

Original Research Article

Effect of Turkish propolis extracts on expression of voltage-gated sodium channel Nav 1.5 and 1.7 α -Isoforms in PC-3 human prostate cancer cells

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Abstract

Purpose: To investigate the effect of dimethyl sulfoxide (DMSO) and water extracts of Turkish propolis (WEP) on mRNA expression of Nav 1.5 and 1.7 α isoforms of Voltage-Gated Sodium Channel (VGSC) proteins in PC-3 human prostate cancer cells.

Methods: DMSO and WEP (20 μ g/mL each) were incubated for 24 h with PC-3 cells and total RNA was extracted using a commercial kit. Real-time polymerase chain reaction (RT-PCR) assay was used to determine mRNA levels of the isoforms of VGSC. Expressions of VGSC were assumed to be 100 % in PC-3 cells incubated without extract.

Results: Both extracts decreased the expression of VGSC isoforms to varying extents. Expressions of Nav 1.5 and 1.7 was 61.43 ± 4.92 and 58.17 ± 2.88 %, respectively for DMSO; while for WEP, the values were 83.54 ± 15.96 and 80.40 ± 13.87 20 μ g/mL, respectively.

Conclusion: This results suggest that DMSO and water extracts of Turkish propolis may have anti-metastatic activity in PC-3 cells due to down-regulation of expressions mRNA of VGSC α -isoforms.

Keywords: Propolis, Voltage-gated sodium channel (VGSC), PC-3 Human prostate cancer cells

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INTRODUCTION

Propolis is a bee product which has been used in folk medicine since ancient times [1]. It is composed of 50 % resin, 30 % wax, 10 % essential and aromatic oils, 5 % pollen and 5 % various other substances, including organic debris. Propolis contains considerable amounts of flavonoids, polyphenolic substances such as quercetin, caffeic acid and caffeic acid phenethyl ester (CAPE), pinocembrin, galangin, p-coumaric acid. These components are potent antioxidants, but there is a problem of standardization [1,2]. Propolis has anti-bacterial,

anti-viral, anti-fungal, immune-modulatory, anti-inflammatory, anesthetic, cytotoxic, anti-proliferative and anti-mutagenic effects [1].

Prostate cancer is one of the most commonly diagnosed cancers in men and the first leading cause of cancer-related deaths in the United States of America and the European Union [3-5]. MR/IR (mortality rate to incidence rate ratio) of prostate cancer is as high as 40 % in Asia compared to 18 % in Europe, 10 % in Northern America and 25 % worldwide [6]. The etiology of prostate cancer is multifactorial, and a number of dietary and lifestyle factors have been implicated

in the development and progression of the disease [5]. In addition, prostate cancer cells usually metastasize mainly to bones, lymph nodes and lungs body [3].

VGSCs are integral membrane proteins which usually mediate fast transport of Na^+ in and out of cells depending on electrochemical gradient. They are functionally up-regulated in metastatic prostate cancer cells like breast cancer, small-cell lung cancer, non-small-cell lung cancer, mesothelioma, melanoma and cervical cancer cells [7]. VGSCs contain α subunit and one or more β subunits. The VGSC α subunit gene family consists of 10 members (Nav 1.1-1.9 and Navx) and β subunits consist of 4 members (β 1- β 4) and one splice variant of β 1A, which have distinct tissue specificities and developmental expression profiles. VGSC Nav α 1.5 mRNA was detected in most human tissues assayed and showed the strongest expression in the heart, and high level in adult and fetal brain, the testes and the prostate. VGSC Nav 1.7 mRNA was also present in most tissues assayed, and the highest expression was found in the testes, adult and fetal whole brain, and placenta [8].

According to the CELEX hypothesis, phytochemicals like resveratrol, curcumin, capsaicin, genistein and ginseng, omega-3 polyunsaturated fatty acids and minerals have anti-cancer effects through down-regulating of VGSC expression, and partly up-regulated VGKC (Voltage-Gated Potassium Channel Protein) expression [9].

In this study, we investigated the effects of propolis extracts on mRNA expression of VGSC Nav 1.5 and Nav 1.7 α -isoforms in PC-3 cells.

EXPERIMENTAL

Chemicals

DMSO, was supplied from Merck (Berlin, Germany), Dulbecco's Modification of Eagles's Medium (DMEM)/Ham's F12 with L-Glutamine, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin and streptomycin were products of Sigma (St. Louis, MO, USA). RPMI 1640, L-Glutamine and Fetal Bovine Serum (FBS) were obtained from GIBCO (Paisley, England). PBS-Dulbecco, Trypsin/EDTA solution and Trypan Blue were supplied by Biochrom AG (Berlin, Germany). Ethanol was obtained from Carlo Erba (Milano, Italy), 100 mM dNTPs from Fermentas Life Sciences (Ontario, Canada), while reverse

transcriptase (AMV) was got from Roche Diagnostics (GmbH, Mannheim, Germany).

Propolis

Propolis samples, produced by honey-bee (*Apis mellifera* L.) in various regions of Turkey, were provided by Fanus Food Company (Trabzon, Turkey) and mixed to obtain Turkish propolis sample. Turkey is rich in *Picea orientalis*, *Fagus orientalis*, *Castanea sativa*, *Rhododendron ponticum*, *Rhododendron luteum*, *Rubus caucasicus* [10].

Analysis of the phenolic compounds (flavonoids) of Turkish propolis was previously carried out in our laboratory. According to HPLC analysis, the main flavonoids of DMSO extracts of propolis were found to be galangin, naringenin, chrysin, kaempferol, quercetin and cinnamic acid derivatives, while caffeic and caffeoyl quinic acids were present in the water extracts [11].

Preparation of DMSO and water extracts of Turkish propolis

Each natural propolis samples were ground (Retsch, ZM 200) then frozen at $-80\text{ }^{\circ}\text{C}$. The ground propolis samples were mixed and bottled in 5 g-portions. The portions were dissolved in 20 mL of DMSO (100 % w/v) or deionized water by continuous shaking in an incubator (Shelleb/Sheldon Mod: 514, USA) at 150 rpm and $60\text{ }^{\circ}\text{C}$ for 24 h. Extracts of 250 mg/mL concentrations were obtained by centrifuging at 4000 rpm for 10 min. Collected supernatants were mixed and stored at $4\text{ }^{\circ}\text{C}$ in the dark. Working solutions at concentration of 20 μg aqueous extract/mL and μg DMSO extract/mL were prepared by diluting stock WEP and DMSO extracts with deionized water and DMSO respectively.

Cell culture

PC-3 human prostate carcinoma cells were obtained from Hematology Department of Gulhane Military Medical Academy in Ankara, Turkey. The cells were cultured in RPMI 1640 medium containing L-Glutamine (GIBCO), 10 % FBS (Sigma) and 1 % penicillin/streptomycin (Sigma) solution at $37\text{ }^{\circ}\text{C}$ in a humidified atmosphere containing 5 % CO_2 . Subcultures were carried out every 3-4 days using 0.25 % trypsin-0.02 % EDTA solution.

Extraction of total RNA

PC-3 cells at a density of 1.5×10^5 cells were placed in T-25 flasks, cultured in duplicate and

incubated for 24 h. Then 0.5 mL of 20 µg/mL WEP, 20 µg/ml DMSO extract of Turkish propolis and 1 ml of FBS were added to 8.5 mL of RPMI-1640 and the cancer cells were incubated for 24 h at 37 °C and 5 % CO₂ after adding the new medium mixture. PC-3 cells which were not treated with any extract were used as a control.

For total RNA extraction from PC-3 cells (Treated and not treated with propolis extracts), QIAGEN (GmbH, Hilden, Germany) OIAamp RNA Blood Mini Kit (Cat.No. 52304) was used. The protocol recommended by the kit manufacturer for RNA extraction from cells samples was precisely followed.

Synthesis of primers and cDNA

All primers were designed by Ocimum Biosolution B.V. (Holland). For Nav 1.5 (hH1): primers forward 5'-CAT CCT CAC CAA CTG CGT GT-3' (570-589) and reverse (hH2) 5'-CAC TGA GGT AAA GGT CCA GG-3' (10559-1078). For Nav1.7 (HNE1) primers forward 5'-TAT GAC CAT GAA TAA CCC GC-3' (474-493) and reverse (HNE3) 5'-TCA GGT TTC CCA TGA ACA GC-3' (843-862). For β-Actin (Hb-actinF) primers forward 5'-CAC TGA GGT AAA GGT CCA GG-3' and reverse (Hb-actinR) 5'-TGT CAA AGT TGA TCT TCA CG-3'.

cDNA was synthesized from 5 µL of total RNA in a final volume of 20 µL reaction using the AMV(Avian Myeloblastosis Virus) Reverse Transcriptase (Roche Diagnostics, GmbH, Mannheim, Germany) with Nav 1.5, Nav 1.7 and β-Actin primers at 2.5 µL from 10 pmol following the manufacturer's protocol.

Real-time PCR

For real-time (RT)-PCR assay, Roche Applied Science LightCycler® DNA Master SYBR Green I kit (Cat. No: 12 015 099 001) was used. cDNA (5 µL) was amplified by RT-PCR with 10x Roche Applied Science LightCycler® DNA Master SYBR Green I and 10 pmol/µL target primers. β-Actin was used as the housekeeping gene. Each sample was analyzed with negative control in duplicate using LightCycler 2.0 System (Roche). The reaction conditions were as follows: 1 cycle 95 °C/30 s, 45 cycle of 50 °C/15 s, 72 °C/20 s,

and the melting curve: 95 °C/30 s, 60 °C/15 s and 40 °C/30 s. PCR amplification was related to a Standard curve. The smallest dilution of cDNA Standard was given the relative value 100 and, following the same reason of dilution, other three points were 10, 1 and 0.1. The results were expressed as percentage of differences relative to normal controls (relative expression) using (Software 4.05).

Data analysis

All data were expressed as arithmetic mean ± standard deviation (SD).

RESULTS

Expression of Nav 1.5 and Nav 1.7 α isoforms of VGSC

As shown in Table 1, both extracts decreased expressions of VGSC Nav 1.5 and Nav 1.7 to different extents. Lower expressions of VGSC Nav 1.5 and Nav 1.7 were obtained in PC-3 cells incubated with 20 µg/mL DMSO extract of Turkish propolis (61.43 ± 4.92 and 58.17 ± 2.88 %, respectively) but higher expressions of VGSC Nav 1.5 and Nav 1.7 were obtained in PC-3 cells incubated with 20 µg/mL aqueous extract of Turkish propolis (83.54 ± 15.96 and 80.40 ± 13.87 %, respectively).

DISCUSSION

The poplar origin of Turkish propolis has been well documented [12,13]. Propolis samples collected in various regions of Turkey have been investigated for antibacterial, antifungal, antioxidant and anti-carcinogenic activities [13,15].

Cinnamic acid and cinnamic acid derivatives, pinocembrin, pinobanksin, chrysin, galangin, vanillin, p-cumaric acid, ferulic acid, caffeic acid, naringenin, CAPE have been detected in Turkish propolis [11,13,14]. In addition, it has been reported that propolis collected from different countries contain phenolic compounds like genistein [16], catechin and resveratrol [17].

Table 1: VGSC Nav 1.5 and Nav 1.7 expression levels in PC-3 cells incubated with 20 µg/mL DMSO and WEP of Turkish propolis

Variable	Water extract (%)	DMSO (%)
Nav1.5 expression	83.54 ± 15.96	61.43 ± 4.92
Nav1.7 expression	80.40 ± 13.87	58.17 ± 2.88

Values are mean ± SD, n=3. Control PC-3 cells not incubated with any extracts were taken as 100 % expression of VGSC Nav 1.5 and Nav 1.7 α isoform

In previous study, it was reported that aqueous and DMSO extracts of propolis had antioxidant properties to different extent [18]. In MTT assay results, aqueous and DMSO extracts showed cytotoxic effects on PC-3 cells depending on the concentration [18]. The results of MTT assay for propolis in PC-3 cells are in agreement with those obtained for Turkish propolis by Turan *et al* [15] and Cuban propolis by Carballo *et al* [19]. Based on results from cytotoxic assays, 20 µg/mL propolis was chosen for Real-Time PCR assay of VGSC Nav 1.5 and 1.7 α -isoforms.

In this study, expression levels of both VGSC Nav 1.5 and 1.7 α - isoforms were reduced by DMSO and WEP in PC-3 cells when compared with control PC-3 cells which were not incubated with extracts. The highest reduction in expression of VGSC Nav 1.5 and 1.7 α isoforms was obtained in PC-3 cells incubated with 20 µg/mL DMSO extract of propolis. This result may be related to synergistic effect of some antioxidant compounds in propolis, especially in the DMSO extract. This study is the first in literature on investigation of expression of VGSCs isoforms in PC-3 cells incubated with extracts of propolis.

Experimental data on VGSC Nav 1.5 and Nav 1.7 α -isoform expressions are in consonance with CELEX hypothesis. The effects of these extracts may be related to flavonoids, polyphenolics, minerals and fatty acid content of Turkish propolis. Previous studies have shown that propolis contains resveratrol, genistein as well as polyphenolic compounds, Zn⁺ other minerals and fatty acids. Fraser *et al* [20] reported that resveratrol dose-dependently inhibited the activity of VGSC and significantly suppressed lateral motility, transverse motility and invasion in strongly metastatic MAT Ly-Lu prostate cancer cells [20].

Since propolis is natural and non-toxic to humans at low concentrations, researchers may find this natural product a suitable candidate anti-metastatic substance a viable substitute for toxic tetrodotoxin in blocking VGSC activity in cancer cells.

CONCLUSION

The findings show that both aqueous and DMSO extracts of Turkish propolis decrease the expression of VGSC Nav 1.5 and 1.7 α -isoforms in PC-3 cells. Further studies to investigate the effects of propolis extracts with different concentrations on expression of all isoforms of VGSC, function of VGSC and protein level in PC-3 and the other cancer cells are required to

ascertain contribution of antitumoral and antimetastatic activities of propolis.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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