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The Effect of Quercetin Nanosuspension on Prostate Cancer Cell Line LNCaP via Hedgehog Signaling Pathway

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Abstract

Background: Prostate cancer (PCa) is the second leading cause of cancer death in American population. In this manner, novel therapeutic approaches for identification of therapeutic targets for PCa has significant clinical implications. Quercetin is a potent cancer therapeutic agent and dietary antioxidant present in fruit and vegetables.

Methods: To investigate the underlying mechanism by which the PCa was regulated, nanoparticles of quercetin were administrated to cells. For *in vitro* experiments, human PCa cell line LNCaP were involved. Cell viability assay and quantitative RT-PCR (qRT-PCR) for hedgehog signaling pathway genes were used to determine the key signaling pathway regulated for PCa progression.

Results: The cell viability gradually decreased with increased concentration of quercetin nanoparticles. At 48 h, 40 mM concentration of quercetin treatment showed near 50% of viable cells. Quercetin nanoparticles upregulates *Su(Fu)* mRNA expressions and downregulates *gli* mRNA expressions in the LNCaP cells.

Conclusions: The results showed that the hedgehog signaling targeted inhibition may have important implications of PCa therapeutics. Additionally, the outcomes provided new mechanistic basis for further examination of quercetin nanoparticles to discover potential treatment strategies and new targets for PCa inhibition.

Keywords: Hedgehog, Prostate cancer, Proliferation, Quercetin nanoparticles, Signaling pathway.

Introduction

Hedgehog (Hh) signal transduction pathway has a main role in the homeostasis, growth, survival, and malignancy (1, 2). Secreted hedgehog molecules attach to the PTC-PTCH1, PTCH2, therefore alleviating PTC-mediated suppression of smoothened (SMO), a putative seven-transmembrane protein. In addition, SMO signaling activates a cascade of intracellular events, provoking activation of the pathway via *Gli* family transcription factors (3). The PTCH1 hedgehog receptor is also a pathway target gene, which forms a negative feedback loops to maintain the pathway activity at a proper level. Hedgehog signaling activation via PTCH1 loss-of-function somatic mutations in human basal cell carcinomas (BCCs) disrupts this feedback regulation, inciting uncontrolled SMO signaling (4). Activation of the Hh pathway is frequently shown by raised levels of

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HIP and PTCH1. In addition to PTCH1 mutation, Hh overexpression and SMO activation can result in activation of the Hh pathway (5). *Su(Fu)* as a negative regulator of the Hh pathway prompting inactivation of this pathway by inhibiting the function of *Gli* molecules.

Prostate development need Hh signaling. Although the initial formation of prostate buds does not need sonic hedgehog signaling (shh), shh is vital for keeping up suitable prostate development, proliferation and tissue polarity (6, 7). The hedgehog pathway activity is low in the normal prostate, while activation of Hh pathway show greatly increased among prostate cancer (PCa) development. Therefore, inhibition of *Gli* function might be a promising prevention and therapeutic target in specific tumors (8).

Quercetin is a principal flavanoid compound that can be found in apples, onions, tea, and red wine, which possesses a wide spectrum of pharmacological properties (9). It has been showed that this compound has growth inhibitory effects on a wide range of cancer cell lines *in vitro* and *in vivo* (10). Quercetin nanoparticles (NQ) are ubiquitous in the environment, which their effectiveness in malignancy treatment has been reported (11). The NQ can cross many barriers, for instance the blood brain barrier, due to their special physicochemical features. And exposing cells to NQ can prompt negative effects such as inducing cell death, particularly in cancer cells (12). Howbeit, quercetins action molecular mechanism on cancer prevention and treatment are not totally described, but the their anti-cancer effects have been shown in different type of cancers including prostate, cervical, pancreatic cancers, and breast (13, 14). In this assessment, utilizing PCa cell lines with wild-type androgen receptor (AR) expression (LNCaP), we have characterized the change in molecular profile and hence the anti-cancer effect induced by quercetin in LNCaP. We recommend that the prostate cancer–preventative effects of quercetin may result from inhibition of the Hh pathway and that they possibly represent an inexpensive, safe, and effective decision for cancer prevention and treatment.

Materials and Methods

Quercetin nanoparticle preparation by microfluidic reactor

High performance liquid chromatography (HPLC)-grade quercetin was purchased from Behansar Pharmaceutical Company (Iran). Polysorbate 40 (Tween 40) and acetonitrile (HPLC grade) were provided from Sigma-Aldrich (Germany). The preparation of NQ by microfluidic reactor was performed according to Azarian et al. (15). Briefly, at predetermined temperatures and certain flow rates of solvent/anti-solvent, quercetin saturated solutions in acetonitrile were injected into the reactor. Quercetin nanosuspension was produced by a microfluidic reactor of polylactide (polylacetic acid) with an interior diameter of 1 mm and inlet angles of 30 °, 60 °, 90 °, 120 °, and 180 °. Four different input variables were considered in this survey (Tween 40 concentrations, anti-solvent flow rate, the angle between two entries (inlet angle), and viz. longitude output arm), which these parameters can affect the precipitation of nanoparticles prepared, possibly. At a constant room temperature of 22±2 °C, different concentrations of Tween 40 were used as the anti-solvent system. Fluid minor volumes were injected by hydrodynamic micro pumps. As an indicator of physical stability of the nanosuspension in order to ascertain parameters impacting the prepared nanosuspension stability, the time of sedimentation was taken. Finally, the produced samples were tightly closed and stored at 22±2 °C along with daily observations to monitor the phase separation of nano dispersions formed.

Size measurement of nanoparticles

To determine the particle size and morphology of prepared nanosuspension after examining the input variables affecting the output, the optimum formulation was studied.

Cell cultures and treatments

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The human PCa cell line LNCaP was obtained from the Cell Bank of Institute Pasteur of Iran, Tehran. The cells were propagated in 100 mm culture dishes at the desired density in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich) at 37 °C and 5% CO_2 until reaching 50–70% confluence.

Cell viability assay

The cell viability assay was conducted by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide). Briefly, in a 96-well culture plate, 5×10^3 cells/ml of LNCaP cells were seeded and incubated overnight at 37 °C and 5% CO_2. Cells were washed twice with PBS after attachment and starved for 6-12 h with serum-free medium (SFM). After starvation, cells were treated with different concentrations of quercetin nanoparticles (10, 20, 40, 80, and 100 μM) for 24, 48 and 72 h. Then, the wells were washed twice with PBS, added 100 ml of MTT (0.5 mg/1 ml) to each well and cells were incubated for 4 h at 37 °C. Then supernatant was removed and 100 μL of DMSO was added to dissolve formazan crystals formed by viable cells. By a spectrometer reader (SCO diagnostic, Germany), optical density (O.D.) was measured at 570 nm. The cell viability was assessed as the percent cell viability compared to the vehicle-treated control cells without quercetin nanoparticles administration, which were determined arbitrarily as 100% viability.

Pyrogenicity test and general safety

Taking advantage of the previous procedure, the pyrogenicity and toxicity of the antigens were checked (19). The amount of endotoxin in the prepared antigens was measured by a commercial Limulus amebocyte lysate kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's recommendations.

Real-time quantitative PCR

Total RNA was isolated from the LNCaP cell line using the RNA extraction kit (GeneAll Biotechnology, South Korea), based on the manufacturer's guidelines. RNA concentration was determined using the NanoDrop 1000 Spectrophotometer (Wilmington, USA). Then the cDNA synthesis was performed from RNA, using the cDNA synthesis kit (Takara Bio, Shiga, Japan), based on the manufacturer's guidelines.

Real-time TaqMan qPCR amplification was conducted with a Rotor-Gene 6000 real-time PCR cycler (Qiagen Corbett, Hilden, Germany) with 5 ng cDNA and 10 pM related primers of *Su(Fu)* and *gli* genes. The data were normalized to the housekeeping β-actin gene (F: 5′- TGGGCATCCACGAAACTAC -3′ and R: 5′- GATCTCCTTCTGCATCCTGT -3′) and calculated as $2^{-\Delta\Delta CT}$ expression. The primers and probes were manufactured by Pishgam (Tehran, Iran) are shown in Table 1.

Table 1. ***Gene-targeted specific primers used in this study.***

Target Gene	Primer	Oligonucleotide sequence (5'-3')	Product size (bp)	Ref.
SUFU	F	CCAATCAACCCTCAGCGGCAGAATG	159	This study
	R	GTAGGTGAGAAAGAGGGCTGTC		
GLI	F	CAGGACCTAAGGACATATCTGGA	77	This study
	R	CTCGGTACCAGAGTGTAACAACC		

Statistical analysis

Data were expressed as mean±standard error of the mean (SEM) and analyzed by SPSS

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software (version 22.0, SPSS Inc, Chicago, IL, USA). Groups were compared with Student's t-test or one-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc analysis. A p value of< 0.05 was considered statistically significant.

Results

To determine the effect of quercetin-induced cell viability, human PCa cell line LNCaP were treated with different concentrations of quercetin. NQ decreased the cell viability gradually with increased concentration of quercetin. At 48 h, 40 mM concentration of quercetin treatment showed near 50% of viable cells ($p< 0.05$), (Fig. 1).

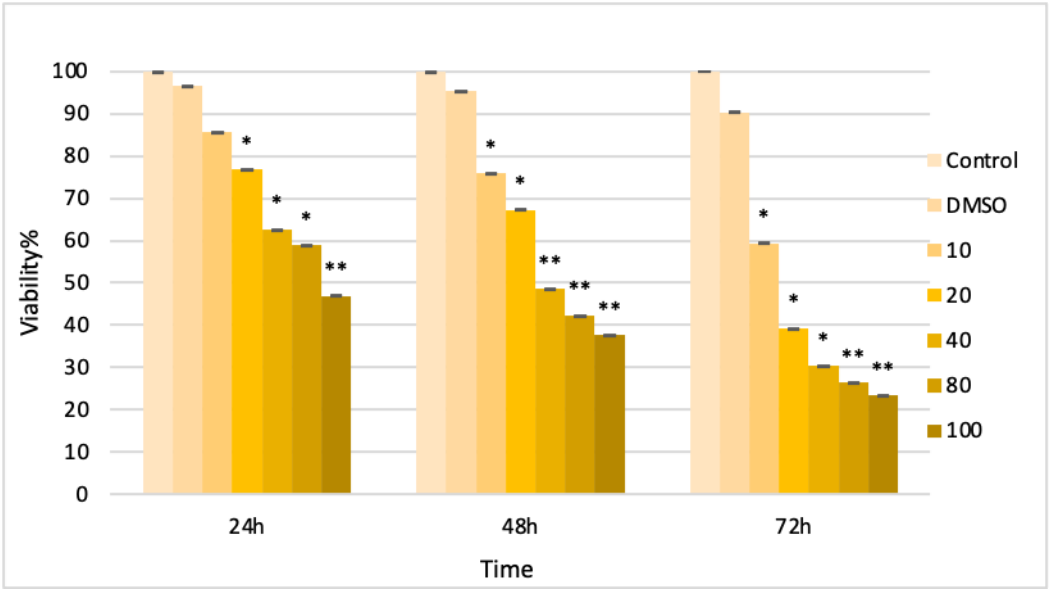

Fig. 1. Effects of quercetin nanoparticles on LNCaP cell viability. The cell viability of LNCaP cells was measured by MTT assay for 24, 48 and 72 h.

In the present study, we determined the levels *Su(Fu)* and *gli* mRNA expression in cancer cell line LNCaP. Detectable levels of *Su(Fu)* and *gli* mRNA was observed in the LNCaP cells. NQ downregulates *gli* mRNA expressions and upregulates *Su(Fu)* mRNA expressions in the LNCaP cells (Fig. 2). Therefore, quercetin inhibits proliferation and cell survival of PCa cells.

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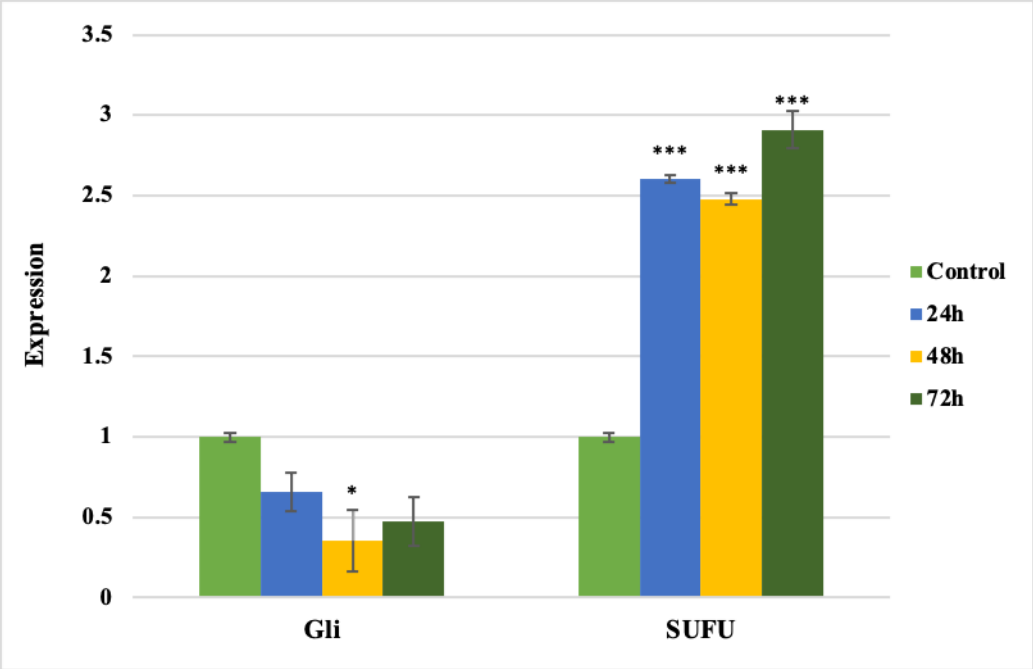

Fig. 2. Quercetin nanoparticle downregulates *gli* mRNA expression. Each bar represents the mean±SEM of three independent observations.

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Discussion

Chemotherapy is regularly utilized alone or in combination with therapeutic modalities to treat advance PCa. In any case, patients frequently develop resistance that can prompt poor therapeutic reaction and disease relapse. Thus, new agents with fewer side effects are needed to improve therapeutic outcome of PCa. Phytochemicals are used extensively for their properties in cancer therapeutic (16). In this manner, natural agents such as flavonoids clinically have been used in many countries to determine their cancer-preventive properties. Quercetin is a compound researched for its antiproliferative and anti-cancerous properties (1, 17, 18). Also, quercetin as a nutritional supplement utilized for prostatitis treatment, which can be an marker for development of PCa (19, 20).

Recently, liposomes and lipid based nanocarriers have shown efficacy in medication and gene therapy (21, 22). The effective and successful utilization of liposome nanocarriers has been catalyzed via targeted delivery and subsequent preferential intracellular uptake, enhancing permeability and retention effect with improved efficacy, selectivity, and overall safety (23). In the current study, quercetin nanoparticles were used to accomplish the promoting effects on PCa suppression through Hh signaling pathway. Hedgehog signaling pathway controls tissue polarity, cell proliferation and cell differentiation within normal development. Abnormal signaling of this pathway has been described in different human cancers, including small cell lung cancer, medulloblastomas, basal cell carcinomas, GI and prostate cancers (4, 24, 25). Our findings in this report indicate that NQ downregulates *gli* mRNA expressions and upregulates *Su(Fu)* mRNA expressions in the LNCaP cells. Therefore, quercetin inhibits proliferation and cell survival of PCa cells by influencing on the Hh signaling pathway. All the results here might serve as a basis for providing the possible treatment of natural anticancer compounds in developing new therapy for PCa in future.

It has been proved that androgens play an important role in PCa progression (10, 26). Hence, the main treatment given to the patients with androgen-dependent disease is androgen deprivation therapy (27). Unfortunately, recurrence of the disease usually happens within 12 to 18 months, leaving patients with castration-resistant PCa (28). Different investigations have reported the anti-proliferative and anti-inflammatory effects of quercetin on human cancer cell lines (29, 30). Apoptosis is one way is to establish tissue homeostasis; however, cancer cells develop mechanisms to elude cell death (31). In spite of primary prostate epithelial cells, PCa cells exposed to quercetin as time and dose dependent manner demonstrated more accumulation of dead cells. The data supports the conditions that the treatment regime of quercetin will be associated by fewer side effects. Quercetin induces intrinsic and extrinsic pathway-mediated apoptosis and modulates the components of insulin-like growth factor signaling in androgen-independent conditions (32). Further, quercetin induces c-jun/sp1-mediated downregulation of AR expression and activity in PCa cells (33). Moreover, by expression repressing in androgen-responsive PCa cells, quercetin attenuated the transcriptional yield of AR (10). These outcomes usually concentrate on utilizing LNCaP and need more data in cell lines that has mutated (DU-145) and lac AR (PC-3) to grasp mechanistic perspective. Therefore, quercetin resensitizes the resistant PCa to anti-androgen treatment. This inhibitory impact of quercetin on AR signaling involves its promising role as a chemopreventive agent or as an adjunct to existing treatment for PCa. Yue et al. (34) revealed that *Su(Fu)* is degraded quickly in certain cancer cells and indicated that Shh signaling promotes ubiquitination of *Su(Fu)*, which results in its destruction in the proteasomes. These outcomes show that Shh signaling controls *Su(Fu)* activity by inducing its turnover by means of the ubiquitin–proteasome system (34). Their information

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demonstrated that the Shh control of *Su(Fu)* stability is physiological, and propose that Shh may overcome the negative inhibition of *Su(Fu)* on *Gli-mediated* target gene expression at least in part by inducing its degradation (34).

To sum up, nanoparticles of quercetin improved the inhibitory role in progression of PCa on cell line LNCaP via Hh signaling pathway. Accordingly, the results recommend that NQ is a promising candidate in PCa therapeutics in future. There could also be a couple of mechanisms by which the Hh pathway is activated in advanced prostate cancers, including over-expression of sonic hedgehog, loss of *Su(Fu)* protein expression, or other alterations. Our investigations predict that targeted inhibition of the Hh pathway could also be an efficient way to stop PCa progression. However, the apoptotic effects and anti-proliferative molecular mechanism of quercetin nanoparticles remains to be determined.

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