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Thymoquinone inhibits the migration of mouse neuroblastoma (Neuro-2a) cells by down-regulating MMP-2 and MMP-9

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[ABSTRACT] Thymoquinone (TQ), an active component derived from the medial plant *Nigella sativa*, has been used for medical purposes for more than 2 000 years. Recent studies have reported that TQ blocked angiogenesis in animal model and reduced migration, adhesion, and invasion of glioblastoma cells. We have recently shown that TQ could exhibit a potent cytotoxic effect and induce apoptosis in mouse neuroblastoma (Neuro-2a) cells. In the present study, TQ treatment markedly decreased the adhesion and migration of Neuro-2a cells. TQ down-regulated MMP-2 and MMP-9 protein expression and mRNA levels and their activities. Furthermore, TQ significantly down-regulated the protein expression of transcription factor NF-κB (p65) but not significantly altered the expression of N-Myc. Taken together, our data indicated that TQ's inhibitory effect on the migration of Neuro-2a cells was mediated through the suppression of MMP-2 and MMP-9 expression, suggesting that TQ treatment can be a promising therapeutic strategy for human malignant neuroblastoma.

[KEY WORDS] Thymoquinone; Neuroblastoma; Migration; NF-κB; MMP-2

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Introduction

Neuroblastoma is the most common extracranial solid tumor in early childhood arising from precursor cells of the neural crest. During the last two decades advances have been made in predicting outcome of the patients and improving therapeutic options [1]. However, high-risk neuroblastoma patients still have a 5-year-survival rate of less than 35% [2-6]. This is due to drug resistance of primary tumors or metastases after relapse. One important criterion in the classification of high-risk neuroblastoma is amplified N-Myc oncogene which is the most crucial prognostic factor in neuroblastoma [1]. Acting as an oncoprotein, the transcription factor N-Myc promotes tumor proliferation, angiogenesis, and metastasis, leading to unrestricted tumor progression and poor outcome [6]. However, the precise mechanism by which N-Myc mediates drug resistance in neuroblastoma is poorly understood. Failure of the current therapeutic regimens to inhibit N-Myc-mediated angiogenic factors contributes to extensive high proliferation and invasive rates, resulting in poor prognosis of neuroblastoma patients [6]. These facts have necessitated the need to develop new drugs.

Malignant neuroblastoma is a highly vascularised solid tumor that requires access to blood vessels for growth, invasion, and metastasis [3]. Angiogenesis is the process of development of intrinsic vascular network, where new capillaries sprout from pre-existing vessels involving the transition from a vascular to vascular phase occurs via neovasculaization, which is a necessary pre-requisite for tumor progression and metastasis [5]. In advanced stages of neuroblastoma, tumor cell secrets matrix metalloproteinases (MMPs) favoring degradation of extracellular matrix and enhancing tumor dissemination [6]. The family of MMPs includes MMP-2 and MMP-9 (gelatinase A and B) that are collagenases. MMP-2 and MMP-9 facilitate invasion and metastasis, and they degrade important constituents of the interstitial stroma and subendothelial basement membrane

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type IV, V, VII, and X collagens and fibronectin [6]. MMP-2 and 9 have been found to be over-expressed in many invasive tumor cells [7]. Several experiments have confirmed the key role of these enzymes in angiogenesis [8]. Emerging treatments with the delivery of anti-migration molecules can thereby arrest the spread of this pediatric tumor [6]. Novel therapeutic approaches with the migration inhibitors are expected to improve patient survivability by reducing morbidity, mortality, and drug-related toxicity [6].

Thymoquinone (TQ) is a bioactive constituent of the volatile oil derived from the seeds of *Nigella sativa* [9]. TQ shows promising *in vitro* and *in vivo* antineoplastic growth inhibition against various tumor cell lines [9-13]. This activity may be attributed to its inhibitory effects on cancer cell growth and its capability of inducing apoptosis [14]. The growth inhibitory activity of TQ is associated with the induction of cell cycle arrest [12] and inhibition of DNA synthesis [9]. The apoptotic activity induced by TQ has been shown to be mediated *via* p53-dependent and p53-independent pathways [15-16]. In addition, TQ has been reported to be active against many multidrug-resistant variants of different human cancer cell lines [9]. More recently, it has been demonstrated that TQ decreases the migration of various types of tumor cell lines including HUVECs [17], MCF-7 and MDA-MB-231 [18] and FG/COLO357 [19]. TQ also inhibits tumor angiogenesis and tumor growth through suppressing AKT and ERK signaling pathways [20].

There is no information on the effects of TQ on migration and invasion of neuroblastoma cells. Therefore, in the present study, the effects of TQ on the adhesion and migration of neuroblastoma cells were investigated in neuroblastoma cells and we also explored its role in down-regulating transcription factor NF-κB (p65) and invasive pathways (MMP-2 and MMP-9) in order to understand the mechanisms responsible for its anti-metastasis effects.

Materials and Methods

Chemicals and reagents

Thymoquinone (TQ), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TQ was dissolved in cell-culture tested, sterile DMSO at a stock concentration of 100 mmol·L^{-1} and stored at −20 ºC until use. Trypsin-EDTA, fetal bovine serum (FBS), antibiotics-antimycotics, Dulbecco's modified Eagle's medium (DMEM), and phosphate buffered saline (PBS) were purchased from Gibco, (Burlington, Canada). Polyvinylidine difluoride (PVDF) membrane was purchased from Millipore (Bedford, MA, USA). Primary antibodies against N-Myc, MMP-2, MMP-9, and NF-κB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β-Actin antibody was purchased from Sigma Aldrich Chemicals Pvt Ltd. (St. Louis, MO, USA). The secondary antibodies, horseradish peroxidase (HRP) conjugated goat anti-mouse IgG, and goat anti-rabbit IgG were obtained from Banglore Genei, (Bangalore, India). All the chemicals used were of extra pure analytical grade.

Cell culture

Neuroblastoma (Neuro-2a) cell line was obtained from National Centre for Cell Science (NCCS), Pune, India. The cells were grown in T25 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics (penicillin 100 U·mL^{-1} and streptomycin 100 μg·mL^{-1}). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Determination of TQ cytotoxicity by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Cell cytotoxicity was assessed by MTT method as described earlier [21]. The cells (approximately 5 × 10^3 cells/well) were seeded into 96-well tissue culture plates. At 24 h after seeding, the medium was changed and the cells were treated with various concentrations of TQ, ranging from 0 to 70 μmol·L^{-1} for 24 and 48 h. Cell viability was quantified by the ability of living cells to reduce the yellow color tetrazolium salt to a purple formazan product. This formazan product was then dissolved in DMSO and absorbance was measured at 570 nm. The cell viability was calculated using the formula:

% Growth inhibition = [A_{570} nm of treated cells/A_{570} nm of control cells] × 100

Adhesion assay

Cell adhesion was assayed as described previously with slight modifications [22]. Microtiter wells were coated with fibronectin overnight. The wells were blocked for 30 min with 0.5% BSA in PBS. The cells were trypsinized and suspended at a final concentration of 5 × 10^5 cells/mL in serum-free medium. Various concentrations of TQ (0, 20, and 40 μmol·L^{-1}) were given to the cells for 24 h before seeding. 100 μL of cell suspension was added to the wells and the plates were incubated at 37 °C for 40 min. Medium was then carefully suctioned out from each well. Each well was washed thrice with PBS. The colorimetric MTT-assay was used to determine the number of remaining cells (adherent cells). The absorbance was measured at 490 nm using a microplate reader. Experiments were performed independently three times.

Wound healing assay

Neuro-2a cells were seeded into a 6-well plate and allowed to grow to 80% confluency in complete medium. The cell monolayers were carefully wounded using a yellow pipette tip that touched the plate as described [23]. Wounded monolayers were then washed several times with PBS to remove cell debris and incubated in medium in the absence or presence (20 and 40 μmol·L^{-1}) of TQ for 24 h. Cell migration into the wound surface and the average distance of migrating cells was determined under an inverted microscope.

Gelatin zymography

Conditioned media from control and TQ-treated cells were subjected to SDS-PAGE on 10% SDS gels co-polymerized