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BASIC MEDICAL SCIENCES / TEMEL TIP BİLİMLERİ

Baicalein Has Protective Effect in H_2O_2-induced L929 Cell Damage

Baicalein, H_2O_2 Kaynaklı L929 Hücre Hasarında Koruyucu Etkiye Sahiptir

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Abstract

Objectives: In this study, it was aimed to investigate the possible protective effects of baicalein in H_2O_2-induced L929 fibroblast cell model by determining the parameters of oxidative stress (SOD) and inflammation process (TNF-alpha, TGF-beta, IL-10).

Materials and Methods: Baicalein was applied at different doses (200, 100 and 80, µM) to L929 fibroblast cells. After 23 hours of incubation, 1 mM H_2O_2 was added and cell viability was test by CVDK8. Then, fluorescent diacetate (FDA)-propidium iodide (PI) staining was performed to determine the ratio of live and dead cells. The levels of TNF-alpha, IL10, TGF-beta and the activity of SOD were determined by ELISA.

Results: After 1 h incubation with 1 mM H_2O_2, the viability decreased by approximately 77%. It was observed that cell viability increased the most in the B100 group, with/without H_2O_2. The cell viability increased after 24 h of baicalein and H_2O_2 co-incubation. The 100 µM baicalein application reduced the damage of H_2O_2 up to 63%. When the levels of TNF-alpha were examined, a significant increase was observed in the H_2O_2-damage group compared to control group. Additionally, the levels of IL-10 and TGF-beta increased in H_2O_2-damage group compared to control group; however, the levels of those cytokines were decreased in baicalein application groups.

Conclusion: Pretreatment with baicalein (especially 200 µM and 100 µM) results in significant findings including the suppression of inflammatory cytokines, TNF-alpha, TGF-beta, IL-10 and increase of SOD parameter, SOD, in L929 cells. Taking everything into account, baicalein which is a natural product may be an alternative therapeutic option for prevention of cellular SOD and inflammatory processes. To better understand the potential beneficial effects of baicalein, molecular mechanisms and cellular targets should be investigated.

Key Words: Baicalein, Hydrogen Peroxide, Inflammation, Oxidative Stress, L929

Öz

Amaç: Mevcut çalışmada, Baicalein'in H_2O_2 ile indüklenmiş L929 fibroblast hücre hasarı modelinde oksidatif stres (SOD) ve enflamasyon süreci (TNF-alfa, TGF-beta, IL-10) parametreleri üzerine olan koruyucu etkilerinin araştırılması amaçlanmıştır.

Gereç ve Yöntem: L929 fibroblast hücrelerine farklı dozlarda (200, 100 ve 80 µM) Baicalein uygulandı. Yirmi üç saatlik inkübasyonun ardından 1 mM H_2O_2 eklendi ve hücre canlılığı CVDK8 ile test edildi. Daha sonra canlı ve ölü hücrelerin oranını belirlemek için floresan diasetat (FDA)-propidyum iyodür (PI) boyaması yapıldı. TNF-alfa, IL10, TGF-beta seviyeleri ve SOD aktivitesi ELISA ile belirlendi.

Bulgular: 1 mM H_2O_2 ile 1 saatlik inkübasyondan sonra hücre canlılığı yaklaşık %77 oranında azaldı. Hasarsız ya da H_2O_2 hasarlı gruplarda, hücre canlılığında artışın en fazla B100 grubunda olduğu görüldü. Baicalein ve H_2O_2'nin birlikte inkübasyonundan 24 saat sonra hücre canlılığının arttığı tespit edildi. 100 µM Baicalein uygulaması, H_2O_2'nin hücreleri öldürme oranını %63'e kadar azalttı. TNF-alfa düzeyleri incelendiğinde H_2O_2-hasarlı grupta kontrol grubuna göre anlamlı artış gözlendi. Ek olarak, IL-10 ve TGF-beta seviyeleri H_2O_2-hasarlı grupta kontrol grubuna göre arttı; ancak bu sitokinlerin seviyeleri Baicalein uygulama gruplarında azaldı.

Sonuç: Baicalein (özellikle 200 µM ve 100µM) uygulaması sonucunda L929 hücrelerinde enflamatuvar sitokinler olan TNF-alfa, TGF-beta, IL-10'un baskılanması ve SOD parametresi olan SOD'nin artması gibi önemli bulgular elde edilmiştir. Tüm veriler ışığında, doğal bir etken madde olan baicalein, hücresel SOD ve enflamatuvar süreçlerin önlenmesi için alternatif bir tedavi seçeneği potansiyeline sahiptir. Baicalein'in potansiyel yararlı etkilerini daha iyi anlamak için moleküler mekanizmalar ve hücresel hedeflerin araştırılmasına yönelik yeni çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: Baicalein, Hidrojen Peroksit, Enflamasyon, Oksidatif Stres, L929

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Introduction

Wound may be the result of an accident; however, it can also occur intentionally for reasons such as surgery. Wound healing following trauma is described as the normalization of cellular, biochemical and systemic processes with the formation of new tissue. It is also an important physiological process that preserves the integrity of the tissue (1). Wound healing consists of three phases; (i) the hemostasis/inflammation phase, (ii) the proliferation phase, and (iii) the remodeling phase (2). Chronic and difficult to heal wounds causes a significant economic burden because of reducing the patient's quality of life (3).

There are many factors that affect wound healing such as infection, oxygenation defects, stress, aging, and smoking. At low concentrations, reactive oxygen species (ROS) are involved in the wound healing process, defense against invading microorganisms, and signals necessary for cell survival. Production of high concentrations of ROS or impaired ROS detoxification is the main cause of chronic non-healing wounds (4). One of the causes of cellular oxidative stress (SOD) is the overproduction of hydrogen peroxide, a kind of ROS. Reducing oxidative balance is very important for the functioning of fibroblasts (5). On the other hand, the inflammatory response that begins after tissue injury accompany with the growth factor, cytokine, and chemokine expressions and those are necessary for wound healing. In the early phase of wound healing, cytokines and chemokines such as interleukine-1-alpha (IL-1α), IL-1-β, transforming growth factor-β (TGF-β), vascular endothelial growth factor, tumor necrosis factor alpha (TNF-α) and IL-8 are substantial and play important roles (6).

Scutellaria baicalensis Georgi. (Lamiaceae), contains baicalein as a major component, is a flowering plant species and its dried roots are used in traditional Chinese medicine for the treatment of cold, diarrhea, dysentery, hypertension, bleeding, insomnia, inflammation and respiratory tract infections (7). Baicalein has lots of beneficial effects including anti-oxidant, anti-inflammatory, anti-cancer, cardioprotective, neuroprotective, and hepatoprotective properties (8-10).

Fibroblasts are one of the key factors in wound healing and remain at the wound site until epithelialization occurs. First, they migrate to the wound site, then proliferate there, after that promote normal wound healing to form new extracellular matrix, and finally induces collagen structures to support other cells associated with effective wound healing (11).

In this study, it was aimed to investigate the possible protective effects of baicalein in H_2O_2-induced L929 fibroblast cell model by determining the parameters of SOD and inflammation process (TNF-alpha, TGF-beta, IL-10).

Materials and Methods

Cell Culture and Viability Test

The mouse fibroblast cell line L929 was purchased from American Type Culture Collection (ATCC, USA). It was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific), 1% antibiotic (Penicillin, Streptomycin, Amphotericin, Gibco, Thermo Fisher Scientific), under 95% humidity and 5% CO_2, and 37 °C temperature. Baicalein (MedChemExpress, Cat.No: HY-N0196), dissolved in 10% DMSO.

Baicalein Administration

For determine the baicalein doses, L929 cells incubated with varying doses (800 µM, 400 µM, 200 µM, 100 µM, 80 µM and 40 µM, 20 µM) for 24 hours. It was observed that the 800 µM dose reduced the viability by 26% and the 400 µM dose by 17% and 20 µM dose by 13%. The 40 µM dose increased the viability by only 5%. Therefore, these doses were not included in the experiment. 200 µM (B200), 100 µM (B100), 80 µM (B80) doses were used in the experiment.

H_2O_2 Administration

L929 cells were incubated for 24 hours with varying doses of H_2O_2 (1 mM, 0.5 mM, 0.4 mM, 0.3 mM, 0.2 mM, 0.1 mM) while determining H_2O_2 damage. It was observed that cell viability decreased by 77% at 1 mM, 78% at 0.5 mM, 78% at 0.4 mM, 83% at 0.2 mM and 88% at 0.1 mM. So, 1 mM dose was chosen for the experiment. The groups with H_2O_2 were named as H+B200, H+B100 and H+B80.

CVDK-8 Cell Viability Test

Cell viability test was performed to see the effect of baicalein on cell proliferation. Cells were first seeded into 96-well plates, 2 µL of baicalein was applied at the specified doses (200 µM, 100 µM and 80 µM) after 70% confluence was detected. Then, 1 mM hydrogen peroxide (H_2O_2) was applied to related wells 23 hours after baicalein application. After 1 hour, 10 µL of CVDK-8 was added to each well. It was incubated at 37 °C for 4 hours for the formation of formazan crystals and the optical density (OD) value was detected with a spectrophotometer (Thermo, MultiSkan GO) at a wavelength of 450 nm.

Semi-Quantitative ELISA Technic

Cells ($2x10^4$) were seeded 48-well plates in 200 µL medium for each well plate. The medium was collected 24 hours after the H_2O_2 damage and the addition of the specified doses of baicalein. In this study, we aimed to measure the levels of following parameters; TGF-beta ELISA Kit (BT Laboratory, Shanghai, China), TNF-alpha ELISA Kit (BT Laboratory, Shanghai,

China), IL-10 ELISA Kit (BT Laboratory, Shanghai, China), SOD ELISA Kit (BT Laboratory, Shanghai, China). The ELISA technic was applied according to the manufacturer's protocol. At the end of the experiments, the microplate was read at a wavelength of 450 nm with ELISA reader (Thermo, MultiSkan GO). The OD values were noted and the calculations were performed.

Immunofluorescence Assay

Cells were seeded 24-well plates in 400 µL medium for each well plate. After the H_2O_2 and baicalein co-incubation, the medium was removed and washed twice with PBS. For fixation, 400 mL of 4% formaldehyde was added to per well and after 4 minutes formaldehyde was removed and cells were washed with PBS. For permeabilization of cells, they were incubated with 99.9% methanol for 20 minutes at room temperature and then washed with PBS. Five ml each of fluorescent diacetate (FDA, ThermoFisher, Cat.No: F1303) and propidium iodide (PI, ThermoFisher, Cat.No: P1304MP) were added to per well, visualized with an inverted microscope (Invitrogen Evos FL) with fluorescence attachment after 10 minutes of incubation.

Statistical Analysis

The results were expressed as means ± SEM. Statistical significance was evaluated by One-Way ANOVA followed by Tukey post-test. All data were analyzed using GraphPad Prism, version 5.0 for Windows (Graph Pad Software, San Diego, California, USA). The probability level of $p<0.05$ was considered statistically significant.

Results

Baicalein Increased Cell Viability with/without H_2O_2-Induced

To test whether baicalein has a cytotoxic effect on healthy L929 fibroblast cells, a viability test was performed. It was observed that high doses of baicalein (200 µM, 100 µM, 80 µM) did not decrease cell viability, on the contrary, it induced cell proliferation (18%, 21%, 13%, respectively). The highest cell proliferation was observed at the dose of 100 µM.

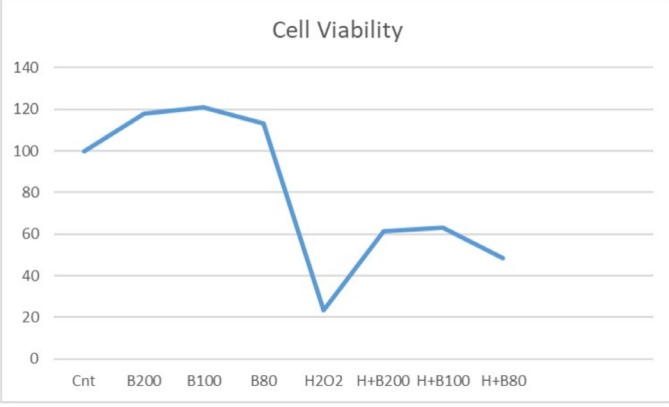

Figure 1: The cell viability assay of experimental groups. The viability of the control group was accepted as 100% and the averages of the experimental groups were calculated

Cnt: Control

After 1 h incubation with 1 mM H_2O_2, the viability decreased by approximately 77%. The cell viability increased after 24 hours of baicalein H_2O_2 co-incubation in all doses especially 100 µM. The 100 µM baicalein application reduced the damage of H_2O_2 up to 63% (Figure 1).

Baicalein Had Anti-Apoptotic Effect on H_2O_2 Damaged Cells

One hundred and ninety-six immunofluorescence assay (IFA) findings to show the anti-proliferative and anti-apoptotic effects of baicalein on L929 cells were similar with the cell viability assay findings. Baicalein given to healthy cells did not seem to decrease cell viability (Figure 2). It was observed that the number of apoptotic cells increased in H_2O_2 damaged group. The number of apoptotic cells decreased due to the increasing dose of baicalein, and the groups with the highest number of viable cells were 200 µM and 100 µM doses (Figure 3). Baicalein has been shown to have an anti-apoptotic effect with/without H_2O_2 in a dose-dependent manner.

Baicalein Effects the Levels of Inflammatory Cytokine

When the levels of TNF-alpha, a pro-inflammatory cytokine, were examined, a significant increase was observed in the H_2O_2 damage group compared to control group. Application of baicalein reduced H_2O_2 damage (Figure 4). Additionally, the

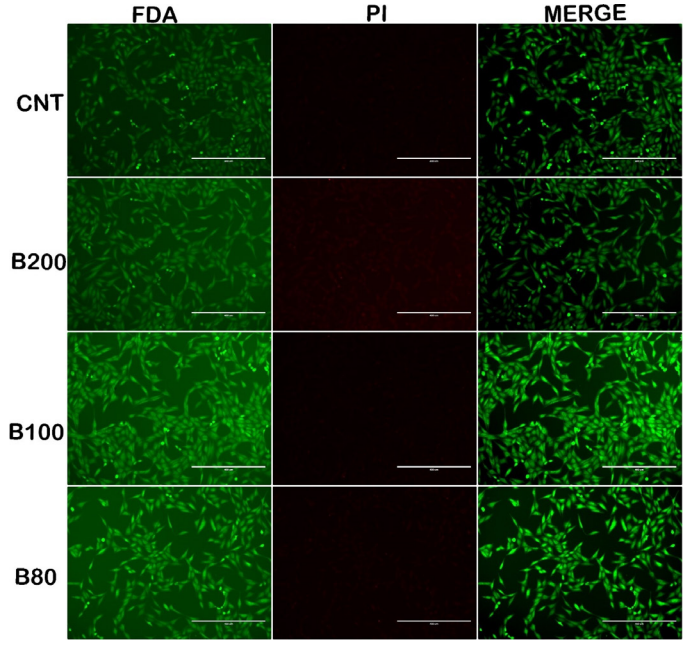

Figure 2: FDA and PI immunofluorescence labeling in L929 cells with baicalein. Red cells (PI positive) indicate dead cell, green cells (FDA positive) indicate live cells

FDA: Fluorescent diacetate, PI: Propidium iodide, CNT: Control

levels of IL-10 and TGF-beta increased in H_2O_2 damage group compared to control group; however, the levels of those anti-inflammatory cytokines were decreased in the baicalein application groups (Figure 5). The activity of SOD enzyme showed a significant decrease in H_2O_2 damage group compared to control. An increase in the activity of SOD enzyme was observed in H_2O_2+baicalein application groups. In B200, B100, and B80 groups, the activity of SOD enzyme increased when compared to H_2O_2 group (Figure 6).

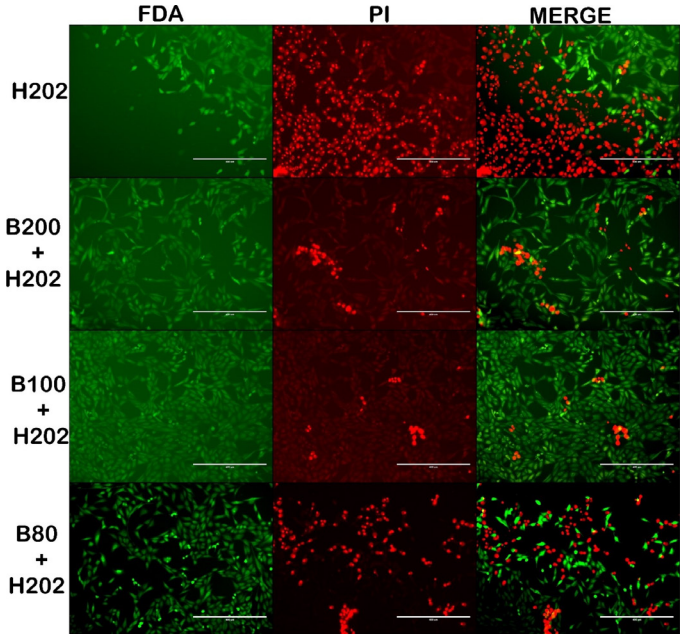

Figure 3: FDA and PI immunofluorescence labeling in L929 cells with H_2O_2 and baicalein. Red cells (PI positive) indicate dead cell, green cells (FDA positive) indicate live cells

PI: Propidium iodide, FDA: Fluorescent diacetate

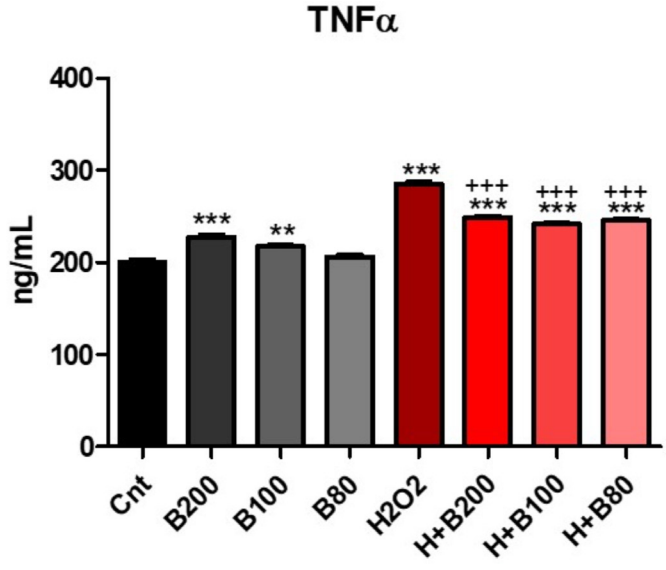

Figure 4: The level of pro-inflammatory cytokine

Control versus all experimental groups; + H_2O_2 versus all experimental groups; +,=p<0.05, ++,**=p<0.01, +++,***=p<0.001

TNFa: Tumor necrosis factor alpha, Cnt: Control

Discussion

To our knowledge, it was shown for the first time that baicalein has a protective effect on H_2O_2-induced L929 cell damage by effecting two important cellular process: SOD and inflammation. In current literature, it is well-known that the anti-inflammatory, antioxidant, anti-angiogenesis, and immunomodulatory effects of baicalein have been proven (12). A study reported that baicalein decreased cell viability in human lung fibroblast MRC-5 cell line (13). In this study, baicalein increased cell viability in L929 fibroblast cells, while it was shown that cell viability decreased in H_2O_2 damage group (5,14,15).

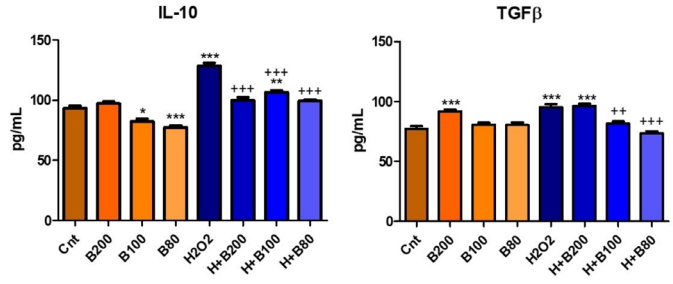

Figure 5: The levels of anti-inflammatory cytokines

Control versus all experimental groups; + H_2O_2 versus all experimental groups; +,=p<0.05, ++,**= p<0.01, +++,***= p<0.001

IL-10: Interleukin 10, TGFB: The transforming growth factor beta, Cnt: Control

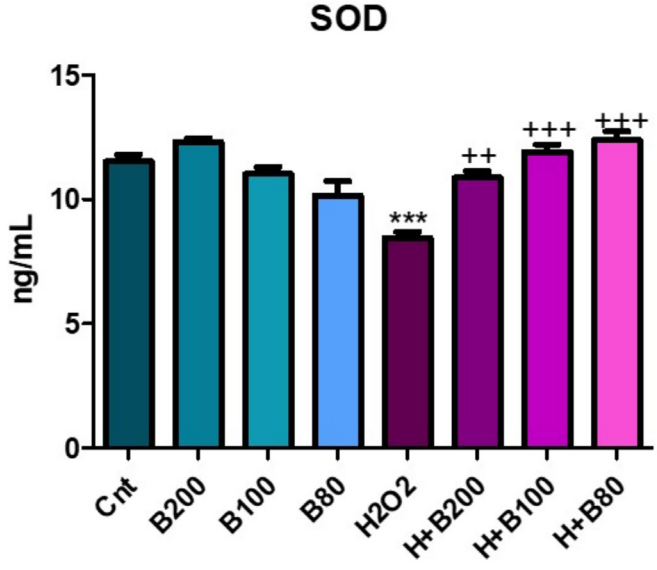

Figure 6: The activity of SOD enzyme

Control versus all experimental groups; + H_2O_2 versus all experimental groups; +,=p<0.05, ++,**=p<0.01, +++, ***=p<0.001

SOD: Superoxide dismutase, Cnt: Control

IFA findings were compatible with the cell viability assay findings. The ratios of FDA, which gives green fluorescent color to living cells, and PI to DNA helix of dead cells, have shown that baicalein has no toxic effects on healthy cells. A study reported similar findings with our study that baicalein did not cause death in Chinese hamster lung fibroblast cells (V79-4) (16). After H_2O_2 penetrates the cells, it produces a highly reactive hydroxyl radical that attacks cellular components including lipids, proteins and DNA (17,18). Membrane lipid peroxidation induced by H_2O_2 is one of the most important damages responsible for the loss of cell viability. It was detected that there were less death cells in H_2O_2 + baicalein treatment groups when compared to control. Consistent with the literature, it can be said that baicalein has a protective effect on H_2O_2 damaged cells (17,19).

SOD is one of the important factors inducing organ damage and associated with over-released of reactive oxygen species such as hydrogen peroxide (20). On the other hand, SOD is an important reactive oxygen species (ROS) scavenger, which can transform superoxide radicals into H_2O_2 and prevent ROS attack to important organs (21). It is known that SOD has therapeutic effects in physiological and pathological cellular conditions including inflammatory diseases, cancer, and neurodegenerative diseases (22). In our study, it was observed that high-dose baicalein application increased the SOD level, and low-dose baicalein slightly decreased the activity of SOD enzyme in related experiment groups. Tan et al. (23) performed a study and reported that pretreatment of baicalein increased the activity of SOD. Additionally, Ye et al. (24) also reported similar findings that baicalein activated apoptosis through induced intracellular ROS generation, and SOD apparently inhibited intracellular ROS production. These information shows us that baicalein has a potential to be effective on cellular SOD parameters.

On the other hand, all kinds of cellular damage including H_2O_2 can cause an inflammation in tissue and cytokine release. In our study, H_2O_2 caused an increase in the levels of TNF-alpha, TGF-beta, and IL-10; while different doses of baicalein application decreased the levels of mentioned cytokines. In current literature, it can be seen that baicalein has anti-inflammatory effects of some experimental models. Lin et al. (25) reported that TNF-alpha and IL-6 were suppressed by baicalein pretreatment; however, IL-10 level was significantly elevated by baicalein in contrast to our study. Generally, studies in literature support the current data about the inflammatory cytokine levels (26-28).

Study Limitations

The study have some limitations. First, I should have used more than one cell culture to compare the exact effects of baicalein on hydrogen-peroxidase damage. Second, I should have tried to determine more inflammatory and oxidative stress parameters. For example, to better explain the anti-inflammatory and anti-oxidant effects, additional parameters including catalase (CAT), lipid peroxidase (LPO), Malondialdehyde (MDA), Interleukin-1-beta (IL-1-B), Interleukin-6 (IL-6), Interleukin-17 (IL-17), and Interleukin-22 (IL-22) should have been determined. Third, seconder cellular signaling pathways related with the inflammation and oxidative stress processes should have been checked.

Conclusion

As a conclusion, pretreatment with baicalein results in significant findings including the suppression of inflammatory cytokines, TNF-alpha, TGF-beta, IL-10 and increase of SOD parameter, SOD, in L929 cells. Taking everything into account, baicalein which is a natural product may be an alternative therapeutic option for prevention of cellular SOD and inflammatory processes. To better understand the potential beneficial effects of baicalein, molecular mechanisms and cellular targets should be investigated.

Ethics

Ethics Committee Approval: This study is a cell culture study and therefore ethics committee approval was not obtained.

Informed Consent: Cell culture study.

Peer-reviewed: Externally peer-reviewed.

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