CYTOTOXICITY OF PROPOLIS AND ITS POLYPHENOLIC COMPOUNDS ON PRIMARY CULTURE OF HUMAN URINARY BLADDER TRANSITIONAL CELL CARCINOMA

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Summary

This study was carried out to find whether propolis and its polyphenolic/flavonoids compounds may induce cytotoxicity in primary culture of human urinary bladder transitional cell carcinoma (TCC) cells as compared to normal urinary bladder epithelial cells. Pieces of TCC or normal epithelial tissue were collected by transurethral surgery from patients in different stages (grade G1,G2, G3) of TCC. Incubation of TCC cells for cytotoxicity testing were carried out with or without diffrent concentration (50, 150, 300 µg/ml) of test components. The cytotoxicity of two preparation of propolis (water and ethanolic extract of propolis; WSDP or EEP) and its polyphenolic compounds (caffeic acid, naringin, chrysin, quercetin) was determined using trypan blue exlusion assay. Findings suggest that EEP is the most effective in inhibition of urinary bladder TCC cell proliferation as compared to WSDP or single flavonoids derived from propolis. All test components showed no cytotoxicity to normal epithelial cells. The result of this study may provide great impact on the potential activity of EEP as an adjuvant to surgery, to suppress or prevent tumor recurrence in urinary bladder since only a few anti-cancer drugs have been effective in tumor control. Since immunomodulation by BCG has been used to impruve the results of surgery it is likely that propolis preparation (EEP) as immunomodulating compound may be a substitute for mycobacterial treatment since propolis preparation or its polyphenolic components have expressed no side effect after treatment in animal models.

Key words**:** Propolis, Flavonoids, Human urinary bladder cancer, Cytotoxicity, Primary culture

Introduction

Bladder cancer is the forth most common cancer among men and the eighth most common among women in the USA (1); bladder cancer occurs about twice as often in males than females. The highest incidence of cancer of the urinary bladder is observed in the developed countries, with the exception of Japan and Russia (e.g. North America 29.2/100,000 *vs*. Japan 8.8/100,000). However the occurrence of urinary bladder cancer among Japanese emigrants in the USA, beyond the second generation, is twice that seen in those remaining in Japan (2).
Several epidemiological studies have related the geografical variation in the incidence of bladder cancer to exposure to known etilogic agents, including smoking, dietary sweeteners, *Schistosoma* infections and some industrial chemicals.Thus, smoking is probably a major contributing factor for the development of urinary bladder cancer. Another 25% of cases of bladder cancer, mostly in men, appear to be caused by industrial exposure to aromatic polycyclic hydrocarbons or polychlorinated biphenyls such as 2-naphthylamine, 4-aminobiphenyl and benzidine (2).

Neoplasms in the urinary-tract epithelium possess several biological characteristics, such as multistage and multifocal carcinogenesis (3). The majority of bladder tumors (90%) have been found to be transitional cell carcinoma (TCC), where variable morphology, natural history and prognosis demonstrate that it is not single disease, but occurs in three distinct forms, each possesing characteristic features such as carcinoma *in situ*; low-grade papillary, noninvasive; and high grade, invasive malignancy (3). Moreover, a high recurrence rate (50-70%) of superficial urinary bladder tumors, even after curative transurethral resection has often been reported (4). These successive, recurrent tumors may increase in their histological grade, and more than 15% of the patients suffer a progression to muscle-invasive disease, with subsequent poor prognosis.

A number of anti-cancer drugs have been used, mainly via local instillation into the urinary bladder, as an adjuvant to surgery, to suppress or prevent tumor recurrence in urinary bladder but only a few anti-cancer drugs have been effective in tumor control. Therefor, a new additional modality is required to achive more satisfactory clinical control for this malignancy.

Large amounts of epidemiological data have supported the inverse relation between the consumption of fruits and vegetables and the incidence of cancer. Thus, a number of enviromental causes have been indentified for urinary bladder cancer, including such dietary factors as low vitamin A intake, infrequent consumption of carrots, milk and cruciferous vegetables, and high consumption of meat, animal fat and coffee.

The flavonoids present in propolis are considered to be rich source of chemopreventive agents, since they have various therapeutic biological activities such as immunomodulatory, anti-bacterial, anti-viral, anti-fungal, anti-protozoal, anti-parasitic, anti-inflamatory, anti-ulcer, anti-allergic, anti-tumor anti-oxidant anti-proliferative, anti-mutagenesis, and anti-angiogenic (5).

This study was carried out to find whether propolis or its polyphenolic/flavonoid compounds interfere with TCC growth ability by induction of cytotoxicity in primary culture of human urinary bladder carcinoma cells of different grades as compared to normal urinary bladder epithelial cells.

Material and methods

Water-soluble derivative of propolis (WSDP) and ethanolic extract of propolis (EEP) Water-soluble derivative of propolis (WSDP) was prepared as we described in (6). According spectrophotometric analisis WSDP contains: flavones and flavonols 2.13%, flavanones and dihydroflavonols 9.06%, total flavonoids 11.19%, total polyphenols 70.48% and EEP contains: flavones and flavonols 1.6%, flavanones and dihydroflavonols 38.60%, total flavonoids 40.20, total polyphenols 84.40%.

Polyphenolic compounds: Polyphenolic compounds, normally present in propolis, we used in the studies were: Caffeic acid (CA) – 3,4-dihydroxycinnamic acid (Aldrich–chemie, Milwaukee, WI, USA), Quercetin dihidydrate (QU) (Fluka, BioChemica, Switzerland), Chrysin and Naringin (Sigma, Germany). All polyphenolic compounds were dissolved in ethanol and further dilutions were made in water. The final concentration of ethanol was less than or equal to 0.1%. Ethanol (0.1%) was also used in the control group. No difference between water as control and 0.1% of ethanol in water was observed in preliminary experiments.

Tumor and normal bladder epithelia-cell samples: Pieces of urinary bladder TCC from the cavity of urinary bladder or pieces of normal epithelial tissue from urinary bladder wall were obtained with informed consent from patients (General Hospital, Urology department, Varaždin, Croatia) by transurethral resection in different stages (grade G1, G2, G3) of bladder TCC. The criteria for the histological grade and clinical stage were as follows. Grade 1 (G1) indicates well-differentiated tumors in cellular atypism and histological features as transitional epithelium. Grade 3 (G3) indicates poorly differentiated tumors lacking in the features of transitional epithelium. Grade 2 (G2) indicates moderately differentiated tumors and medium features between G1 and G3.

Tumor- and normal bladder epithelia-cell suspensions: Single cells suspension from pieces of either TCC cells or normal epithelial tissue from the urinary bladder was prepared according standard laboratory procedure (6).

Primary cell culture and reagents: Incubation for cytotoxicity testing were carried out in RPMI-1640 medium with 20% FCS at 37˚C with 5% CO2 with or without diffrent concentration (50, 150, 300 μg/ml) of test components. The cytotoxicity of two preparation of propolis (WSDP or EEP) and their respective polyphenolic compounds (caffeic acid, naringin, chrysin, quercetin) was determined using trypan blue exlusion assay.

Statistics: Differences between sample values were evaluated by Student's *t* test.

Results

Water and ethanolic extract of propolis and its polyphenolic compounds inhibite growth of primary culture of human urinary bladder TCC cells

Our first aim was to investigate whether treatment with WSDP or EEP of propolis or with respective propolis' polyphenolic compounds induce cytotoxic effect to primary culture of human urinary bladder TCC cells. As shown in Figures 1, 2 and 3 propolis and its polyphenolic compounds induce cytotoxicity of primary culture of human urinary bladder cancer cells in all histopathological grades of cancer. Comparing the cytototoxic efficacy of test components in different stages of urinary bladder TCC cells, the greates cytotoxicity was achived in G1 stage (Figure 1). Majorority of test components exhibited a time- and dose-depedent cytotoxicity (data not shown). Thus WSDP treatment at concentration of 75, 150, and 300 mg/ml resulted in 6-14% greater cytotoxicity than that in control tumor cells in G2 histopathological cancer grade after 24 h; EEP 23.5-37.16%, CA 9.61-16.76%, naringin 3.76-22.66%, chrysin 1.13-14.07% and QU 8.77-21.45%. In stage G3 test components at doses of 75, 150, and 300 mg/ml increased cytotoxicity to primary culture of human urinary bladder TCC cells in relation of control tumor cells after 24 h incubation of primary culture as follows: WSDP 0.25-25.71%, EEP 27.13-38.46%, CA 13.5-18.18%, naringin 3.7-20%, chrysin 0-16.16%, and QU 22.22-40%, respectively. The most effective agent in these studies was EEP (Figure 1). Test components exhibited no cytotoxic effect on normal urinary bladder epithelial cells.

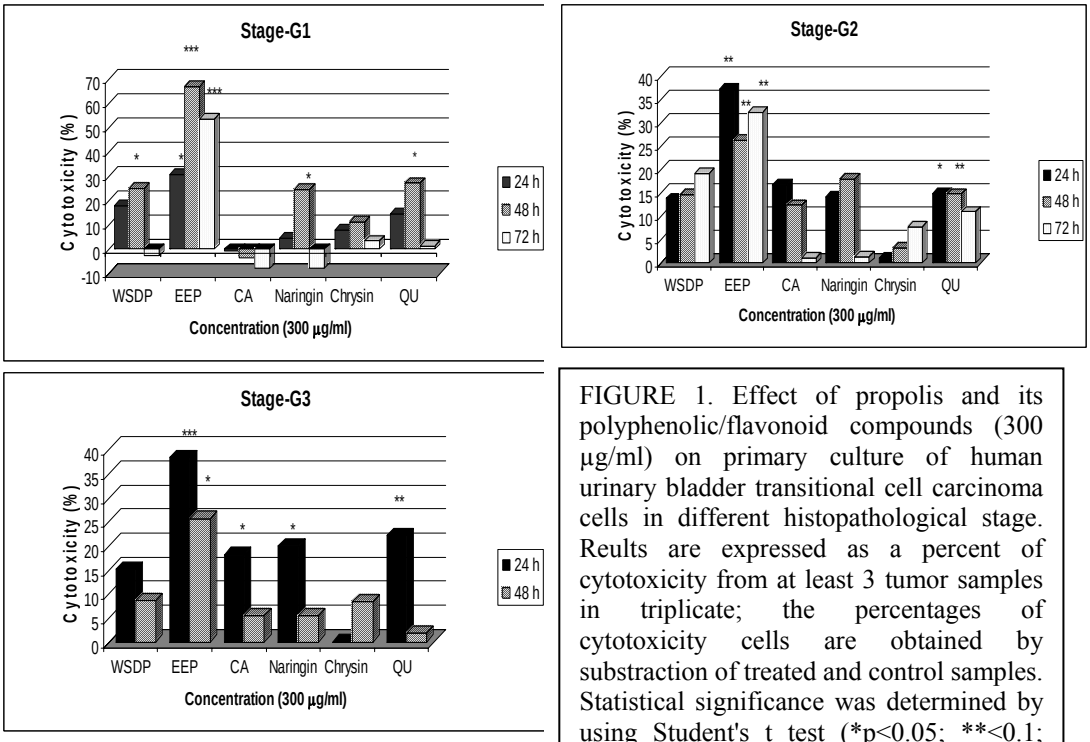

FIGURE 1. Effect of propolis and its polyphenolic/flavonoid compounds (300 µg/ml) on primary culture of human urinary bladder transitional cell carcinoma cells in different histopathological stage. Reults are expressed as a percent of cytotoxicity from at least 3 tumor samples in triplicate; the percentages of cytotoxicity cells are obtained by substraction of treated and control samples. Statistical significance was determined by using Student's t test (*p<0.05; **<0.1; ***<0.001).

Discussion

This study is the first according to the literature available, to demonstrate that treatment of primary culture of human urinary bladder TCC cells with either preparation of water and/or ethanolic extract of propolis or with their polyphenolic/flavonoid compounds caused direct toxicity *in vitro* as compared to untreated control cells. These results demonstrated that test components induced time- and dose-depedent cytotoxicity on urinary bladder TCC cells. The test components exhibited toxicity on primary culture of human urinary bladder TCC cells, and no toxicity to normal urinary bladder epithelial cells. EEP was the most effective agent in these studies.The percent (%) of cytototoxicity of EEP was reversible to the histopathological cancer grade. Moreover, it is likely that synergistic action of different flavonoids present in EEP were more effective than any single compound. These results are in line with our earlier studies using animal cancer model (6-8). In addition, the cooperative action of different isoflavones on cell growth, DNA synthesis, and apoptosis was also shown (9). Cell proliferation is thought to play an important role (4) in multistage carcinogenesis, including bladder tumorigeneis.

The result of the present study indicate that two preparation of propolis (WSDP and EEP) and their polyphenolic/flavonoid components could effectively inhibit 3 histopathological grades (G1, G2, G3) of human bladder cancer. The greates percent of cytotoxicity was in grade 1 (G1) while the effect of test components in grade 2 (G2) or 3 (G3) was essentially similar. It is possible that propolis and its flavonoids might induce cell diferentiation as suggested by Csokay et al. (10). Several biochemical targets have been proposed to explain the cytotoxix effect of propolis and its polyphenolic comounds. For example, quercetin and other flavonoids components derived from propolis are specific inhibitor of tyrosine kinase responsible for tumor growth (11); rapid reduction IP3, downregulation of the c-myc and Ki-ras oncogenes are part of the antiproliferative and apoptotic action of quercetin (11). Avila et al.(12) demonstrated that quercetin as a modulator of the cellular neoplastic phenotype may effect on the expression of mutated H-ras and p53 in rodent and human cells.

Transitional cell carcinoma (TCC) is a tumor that occurs mostly in the urinary bladder and has been linked to multiple and accumulated aberrations in oncogenes (e.g. *H-ras* mutations) and cancer –suppressor genes (e.g. *p53* inactivation), as well as to the allelic loss of specific chromosomal loci (e.g. cromosomes 9q and 11p) (13). Mutational activation of the *H-ras* oncogene was first reported (13, 14) in human T24 transitional cell line that played essential role in urothelial carcinogenesis. Thereafter, oncogenic activation of H-Ras is an important tumorogenic factor for bladder tumor which confirms molecular epidemiological studies. These studies conducted within different geographic regions or in different races and tumor stages/grades have revealed that up to 84% of bladder transitional cell carcinoma carried activated H-Ras. Quercetin, except that down-regulate the expression of the mutant p53 gene in cancer cell lines, also causes G1 phase arrest in several cancer cell lines, binds to estrogen II receptor sites, reduces expression of ER negative cells, inhibits DNA topoisomerase I and II and angiogenesis. Moreover, quercetin and other flavonoids have antiinflamatory, antioxidative, anticancer, antioxidative, antiestrogenic, and immunomodulatory activities (5-8). Therefore, flavonoids and caffeic acid analogues are particulary affective at inhibithing the prooxidant enzymes xanthine oxidase, COX or LOX (15, 16) which is important strategy for preventing cancer. Ethanol (EEP) and ether extracts of propolis (REP), caffeic acid phenethyl ester (CAPE), quercetin, reservatrol, and genestein, active components of propolis have also anti-inflammatory and anti-angiogenic activities in urinary bladder cancer (5, 16).
The incidence of bladder cancer has increased, and responses to therapy have been limited. Several studies have shown the effectiveness of immediate post-resection chemotheraphy with agents such as doxorubicin and mitomycin C (17). We here reported that water and/or ethanolic extract of propolis or their polyphenolic/flavonoid compounds may induce cytotoxicity in primary culture of human urinary bladder cancer cells and have selective effect as compared to normal urinary bladder epithelial cells.

Although bacillus Calmete-Guerin is one of the most effective agents for preventing recurrence, it cannot be used immediatelly after resection secondary to the systemic absortion. Propolis and its polyphenolic/flavonoids components, are not harmful if absorbed and they reportedly do not effect normal tissue, making it an ideal potential agent. Our previous data *in vivo* and numerous literature data about chemopreventive mechanism and local effect of these components to tumor cells (5, 18) suggest that propolis and its compounds could be potential candidates in therapy of urinary bladder cancer. Widely available, safe and inexpensive, propolis and its compounds may prove to be considerable benefit in the prevention and treatment of urinary bladder cancer.

In conclusion, these results suggest that propolis and its polyphenolic/flavonoid components may become an attractive and promising treatment for urinary bladder cancer but further animal and human in vivo studies are warranted to evaluate the safety and clinical utility of these test comonents in patients with urinary bladder cancer.

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