). *** Represents the Statistical significance for the difference between the data of experimental groups and control group (P ≤ 0.001). ### Represents the Statistical significance for the difference between the data of experimental groups and NNK group (P ≤ 0.001).
control group. There was not any meaningful statistical difference between TGF-β1 levels of other groups compared to control ($P > 0.05$). Based on data demonstrated in Fig. 3-D, there was a significant reduction in TIMP-1 levels of all groups compared to control ($P \leq 0.001$) except NS+E group ($P > 0.05$). Additionally, there was a significant increase in TIMP-1 expression of all groups versus NNK ($P \leq 0.001$).

3.1. Texture appearance and shape features of lung tissues

There was significant influence of preformed interventions on histology and physical appearance of lung tissues of experimental groups. The natural structure and morphology of lung tissue was particularly preserved in intact models, however, a wide range of disparity in appearance was observed in experimental groups. Preformed interventions resulted in variation in lung size and volume, different degrees of visible acute inflammation, surface fractions, damaged parts, and recognizable black spots (Fig. 4).

3.2. IHC expression of VEGFR-2 and TIMP-1 protein in lung tissues of experimental groups

A significant variation was present in the expression of VEGFR-2 staining and its fluorescence intensities within lung tissues of experimental groups (Fig. 5-A and C). Although this marker was positively observed in the cytoplasm of endothelial cell of all groups induced by NNK, the highest rate of positivity was noted for NNK group in 94.33%. The lowest rate of expression was observed in 31.5% and 35.19% of the samples of control and solvent groups, respectively. The expression profiles of all groups are presented in Fig. 5-B. Lung tissues seemed normal in control and solvent groups. Photomicrographs exemplified strong over-expression of atypical and abnormal cell accumulation and inflammation in different areas. Additionally, in lung tissues of NNK group some air spaces seemed irregular in size. In E+NS group, lung tissue seemed normal and around the bronchioles and arteries some slight inflammation was observed. Areas of severe inflammation and decreased volume of alveoli were visible in the lung tissue of NNK+E group. Additionally, in NNK+E+NS group despite sites of severe inflammation, parenchyma structure was preserved (Fig. 4-A).

Regarding TIMP-1, it was found in all lung tissues, primarily associated with sub-epithelial fibroblast and myofibrils foci (Fig. 6-A) that some partially occupied alveolar spaces. TIMP-1 was also detected in alveolar epithelial cells. No signal of abnormality related to this marker was observed in lung tissues of control and/or solvent groups. NS+E and NNK+NS+E groups demonstrated the most intense extracellular staining of TIMP-1 among other experimental groups and this marker was mainly found in the elastic lamina of most vessels, revealing the characteristic duplication of this structure in pulmonary fibrosis (Fig. 6-A). Less intense staining was noticed in thickened alveolar septa. In contrast, in NNK-induced lungs, few positive staining was observed even in the elastic lamina of vessels. In some areas of lungs underwent interventions (NNK+E and NNK+NS), numerous interstitial cells, mainly macrophages were stained (20% and 40% respectively), however, the values were significantly lower than those of intact lungs. Tissue samples of NNK induced group showed negative TIMP-1 antibody versus intact models. In NNK group, despite easily found alveolar macrophages, some irregular airspaces, thickened or broken septa, thick collagen fibers with edematous or cell infiltrated were observed, as well as irregular distribution (Fig. 6-A). Disparities between TIMP-1 protein fluorescence intensities in lung tissues of different groups are demonstrated in Fig. 6-C.

4. Discussion

NNK, the classic agonist for nicotinic acetylcholine receptor (nAChR), extensively enters the bloodstream to distribute to the other tissues through rapid absorbing [23]. Lung is known as the potential cancer susceptible tissue for any kind of tobacco exposure, especially IP route which has the highest systemic carcinogen exposure compared with inhalation (INH) and PO. In vitro, this organ had the highest levels of NNAL among the other tissues regardless of the route of induction, indicating that the major NNK metabolism takes part in the lung tissue [2].

It seems that different routes of NNK exposure in rodent models activate some similar signaling pathways of lung cancer. NNK increases α7-nAChR-mediated expression of contactin 1 that is induced by VEGF-C/Flt-4/Src/p38 MAPK/C/EBPζ pathway. Following down-regulated E-cadherin and activated RhoA, NNK will result in tumor invasion and metastasis. This carcinogen regulates the metabolism pathway of arachidonic acid to synthesize more TxA2, which in the next step will link to its corresponding receptor TP, thereby downstream PI3K/AKT/CREB pathway that ends to angiogenesis and cell survival. Through NF-kB and ERK1/2 pathways, carcinogen NNK induces HO-1 expression that leads to tumor invasiveness, advanced stages of lung cancer, and poor prognosis rate [24]. Although we cannot firmly announce the

![Fig. 4. The appearance of lung tissues of intact and experimental groups.](image-url)
presence of tumor glands following 12/5 mg/kg of NNK injection in this study due to the short time between dosing and tissue collection, but the gene expression assessments and histological observations firmly suggested the genotoxicity and tissue damage in NNK-induced groups, indicating the activated tumor signaling pathways.

Based on results (Fig. 3-A and B) there was a significant increase in EGFR and VEGFR-2 gene and protein expression following 12/5 mg/kg of NNK induction versus control. Up regulation of EGFR was reported in nicotine-induced cervical cancer cell lines that ended in G1/S cell cycle progression through RAS-RAF-MEK-ERK MAPK and PI3K-AKT-mTOR pathways. Activation of EGF induces EGFR dimerization and transphosphorylation of the C-terminal domain, consequently leads to cancer progression [25]. In response to some external pathogenic cell stimulation, such as the proper dosage of NNK, the activated inflammatory response will provide adequate signaling pathways in defense of the host [26]. The NNK-mutated EGF and VEGF can impart with

Fig. 5. A: IHC florescence expression (staining) of VEGFR-2 in lung tissues of rats. The chosen fields were randomly obtained in x200 magnification. Primary antibody is shown in red, PI stained nuclei in green, and marker expression is shown by white arrows within merged photos. Scale bar 100 µm. B: Densitometry scores of VEGFR-2 protein expression in lung tissue of studied groups. C: Fluorescence intensity profile plot calculated on images obtained from samples immunostained for VEGFR-2 protein. Various degrees of fluorescence localization along a line drawn on IHC photos of experimental groups are shown by different colors.
ligand-independent signaling leading to the up-regulated pro-oncogenic processes including chronic cell cycle proliferation [27]. Thus, in this study, the concurrent over expressed EGFR and VEGFR-2 levels in lung tissue of NNK groups, beside downregulation of TGFβ-1 and TIMP-1 (Fig. 3-C and D), strengthen the efficiency of the applied dosage of NNK to activate initial activated signaling pathways of lung cancer. The reduction or dysregulation of TGFβ-1 has been mainly reported in primary cancers than in metastatic cancer models. In this regard, lower expression of TGFβ-1 was reported in the primary stage of colon cancer [28] and brain tumors [29] in which TGFβ-1 reduction was the result of receptors genetic alteration and changes in transcription regulation that affect the stability of this protein. Putting it all together, the significant...

Fig. 6. A: IHC florescence expression (staining) of TIMP-1 in lung tissues of rats. The chosen fields were randomly obtained in x400 magnification. DAPI stained nuclei is shown in blue, primary antibody is shown in green, and marker expression is shown by white arrows within merged photos. Scale bar: 50 μm. B: Densitometry scores of TIMP-1 protein expression in lung tissue of studied groups. C: Fluorescence intensity profile plot calculated on images obtained from samples immunostained for TIMP-1 protein. Various degrees of fluorescence localization along a line drawn on IHC photos of experimental groups are shown by different colors.
decrease of TIMP-1 in NNK group compared with control approves the tumorigenic effects of 12/5 mg/kg of NNK injection. In human models of cognitive heart failure, reduced secretion of TIMPs by alveolar macrophages was also observed. It seems that, NNK via modulating cytokines synthesis, attenuates immune system, suppresses IL-2 production, and results in inflammation and lower levels of TIMPs [30].

Under normal circumstances, there is a natural balance between angiogenic/angiostatic markers that cancer incidence disrupts this order. Exercise-induced modulation may be an adjunctive anti-cancer strategy to inhibit tumor initiation and progression. Following this realization, in this study, exercise intervention significantly suppressed VEGFR-2 as well as EGFR (Fig. 3-A and B) gene and IHC expressions in hypoxic and necrotic stress of pathologic condition of NNK+E group compared to NNK group. The exercise adaption and the hypoxia occurred following 12 weeks of swimming program in this study, through decreasing blood flow, might have led to low perfusion of the lung tissue, resulting in a decreased EGFR in NNK-exposed rats (NNK+E) compared to NNK [31].

In comparison with NNK groups, intervention with exercise and N. sativa remarkably decreased the pathologic expressions of VEGFR-2 and EGFR (Fig. 3-A and B). N.sativa induces its protective antioxidant role via its phytochemical constituents as strong superoxide radicals. Based on our observations, lower levels of angiogenic factors in intervention-preformed groups compared to NNK must be happened through de-activated VEGFR-EGFR/MAPK/ERK1/2 signaling pathway by N.sativa nanocapsule treatment [30]. In this manner, it has been reported that thymoquinone (TQ) administration inhibited EGFR and VEGFR phosphorylation and interestingly down-regulated the expression of transcription target as a result of the inhibition of EGFR/ERK1/2 signaling [32]. It is clear TGF-β can be produced by the tumor cell itself or other cells in the tumour microenvironment [5]. The mechanical load following submaximal swimming, possibly has induced the overexpression of TGFβ-1 in NNK+E group compared to NNK group. Similarly, TIMP-1 protein level recovered by submaximal swimming and N.sativa nanocapsule. It has also been reported that, in nicotine-exposed rats, 24 weeks of endurance exercise reduced pulmonary elastase following the increased lung superoxotase and TIMP-1 levels [33]. In Fig. 3-D, we reported that single and dual administration of swimming exercise and N.sativa nanocapsule significantly recovered TIMP-1 levels versus control. It seems that combination of exercise with N.sativa (NNK+NS+E) was more effective in recovering TIMP-1 levels to the base values than separated application of any of these interventions. It seems that, these interventions via similarly acting as anti-cancer multi kinases drugs, upregulated TIMP-1 levels. To firmly discuss the reasons of altered amounts of TGFβ-I and TIMP-1 levels, the circulation levels of this marker necessarily must be measured. Potential limitations of our study result from the experimental design and the performed measurements. However, the ambivalent alterations of TGFβ-I along with the stimulated angiogenic factors: EGFR and VEGFR-2, prove the effective role of our interventions in moderating primary lung carcinoma.

In the absence of carcinogen NNK, the altered amounts of angiogenic markers after submaximal swimming and N.sativa nanocapsule, must be considered as the result of beneficial physiological adaptation in lung tissue. Regarding our data, VEGFR-2 and TGFβ-I levels of NS+E groups significantly increased versus control group (Fig. 3-A and C). As clearly explained before, regardless of type, mechanical stimulation of endurance exercise revives and upregulates angiogenesis markers and cytokines as a natural mechanism in any healthy or damaged tissue. On the other hand, it seems that TGFβ-I levels in NS+E group might be affected following the increased NADPH oxidase expression and the reduced amounts of superoxide dismutase at 12 weeks of submaximal swimming in the present study. Additionally, following submaximal exercise, the increased HIF-1 levels will result in regulation of this marker in rats [7–9,34]. Future studies are needed to investigate the exact coordination of biological mechanisms involved in the present study.

5. Conclusions

According to the findings of the current study, certain dose of N. sativa nanocapsule besides preforming 12 weeks of submaximal swimming modulated pathologic conditions of primary lung cancer through restoring the balance of angiogenic/ angiostatic markers and consequently blocking the signal pathways leading to malignancies. However, this area of investigation remains in its infancy. More studies are needed to understand the mechanisms underlying the submaximal swimming exercise and N.sativa nanocapsule as a potentially effective adjuvant treatment strategy. There now exists an unprecedented opportunity for exercise-oncology researchers to investigate the key questions that can unlock the potential therapeutic promise of exercise in cancer initiation and progression.

Statements of ethics

All the animal study protocol was approved by the HRI institutional Ethics Committee of Babol University of Medical Sciences and conformed to I.R.I national institutes of health guidelines (ethics code: MUBABOL.HRI.REC.1395.109).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

Zahra Abrishami Kia was involved in the designing, execution of experiments, data analysis and proofreading of the manuscript. Seyede Tayebeh Sadati Bizaki, was involved in the designing of the study and execution of experiments. Dr. Shadmehr Mirdar Harijani, was involved in monitoring the methodology. Elham Asaa’di Ghareh Tapeh, was involved in data analysis. Nayyereh Katal was involved in execution of experiments. Roya Gorji Baziary was involved in data analysis.

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