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Genotoxic potential of Turkish propolis in peripheral blood lymphocytes

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Propolis is a natural product that is collected by the honeybee (*Apis mellifera* L.) from plants. The *in vitro* genotoxic potential of propolis in human lymphocytes was investigated. Blood samples were obtained from ten healthy (five female and five male), non-smoking and alcohol volunteers, which were incubated and exposed to increasing concentrations of propolis (5, 25, 50 and 250 mg/ml). The mean sister chromatid exchange (SCE) rates were 10.398 ± 1.47–21.522 ± 2.08. The differences between the control and exposed cells were statistically significant ($p < 0.05$). Increasing SCE rates showed that propolis could have genotoxic effects in high concentrations. SCE rates of women donors exceeded those of men donors. Women donors had the highest SCE rates (25.674 ± 8.71, 22.456 ± 7.97 and 15.756 ± 5.09 for mean of SCE).

1. Introduction

Propolis is a natural product that is collected by the honeybee (*Apis mellifera* L.) from plants. It is used to block holes and cracks, to repair combs, to strengthen the thin borders of the comb and to reduce the hive entrance in the fall make it easier to depend in hive (Ghisalberti 1979).
Propolis contains a variety of substances including phenolic compounds such as flavonoids, organic acids and their esters, alcohols and trace elements. In general, propolis contains 45–55% resin, 23–35% waxes and oil acids, 10% essential oils, 5% pollen and 5% other organic substances and minerals (Walker and Crane 1987). Propolis is alleged to exhibit a broad spectrum of activities including antibacterial (Kujumgiev et al. 1993; Sforcin et al. 2000; Popova et al. 2005), antifungal (Ota et al. 2001; Sawaya et al. 2002), antiviral (Manolovan et al. 1985; Amoros et al. 1994), antimicrobial (Metzner et al. 1979), antiinflammatory (Miyataka et al. 1997), local-anesthetic (Paintz and Metzner 1979), antioxidant (Orhan et al. 1999), immunostimulating (Dimov et al. 1991) and cytostatic (Banskota et al. 2000) properties.
The SCE analysis method is a cytogenetic test that is used both *in vivo* and *in vitro* to examine DNA damage induced by mutagens and carcinogens. The SCE analysis is used to determine mutagenic effects of chemicals forming chromosome damages by mutagens. The SCE assay in human peripheral lymphocytes is widely used to detect occupational and environmental exposures to genotoxic compounds (Hsu et al. 1997). Determination of SCE frequencies have enjoyed widespread use as an indicator for genotoxicity following *in vitro* or *in vivo* exposure to genotoxins (Husum et al. 1985). A SCE, which arises spontaneously from the reciprocal exchange of DNA between two sister chromatids of a duplicated chromosome in proliferating cells, is a common mutagenicity test and is evidence of damage to the genome (Latt et al. 1981; Tucker et al. 1993). It has been shown to be a highly sensitive parameter for evaluating human exposure to mutagenic and carcinogenic agents (Perry and Evans 1975; Bonassi et al. 1999).
We report a study of healthy persons, evaluating rates of SCE in their peripheral blood lymphocytes under different concentrations of propolis, compared to a control group.

2. Investigations and results

The *in vitro* genotoxic potential of propolis in human lymphocytes was investigated. Blood samples were obtained from ten healthy (five female and five male), non-smoking and alcohol volunteers, which were incubated and exposed to increasing concentrations of propolis (5, 25, 50 and 250 mg/ml).
The mean values of the SCE rates after addition of different concentrations of propolis were between 10.398 ± 1.470 and 21.522 ± 2.088 (Table 1). SCE rate after addition of 0.5 ml propolis (21.522 ± 2.088) was approximately 3 times that of in the control (7.564 ± 1.02). Increasing propolis concentrations caused increases in the rate of SCE.
Mean of SCE, sex and age donors are shown in Table 2. The mean values of the SCE rates of donors were between 9.322 ± 3.78 and 25.674 ± 8.71. In this study, donors usually were between 20 and 30 years old. Only one donor was 42 years old. He had the most SCE frequency among men (14.374 ± 6.12 for mean of SCE). SCE rates of women donors exceeded those of men donors. Women donors had the highest SCE rates (25.674 ± 8.71, 22.456 ± 7.97 and 15.756 ± 5.09 for mean of SCE).

Table 1: Mean SCE rates (%) of control and different propolis concentrations ($p < 0.05$)

Donors (n)	Control	Propolis concentration			
		0.01 ml	0.05 ml	0.1 ml	0.5 ml
1	11.11	17.39	26.08	27.27	30.43
2	6.52	8.69	13.04	15.21	19.56
3	4.65	6.97	9.09	11.62	14.28
4	10.25	12.19	14.58	19.04	22.72
5	4.87	6.66	9.52	13.95	15.38
6	13.81	19.44	29.11	31.39	34.62
7	8.21	10.20	11.97	18.79	22.70
8	5.47	8.02	10.43	13.71	16.36
9	3.72	5.45	8.06	12.65	17.24
10	7.03	8.97	12.88	17.97	21.93
Mean SCE*	7.56 ± 1.02	10.39 ± 1.47	14.47 ± 2.28	18.16 ± 2.05	21.52 ± 2.08

* Values represent mean ± SE (Standard Error)

Table 2: Sex, age and mean SCE rates (%) of subjects ($p < 0.05$)

Donors (n)	Sex	Age	Donors mean SCE (%)
1	Female	25	22.45 ± 7.97
2	Female	23	12.60 ± 5.18
3	Male	32	9.32 ± 3.78
4	Female	27	15.75 ± 5.09
5	Male	26	10.07 ± 4.53
6	Female	28	25.67 ± 8.71
7	Male	42	14.37 ± 6.12
8	Male	28	10.79 ± 4.34
9	Male	26	9.42 ± 5.51
10	Female	25	13.75 ± 6.19

3. Experimental

3.1. Propolis samples

Propolis sample was collected from Bursa city in Turkey (North-West Anatolia). Hand collected propolis sample was kept desiccated in the dark until processing. A voucher specimen is deposited in the Department of Medical Genetics, Faculty of Medicine, University of Erciyes, Turkey.

3.2. Subjects

Ten healthy subjects (five female and five male) were examined. Peripheral blood was obtained from people who did neither smoke, nor drink alcohol and had no viral infection. Heparinized blood samples (10 ml) were collected for peripheral lymphocyte cultures.

3.3. Chemicals

RPMI 1640 medium (Biological Industries), fetal calf serum, (Biological Industries), phytohaemagglutinin (PHA) (Biological Industries), colcemid (Sigma), Hoechst 33258 stain (Sigma), 5-bromo-2-deoxyuridin (BrdU) (Sigma), L-Lysin (Sigma Chemie, Germany) and Giemsa stain (Merck) were used for SCE in peripheral blood culture.

3.4. Water-soluble derivative of propolis sample

A water-soluble derivative (WSD) of propolis was prepared using the method described by Nikolov et al. (1987). In short, Turkish propolis was extracted with 96% ethanol, which was filtered and evaporated to dryness in a vacuum evaporator. The resultant resinous product was added to a stirred solution of 8% L-Lysine and freeze dried to yield the WSDP, a yellow brown powder. The WSDP was stored under sterile conditions at 4 °C until used.

3.5. Sample preparation for sister chromatid exchange assay

Heparinized blood samples (10 ml) were collected from the subjects for peripheral lymphocyte cultures. The growth medium containing 80 ml RPMI 1640 with Glutamax and 25 mM HEPES (Gibco) was supplemented with 3 ml PHA, 20 ml fetal calf serum and 1 ml penicillin/streptomycin. Using an aseptic technique, 0.5 ml blood, 100 µl of BrdU and different concentrations of propolis (0.01, 0.05, 0.1, 0.5 ml) were added to a different culture medium of 5 ml. After mixing the contents of each culture tube by gently inverting them a few times, the culture tubes were incubated at a slanted position at 37 °C for 72 h. After a culture period of 70 h, 0.1 ml colcemid solution (10 µg/ml) was added to each culture tube and mixed by shaking gently. The tubes were incubated at 37 °C for an additional 25 min. The tubes were centrifuged at 2000 rpm for 4 min and the supernatant was discarded. The pellet was resuspended using 10 ml of a hypotonic solution (0.075 M KCl) and the tubes were incubated at 37 °C for a further 4 min. After the tubes were centrifuged at 2000 rpm for 4 min and the supernatant was discarded, the pellet was resuspended using 5 ml of Ibrainow solution (92 parts of tap water, 5 parts of glacial acetic acid, 3 parts of absolute methanol). The tubes were centrifuged at 2000 rpm for 4 min and the supernatant was discarded, the pellet was resuspended using 10 ml of fresh fixative (three parts of absolute methanol to one part of glacial acetic acid). The tubes were centrifuged at 2000 rpm for 4 min and the supernatant was discarded. This procedure was repeated thrice. The pellet was resuspended and 0.5–1 ml of fresh, cold fixative solution was added to the tubes. Then 3 or 4 drops of the cell suspension were dropped on to a cold-wet glass slide and placed on a hot plate to dry.

3.6. Staining and examination of sister chromatid exchange

The slides were put in phosphate tampon at room temperature for 5 min. The slides were treated in Hoechst 33258 (5 µg/ml) at room temperature for 20 min. The slides were washed with phosphate tampon. The slides were covered with phosphate tampon and treated at 366 nm of UV for 25 min. The slides were washed with $2 \times$ SSC (0.03 M tri sodium citrate and 0.3 M sodium chloride). The slides were incubated with $2 \times$ SSC at 37 °C for 15 min. The slides were washed with tap water. The slides were stained with 3% Giemsa for 20 min.

The scoring unit for SCE was the average of the chromatid changes per metaphases after 50 metaphases were counted for each concentration and donor.

3.7. Statistical analysis

Mann-Whitney test was used for the analysis of SCE.

4. Discussion

There are many factors effecting the SCE frequency in lymphocytes; age, gender, smoking and alcohol consumption, viral infection as well as X- and gamma ray exposure. Besides that, culture time and materials like medium, BrdU, serum used in SCE assays, are known to influence SCE frequency (Bender et al. 1992; Perry and Wolff 1974). In our study, culture time was 72 h and culture materials like medium, BrdU, serum were used in equal amounts at all cultures. Only propolis was used in different concentrations.

It has been reported that SCE frequency is increased by the excessive use of tobacco and alcohol (Sarto et al. 1985; Van Rensburg et al. 1989; Lazutka et al. 1994). Taguchi and Shiraishi (1989), showed that cells exposed to X-rays, show increased SCE frequency. Furthermore, it is reported that SCE frequency increases in cells of persons who are suffering from a viral infection (Koshikawa et al. 1998). In this study, only donors who did neither smoke nor drink alcohol were included. The volunteers were not exposed to X-rays and had no viral infection.

Age and sex have been shown to significantly modify SCE and micronucleus (MN) frequencies, in fact, females and older subjects exhibit higher mean values of both in comparison with males and younger persons (De Arce 1981; Hedner et al. 1982; Bender et al. 1988; Barale et al. 1998). The SCE frequency showed an increase in women between 30 and 40 years (Carrono and Natarajan 1988). Several studies support the genotoxic action

of sex hormones. Increased levels of spontaneous SCE and chromosome aberrations are detectable in lymphocytes during menstrual cycle, principally around ovarian time (Joseph-Lerner et al. 1993; Landi and Barale 1999). In this study, most donors were between 20 and 30 years old. Only one donor was 42 years old. He has the highest SCE frequency among men as expected and SCE rates of women donors exceeded those of men donors.

Considering the genotoxic effects of high doses of propolis shown here, care should be taken with the propolis dose to be used *in vivo*. The increasing SCE frequency in peripheral blood lymphocytes indicates that propolis may be a genotoxic and mutagen agent. Ozkul et al. (2005), reported that propolis increased MN in peripheral blood lymphocytes. If propolis increases MN rate, it may increase SCE rate. These results are consistent with our study.

Propolis is a natural product showing both useful features and harmful features like genotoxicity. However the complex content may also result from synergistic effects. The chemical composition of the propolis sample used in this study, was determined by Ozkul et al. (2005). In their study, the propolis sample was characterized by the presence of 23 compounds some of them identified for the first time in propolis. Further study are needed to determine the effects of different compounds isolated from propolis on SCE assay results.

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