Evaluation of Effect of Propolis on MC3T3-E1 Osteoblasts: A Preliminary Study

Propolisin MC3T3-E1 Osteoblastları Üzerine Olan Etkisinin Değerlendirilmesi: Öncü Çalışma

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ABSTRACT Objective: Propolis has been used for centuries by many cultures for its antiseptic, antimicrobial, and detoxifying properties. It is a sticky substance that bees make from tree and other plant resins. Propolis contains flavonoid compounds known for their anti-inflammatory and antioxidant activity as well as tissue strengthening and regenerative effects. The osteoblastic cell line MC3T3-E1 has been established from a mouse calvaria. This cell line is stated to be a useful model for examining the process of osteoblastic development in vitro. In this study we aimed to evaluate effect of propolis on osteoblast cells. **Material and Methods:** Propolis was prepared at three concentrations as follows: 5%, 10%, and 20%. The effect of propolis on MC3T3-E1 osteoblasts was determined by 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) and flow cytometry analysis. **Results:** Results were evaluated at 24h and 72h. At 24 h propolis showed proliferative effect, among solutions 5% propolis extract was found the most proliferative solution. On the other hand, at 72h, all concentrations showed cytotoxic effect, whereas 5% propolis showed minimum cytotoxicity even than the control group. According to flow cytometry analysis, 5% propolis showed lesser apoptosis compared to control group and other test solutions. **Conclusion:** These findings indicate that propolis has proliferative effect on osteoblasts in short term, on the contrary it showed cytotoxic effect at long term period.

Key Words: Propolis; osteoblasts; cell culture techniques

ÖZET Amaç: Propolis yüzyıllardır antiseptik, antimikrobiyal ve detoksife özelliklerinden dolayı kullanılmaktadır. Arıların ağaç ve diğer bitki rezinlerinden elde ettiği yapışkan bir maddedir. Propolis doku direncini ve rejeneratif etkinliği arttıran antiinflamatuar ve antioksidan özelliklere sahip flavonoidleri içerir. Çalışmada kullanılan MC3T3-E1 hücre hattı fare kalvaryasından elde edilmiştir. Bu hücre hattının in vitro osteoblastik çalışmalarda oldukça kullanışlı olduğu ifade edilmektedir. Çalışmamızda propolisin osteoblast hücre hattı üzerine etkisi incelenmiştir. **Gereç ve Yöntemler:** %5, %10 ve %25 olmak üzere üç farklı konsantrasyonda Propolis solüsyonu hazırlandı. Propolisin MC3T3-E1 osteoblastları üzerine olan etkisi 3-(4,5-dimetil-thiazoil)-2,5-difenil-SH-tetrazolium bromid (MTT) ve akım sitometri analizi ile değerlendirildi. **Bulgular:** Analiz sonuçları 24. ve 72. saatlerde değerlendirildi. 24. saatte propolis proliferatif etki gösterdi, %5'lik solüsyon diğer solüsyonlar arasında en proliferatif etkiyi göstermiştir. Öte yandan 72. saatte tüm konsantrasyonlar sitotoksik etki gösterirken, %5'lik propolis solüsyonu minimum sitotoksik etki göstermiştir. Akım sitometri analizine göre %5 propolis solüsyonu diğer solüsyonlar arasında en az apoptozise neden olmuştur. **Sonuç:** Bu sonuçlara göre kısa dönemde propolis proliferatif etki gösterirken uzun dönemde sitotoksik etki göstermektedir.

Anahtar Kelimeler: Propolis; osteoblastlar; hücre kültürü teknikleri

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Propolis is a strongly adhesive, resinous substance collected, transformed, and used by bees to seal holes in honeycombs, smooth out internal walls, and protect the entrance against intruders.[1] Propolis

contains considerable amounts of polyphenol substances such as quercetin, caffeic acid and derivatives, pinocembrin, galangin, etc., which may act as potent antioxidants.[2,3]

Modern herbalists recommend propolis for its anti-bacterial, anti-fungal, anti-viral, hepatoprotective and anti-inflammatory properties, to increase the body's natural resistance to infections and to treat gastroduodenal ulcers. Applied externally, propolis relieves various types of dermatitis caused by bacteria and fungi.

The osteoblastic cell line MC3T3-E1 was established from C57BL/6 mouse calvaria and selected based on high alkaline phosphatase (ALP) activity in the resting state. The cells can differentiate into osteoblasts and osteocytes and have been demonstrated to form calcified bone tissue *in vitro*. Mineral deposits have been identified as hydroxyapatite. MC3T3-E1 cells exhibit properties of osteoprogenitor cells and preosteoblasts in the active growth stage; once the cells stop growing, they differentiate to develop markers of mature osteoblasts, including expressing high levels of ALP and forming mineralized bone matrix.[4-7] Thus, this cell line seems to be a useful model for examining the process of osteoblastic development *in vitro*.[7]

The aim of the present study was to determine the effect of propolis on MC3T3-E1 osteoblast cultures and to investigate the appropriate concentration of propolis for an osteoblastic-like cell line. Thus, our findings would be beneficial for further studies on the potential roles of propolis on bone tissue.

MATERIAL AND METHODS

SOURCE OF PROPOLIS

The propolis samples were produced by honeybees (*Apis mellifera L.*) in the region of Yomra, Trabzon, Turkey, rich in *Picea orientalis*, *Fagus orientalis*, *Castanea sativa*, *Rhododendron ponticum*, *Rhododendron luteum*, and *Rubus caucasicus*.[8] The samples were provided by the Trabzon Agricultural Development Cooperative.

PREPARATION OF DIMETHYL SULFOXIDE EXTRACTS OF PROPOLIS

In preexperimental studies, alcoholic extracts showed cytotoxicity for cancer as well as control cell lines. Therefore, we used an alternative extraction procedure with dimethyl sulfoxide (DMSO) as the solvent. Each natural propolis sample was ground (Retsch, ZM 200) and bottled in 10 g portions. Portions were dissolved in 5 ml DMSO by continuous mixing for 72 h, and 60% ethanol was added to achieve 50 ml solution. Following this procedure, the solution was filtered. The filtered solution was maintained at 60°C in a water bath for 2 h to remove alcohol. Final volumes were completed to 50 ml using deionized water to give a 300 mg/ml stock concentration of propolis extracts. Working solutions at concentrations of 50 (5%), 100 (10%), and 200 (20%) mg/ml were then prepared.

CELL CULTURE

Murine osteoblastic MC3T3-E1 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in a 37°C incubator with humidified air and 5% CO_2 atmosphere with a modified minimal essential medium (a-MEM; Gibco BRL, Grand Island, NY). Unless otherwise specified, the medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. When cells reached confluence, they were harvested using a 0.05% trypsin-0.02% EDTA solution. The cells were then seeded in 6-well plates, grown to 90% confluence, and treated with culture medium containing 10 mM β-glycerophosphate and 50 mg/mL ascorbic acid to initiate *in vitro* mineralization. Cell culture medium was changed every 2-3 d. After 6 d or 14 d, to measure cell viability, alkaline phosphatase activity, and collagen content, or calcium deposition and osteoprotegerin secretion, the cells were cultured with medium containing dRib and/or kaempferol for 2 d.[5,6] Kaempferol was purchased from Wako Pure Chemicals (Osaka, Japan).

MTT ASSAY

The 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) assay was performed.[6,7] Target cells were resuspended in medium at 1x105

cells/mL, and 100 µL of 105 cells/mL cell suspension were distributed in each well of the 96-well plates and allowed to adhere for 24 h. Wells containing 200 µL medium alone without cells and reagents were used as the negative controls. After treatment for the stated incubation doses of propolis extracts, and for the stated times, 20 µL MTT solution (5 mg/mL) was added to each well, and the microplates were further incubated at 37 °C for 4 h. The unreactive supernatants in the well were discarded, and 100 µL acidified isopropanol (0.04 N HCl in isopropanol) was added to the cultures and mixed thoroughly to dissolve the dark-blue crystals of formazan. The absorbance values of each well were determined with a microplate enzyme-linked immunoassay reader equipped with a 570 nm filter. The negative control well was used for the baseline zero absorbance.

The results are presented as the percentage viability, determined as (1-absorbance of the experimental well/absorbance of the positive control well) x100. Each experiment was repeated three times.

FLOW CYTOMETRY

Apoptosis analysis: Cells were plated at a density of 5×10^4 cells/well into 6-well plates. Apoptosis of the MC3T3-E1 cells was evaluated with flow cytometry. Cells were collected and detected using the annexin V-FITC/PI apoptosis detection kit (Molecular Probes, City, State) as described by Xu et al.[9] Briefly, cells were collected and resuspended in 1xcold binding buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 2.5 mM CaCl2, 1 mM MgCl2, 4% BSA) for analysis. Cells were also stained with PI to detect late apoptosis cells. Ten thousand cells were subjected to flow cytometric analysis.

STATISTICAL ANALYSIS

MTT assay and flow cytometry analysis were done at 24 h and 72 h. Each experiment was repeated independently three times. Analyses of variance (one-way ANOVA) were performed using SPSS 17.0. A P value less than 0.05 was considered significant.

RESULTS

EFFECT OF PROPOLIS ON MC3T3-E1 CELLS

At 24 h, the proliferation rate of the cells exposed to 5% propolis was significantly higher than that of cells exposed to 10% and 20% propolis. The proliferative effect of propolis was observed at all doses: 5% propolis (386%), 10% propolis (217%), and 20% propolis (105%). The proliferation data showed that there was a statistically significant difference among the groups ($p<0.05$) (Figure 1).

At the 72 h time period, all propolis solutions showed cytotoxic effects as follows: 5% propolis showed 10%, 10% propolis showed 22%, and 20% propolis 32%, respectively (Figure 2). With flow cytometry, apoptosis was observed as follows: 5% propolis showed 0.8%, 10% propolis showed 28.1%, 20% propolis showed 34.7%, and the control group showed 7.4%. According to these results, the best condition was achieved by using 5% propolis solution for 24 h and 72 h (Figure 3).

EFFECTS OF PROPOLIS ON APOPTOSIS OF MC3T3-E1 CELLS

There was no PI positive group, which means apoptosis was not seen with the flow cytometer at 24 h.

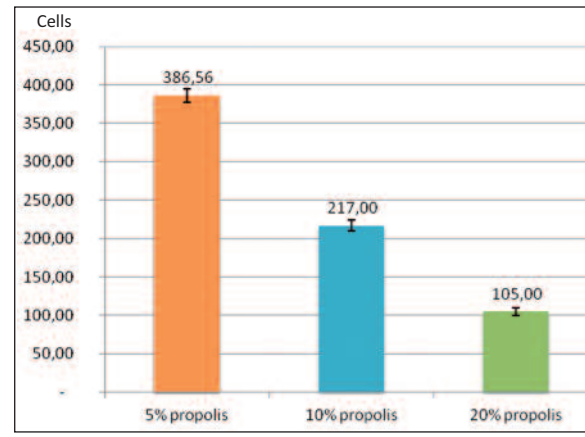

FIGURE 1: Proliferation of MC3T3-E1 cells assessed by MTT analysis at 24h. MC3T3-E1 cells exposed to 5, 10, 20 % propolis solutions. The effect of propolis on cell proliferation was assesed by MTT assay at 24 h. Data was expressed as the mean of triplicate experiments (n=3). At 24h proliferative action of cells exposed to 5% propolis was significantly better than other concentrations.
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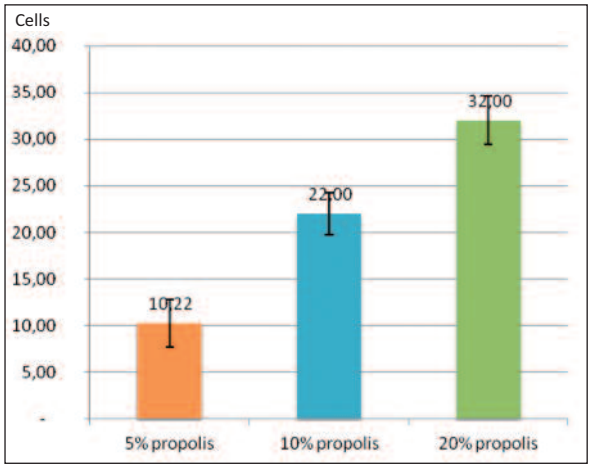

FIGURE 2: Cytotoxicity MC3T3-E1 cells assesed by MTT analysis at 72h. MC3T3-E1 cells exposed to 5, 10, 20 % propolis solutions. The effect of propolis on cell cytotoxicity was assesed by MTT assay at 72 h. Data were expressed as the mean of triplicate experiments (n=3). At 72h all experimental solutions showed cytotoxic effect, where as 5% propolis was least cytotoxic. (See color figure at http://www.turkiyeklinikleri.com/journal/dis-hekimligi-bilimleri-dergisi/1300-7734/)

The number of apoptotic cells (including early apoptotic and late apoptotic cells) on day 3 was significantly higher than that on day 1 ($p<0.05$) (Figure 4).

DISCUSSION

In the dental literature, the effect of propolis on periodontal ligament cells has been studied generally.[10-17] These studies revealed propolis's favorable effects on periodontal ligament (PDL) cells. Due to these results, researchers proposed using propolis as a storage medium following avulsion. Only one study examined human cartilage and condrocytes.[18]

Osteoblasts are the bone-forming cells of the skeleton. MC3T3-E1 cells, an osteoblast-like cell line that retains various osteoblastic cell characteristics, may provide a very useful system for studies of differentiation of bone cells and the mechanisms of bone regeneration.[4,19] Thus, this cell line is a well-accepted model of osteogenesis *in vitro*. Li et al. investigated echinacoside's bioactivities on the proliferation, differentiation, and mineralization of the osteoblastic cell line MC3T3-E1.[20] Their results showed that echinacoside caused a significant increase in cell proliferation, ALP activity, collagen I content, osteocalcin levels, and mineralization in osteoblasts, suggesting that echinacoside has a stimulatory effect on osteoblastic bone formation or has potential activity against osteoporosis. Chen et al. investigated the beneficial effects of dietary genistein on MC3T3-E1 cells.[21] They suggested the beneficial skeletal effects of genistein, as levels achievable with diet were obtained. Genistein inhibited interleukin-6 synthesis in osteoblastic

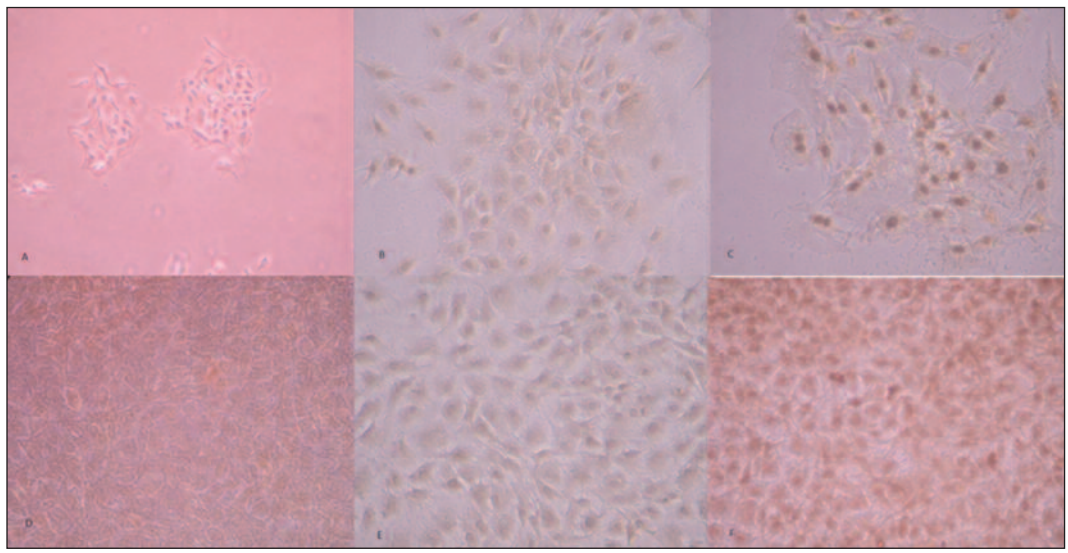

FIGURE 3: Images of culture flasks under inverted microscope. A- 5% propolis solution at 24 h (X40 magnification); **B-** 10% propolis solution at 24 h (X100); **C-** 20% propolis solution at 24 h (X100); **D-** 5% propolis solution at 72 h (X20); **E-** 10% propolis solution at 72 h (X100); **F-** 20% propolis solution at 72 h (X100). (See color figure at http://www.turkiyeklinikleri.com/journal/dis-hekimligi-bilimleri-dergisi/1300-7734/)

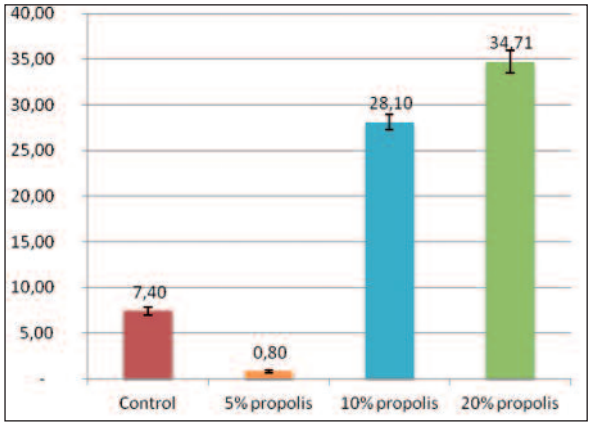

FIGURE 4: Apoptosis of MC3T3-E1 cells assesed by flow cytometery at 72h. MC3T3-E1 cells exposed to 5, 10, 20 % propolis solutions. Cells at 72h were stained with PI and analyzed by flow cytometry. Data was expressed as the mean of triplicate experiments (n=3). Best result was obtained by 5% propolis solution according to control group. This means that 5% propolis has protective effect on cells.
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MC3T3-E1 cells. Xu et al. investigated whether and to what extent low-dose irradiation affected the proliferation, differentiation, and mineralization of osteoblasts *in vitro*.[22] They concluded that the effects of low-dose irradiation on osteoblast proliferation and differentiation differ from those of high-dose X-irradiation, which suggested that low-dose irradiation could promote fracture healing through enhancing the differentiation and mineralization of osteoblasts.

Until now, the effect of propolis on osteoblast cells *in vitro* has not been evaluated. In this study, the cytotoxic effect of propolis on osteoblast cells was examined *in vitro* at two time intervals. Results were assessed with MTT and flow cytometry assays. Propolis solutions showed a proliferative effect at 24 h, whereas at 72 h a cytotoxic effect was observed. All propolis solutions showed a proliferative effect; among them, 5% propolis had the best results. In contrast, favorable results were not obtained for a long time period as the cytotoxic effect occurred for all test solutions, whereas 5% propolis showed lower cytotoxicity than even the control group.

The plant species available in a geographic area determine the amounts of important compounds present in propolis.[23] A study of New Zealand propolis revealed that important dihydroflavonoids, pinobanksin and pinocembrin, made up approximately 70% of the flavonoids in the samples analyzed. A similar study of Brazilian, Uruguayan, and Chinese samples showed dihydroflavonoids comprise less than 10% in all but one sample (which had 50%). Turkish *C. sativa propolis* mainly contains important flavonoids (31.80%) such as galangin, quercetin, kaempferol, apigenin, pinobanksin, pinocembrin, and pinostobin. These flavonoids are very important in herbal medicine.[24] The flavonoids concentrated in propolis are powerful antioxidants, and they have been shown to scavenge free radicals and thus protect against lipid peroxidation in the cell membrane. Cell protective effects may contribute to the flavonoid content of propolis.

Propolis has numerous constituents. Here, propolis's total effect was investigated; the constituents were not studied separately. The mechanism of action is beyond the scope of this study; thus, we cannot explain how to show these effects. Further detailed studies will clarify this question.

The present *in vitro* study showed that propolis extract provides a proliferative effect on mouse MC3T3-C1 cells for 24 h and minimum cytotoxicity for 72 h for the 50 mg/ml propolis solution. During a short time period, dose-dependent proliferation was observed, whereas a cytotoxic effect was observed for a longer time period.

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