A PILOT STUDY ON THE IMMUNOMODULATORY EFFECT OF BULGARIAN PROPOLIS

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ABSTRACT
Propolis is a product from the honey bee A. mellifera with various pharmacological properties. Its immunomodulatory activity is in the focus of the current research. Peripheral blood mononuclear cells (PBMC) from heparinized venous blood of healthy donors (n=6) were cultured for 24 h in the presence of propolis from the Eastern Rodopi Mountain (ethanol extractions with concentration 0, 01; 1; 2.5; 5 and 10 mg/L) or CAPE in concentration 2, 4, 8 and 16 mg/L. PBMC cultured in serum free RPMI only were used as controls. The percentage of T helper/inducer (CD4+CD3+), T cytotoxic (CD8+CD3+), B (CD19+CD3-) and NK (CD56+CD16+CD3-) lymphocyte subsets, as well as the proportion of apoptotic (Annexin V+) cells within each subset were determined before and after the cultivation by flow cytometry (FACS Calibur, BD). The percentage of CD19+ cells decreased in high concentrations of both of substances, but in low concentrations they had a protective effect on the proliferation and B cell activity. Low doses had no effect on the percentage of CD4+ and CD8+ T cells. The high concentrations of propolis (10 mg/L) and CAPE (16 mg/L) induced apoptosis in a large portion of all of cells types. All concentrations tested had no negative effect on the proliferation and vitality of NK cells. Our results evidence that high propolis concentrations are toxic for human PBMC, but low concentrations modulate cellular immunity.

Keywords: propolis, PBMC, CD-molecules

Introduction
Propolis is a product of the vital activity of the honey bee Apis mellifera. It possesses a variety of pharmacological properties – antibacterial and antiproliferative activity, antitumor and antioxidant, local anesthetic and anti-inflammatory effect. The immune modulating activity of propolis has been an object of intensive research for the last several years. Propolis and its more than 300 components affect cellular and humoral immunity by activating different molecular mechanisms.

The effect of propolis on natural resistance is investigated in vivo. Phagocytosis and cytotoxicity are activated on the cellular level (1). It activates NK-cells in rats, treated with propolis. It also stimulates macrophages to produce cytokines (TNF-α and IL-12) and intensifies the cytotoxic activity of NK-cells (10, 18). Cell-mediated immunity is activated by the cytokines, released from macrophages after propolis treatment. It activates the proliferation of Th-cells and stimulates Tc and Treg lymphocytes (20).

Propolis has an effect on humoral immunity by enhancing antibody production in rats, immunized with bovine serum albumin. Caffeic acid and quercetin, used in the same experiment have no effect on antibody production. That is a result of the synergetic effect of the propolis components as a whole (19).

Extracts of particular propolis components (Artepillin C) modulate the immune response, changing the ratio CD4+/CD8+ lymphocytes, as well as the total number of Th. Thus they affect simultaneously both cellular and humoral immunity (7). Other investigators prove the immune modulation induced by propolis. It stimulates T – lymphocyte blastogenesis and the secretion of IL-2, IL-4 and IFN-γ, but it has no effect on B-lymphocyte proliferation, induced by
lypopolysaccharide in Balb/c mice (14).

There has been no systematic and profound research concerning the therapeutic properties of Bulgarian propolis and its ability to modulate the immune response in vitro. There are analyses of Chinese, Brazilian, Scandinavian and some other types of propolis both in vivo in animal models and in vitro in normal and tumor cell lines, but no scientific data for the Bulgarian product is available yet. Propolis and its components are tested mainly in the area of microbiology and parasitology (6, 8, 11, 15, 16).

The present investigation was motivated by the contradictory data about the immune modulating activity of propolis, and the lack of information in this aspect about Bulgarian propolis. Our study is the first aimed to determine the effect of the Bulgarian propolis on the differentiation of human lymphocytes in vitro.

Materials and Methods

Samples

Mononuclear cells from peripheral blood (PBMC) from healthy volunteers (men and women, n=6), isolated by gradient centrifugation on Histopaque -1077 RPMI (Sigma-Aldrich, Cat. N: H8889) were used. The research is approved by the Ethics Commission of Medical University. Declarations of informed consent were signed by all the examined individuals.

Cell cultivation

PBMC were resuspended with density 1x10^6 /ml in RPMI serum free medium (Sigma-Aldrich, Cat. N: R6504), containing 100 U/ml penicillin, 100 mg/ml streptomycin. The tested components were added to the culture medium in the beginning of the experiment. Propolis from the Eastern Rodopi Mountain was collected in 96% ethanol, according to a standard protocol of Pharmacopea Europeica was used. It was extracted in 96% ethanol, according to a standard protocol of Pharmacopea Europeica was used. It was applied in concentration: 0.01, 1, 2.5, 5 and 10 mg/L. Phenethyl ester of caffeic acid (CAPE) – a chemically synthesized component of propolis (Sigma, Cat. № C8221-IG) in concentration 2, 4, 8 and 16 mg/L was also used.

PBMC, cultured in medium without the tested components, served as a negative control, and PBMC, stimulated with Phytohemagglutinin PHA-P (Sigma-Aldrich, Cat. N: L8754) in final concentration 2 μg/ml were used as a positive control. The cells were cultured for 24 hours in an incubator Heraeus (Germany) at 37°C, in 5% CO2 and high humidity.

Flow cytometric analysis of apoptosis in subpopulations of human PBMC

Annexin V-FITC (BD. Apoptosis Detection Kit I (BD Cat. № 556547) and antibodies against surface lymphocyte markers were used before and after the cultivation in order to analyze the apoptosis of lymphocyte subpopulations. All additional monoclonal antibodies were purchased from BD Biosciences. The procedure was performed according to a modified protocol recommended by the manufacturer. PBMC were washed and then resuspended in Annexin V connecting buffer (1.4 M NaCl, 25 mM CaCl2) to concentration 1x10^6 cell/ml. 100μl the suspensions were incubated for 30 min. on ice, in the dark with one of the following combinations: Annexin V-FITC/CD56+CD16-PE/CD45-PerCP/CD19-APC; Annexin V-FITC/CD69-PE/CD3-PerCP/CD8-APC. The marked cells were analyzed within an hour, using flow cytometer FACS Calibur and software BD CellQuest Pro 4.0.2. At least 20 000 lymphocytes or 10 000 T lymphocytes were collected for each analysis.

Results and Discussion

After 24 hours of propolis and CAPE treatment, lymphocyte populations in cultured PBMC revealed different sensitivity. The increasing concentrations of the investigated components decreased cell vitality. The influence was concentration-dependent. The low concentrations of propolis (1mg/L) and CAPE (2 mg/L) had no effect on cell vitality and even had a proliferative effect on the cells.

The following changes were observed:

1. Effect of propolis on lymphocyte populations

Propolis had a proliferative effect in low concentrations of 1 and 2.5 mg/L on CD19+. In 5 mg/L a pro-apoptotic effect was detected – there were 17.81% Annexin V positive cells in comparison with untreated cells. In the highest concentration of 10 mg/L approximately 56.08 % of cells were apoptotic (Fig. 1).

The resistance of CD19+ cells to propolis treatment was similar to that of the total population of CD3+ cells. The percent of apoptotic CD8+ cells, treated with concentrations 1, 2.5 and 5 mg/L propolis was respectively 3.81%, 8.11%
and 1.25% (Fig. 2). The concentration of 10 mg/L induces apoptosis in 80% of Tc cells.

In order to investigate the activation status of CD4<sup>+</sup> and CD8<sup>+</sup> cells we analyzed the expression of the early activation marker CD69 within the CD3 population. After propolis treatment no increase in activated T-lymphocytes was observed. Concentration 2.5 mg/L propolis had no effect neither on CD4<sup>+</sup> (7.4% compared to 6.5% in control cells), nor on CD8<sup>+</sup> T- cells (3.6% compared with 2.4%) (data not shown).

### 2. Effect of CAPE on lymphocyte populations

After CAPE treatment of the cells the tendencies observed were similar to those determined after propolis treatment, but the effect was more intense.

In low concentrations (2 mg/L CAPE) there was a proliferative effect; in 4 mg/L the number of apoptotic cells was similar to the control cells - 1.68 %, but after raising the concentration (8 and 16 mg/L) the vitality of B-lymphocytes in the cell culture was lowered. In concentrations 8 and 16 mg/L the percent of apoptotic cells was 46.97% and 61.01% (Fig. 1).

The large proportion of survived cells after propolis treatment, was observed in the CD56<sup>+</sup> population. The low concentrations had a proliferative and almost no pro-apoptotic effect. Only in the highest concentration of propolis (10 mg/L) apoptosis was observed in 54.15% of NK-cells (Fig. 4). These cells revealed considerably higher resistance in comparison with CD4<sup>+</sup> and CD8<sup>+</sup> cells.
After CAPE treatment the resistance of NK-cells was lower than after propolis treatment. Still in 4 mg/L CAPE the vitality of NK-cells decreased to 46.77%. The next concentration increased the percent of apoptotic cells, but nevertheless, the values were lower than the apoptotic CD3+ cells – 53.5% and 61.48% (Fig. 4).

The alteration in CD69 expression after CAPE treatment influenced stronger CD8+/CD69+ cells than CD8-/CD69+. The effect was more pronounced in the lowest concentration - 2 mg/L CAPE activated CD4+ (CD69+/CD8-/CD3+) in 14% compared to 6% activation of control cells. It had no effect on CD8+ cells (5.3% compared to 2.4%). Higher CAPE concentrations (4 mg/L) also had no activating effect (data not shown).

Within all cases of CAPE treatment an increase of apoptotic cells was observed in parallel with concentration increase. The effect was stronger than after propolis treatment.

A lot a harmful influences with endo- or exogenic origin cause pathological alterations in the human body. The immune system is activated by these stimuli and switches on the mechanisms of innate and adaptive immunity. There is a balance between these two types of immune response that sustains the immunological homeostasis. Some scientists prove that propolis ethanol extract stimulates non-specific immunity, activates humoral immunity and enhances cell-mediated immune response (11, 13, 17).

In our previous research we detected the antiproliferative and pro-apoptotic effect of Bulgarian propolis on the fibroblast cell line McCoy Plovdiv (3, 21). The fact that propolis induces apoptosis in fibroblasts provokes our interest in its modulating influence on the immune system by affecting some cell populations and switching the immune response to different directions.

The evidence concerning the influence of propolis on the immune system is quite contradictory. Some authors proved that the primary effect of propolis and its components is on cell resistance, rather than on humoral immunity (14, 19). Park J. et al. determined that propolis stimulated T-lymphocyte proliferation in Balb/c mice, but it had no effect on B-lymphocyte blastogenesis, induced by lypopolysaccharide. It led to a decrease in their number in relation to the total number of lymphocytes. In other in vivo experiments propolis stimulated humoral immunity, causing antibody production in bovine serum albumin immunized rats. Two of propolis components - caffeic acid and quercetin, used in this experiment, had no effect on antibody production, which can be explained with the synergetic effect of the whole compound (19).

There are different explanations for the contradictory results of the natural honey product on the immune system. Particular types of antigens bind and activate different populations of T lymphocytes, which have different immune functions. In order to ensure an effective immune response a restricted or an intensified proliferation occurs. The influence of different components on various subpopulations can be used for inducing an optimal CD4/CD8 ratio and thus optimal protection (5).

In our experiment the high resistance mostly of CD56+ and CD3+ lymphocytes determines more efficient influence of the natural honey product on cell immunity. The low concentration of propolis has a stimulating effect on T-cell immune response. On the other hand, the positive effect of the low concentration on B-lymphocytes determines its potential to stimulate antibody production. The high concentration of propolis induces apoptosis in B-lymphocytes. Probably the increase of T, in relation to B-lymphocytes is a result of predominant apoptosis of B cells, but not of proliferation of T-cells. The highest propolis concentration (10 mg/L) and CAPE (16 mg/L) have a toxic effect and cause cell death. The activation of T lymphocytes leads to a series of interactions and events, including activation of transmembrane signals and expression of cytokine genes. The cytokines bound to specific receptors on the surface of target cells regulate the growth or/and differentiation of the cells and thus optimize the immune response. The development of cellular or humoral immunity depends on the cytokine production mainly from CD4+ and CD8+ cells (4). In our preliminary research we found that similarly treated PBMC had an ability to secrete cytokines. After propolis treatment an inhibition of IL-6 was determined in combination with increased IL-2 production. Probably the stimulating effect of IL-2 is directed to NK-cells and T-cells. We suppose that as a result of intermolecular interactions propolis activates Th cells and stimulates cellular immunity in vitro.

The low concentrations are favourable for the
proliferative potential of all cell populations and they even have protective influence on B-lymphocytes.

After propolis treatment no clear tendency for early lymphocyte activation in comparison with phytohemagglutinin stimulated control cells was determined. The analysis of the lymphocyte profile after CAPE treatment revealed high expression of CD69 in CD4⁺ cells in relation with CD8⁺. These facts indicate an activating effect of propolis component affecting on CD4⁺ cells and thus influencing both cell and humoral immune response.

CAPE is one of the main propolis components that has proven anti-inflammatory properties. It is shown that this phenolic compound is a potent inhibitor of mitogene-induced T-cell proliferation, lymphocyte production (2), and NF-κB activation in vitro (9). Marquez N. et al. investigated the effect of CAPE on Jurkat cells and discussed it as a powerful inhibitor of early and late T-cell activation. They proved that CAPE inhibited the NF-κB and NFAT (nuclear factor of activated T-cells) transcriptional activity. This signal pathway is important for the regulation of cytokine gene expression and immune response. Since activated T-cells play a crucial role in the onset of several inflammatory diseases, the inhibition of transcription factors NF-κB and NFAT represents a rationale for the development of novel and safe anti-inflammatory agents (9).

Our study proves a positive effect of the natural honey product on T-cell activity and an antiapoptotic effect on NK cells. That is a prerequisite for its application in the therapy of diseases requiring active cell-mediated immune response.

The high content of CAPE in Bulgarian propolis (6) presumes its participation in the modulation of both cellular and humoral immunity. All our experiments reveal that the effect after CAPE treatment is more intensive than after propolis treatment. In low concentration, CAPE activates T-lymphocytes, shows antiapoptotic effect on B-lymphocytes and does not influence NK-cells. In all cell populations we determined a high level of apoptosis in comparison with propolis treatment. The results might be explained by the fact that CAPE is a chemically synthesized product and in lower concentration it has stronger effect than the complex product.

This is the first study on the immune modulating activity of the Bulgarian propolis. It proves that propolis has an effect on lymphocyte differentiation and in low concentrations can modulate the cellular immune response.

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REFERENCES


