

# Effect of whey protein derivatives on cell viability, cell migration and cell cycle phases in MCF-7 cells

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## ABSTRACT

**Objective:** This study aimed to obtain protein derivatives after treatment of whey proteins with hazelnut oil and olive oil and determined their effects on MCF-7 cells.

**Materials and Methods:** Whey proteins obtained from 6% whey powder were treated with hazelnut oil (HO) and olive oil (OO) at a protein to lipid ratio of 1:10 at 60°C for 120 minutes. The protein derivatives formed with whey protein and HO or OO were applied to MCF-7 cancer cells and healthy fibroblasts. The effects of protein derivatives on cell viability, apoptosis, reactive oxygen species (ROS) production, wound healing, cell cycle phase distribution and cell cycle related proteins Akt and p21(Waf1/Cip1) expressions were investigated.

**Results:** Cell viability decreased significantly after 24 h of incubation with WP:OO. The percentage of apoptotic or necrotic cells varied between 5-10% and no statistically significant effect was observed. There was no statistically significant difference in ROS production and colony formation between controls and WP:HO or WP:OO groups. Treatment of cells with WP:OO for 24 h significantly decreased cell migration compared to the control group. G2/M phase was significantly suppressed in WP:OO group compared to the control group. WP:OO also increased the expression of p21(Waf1/Cip1) significantly when compared with the control group.

**Conclusion:** Our results showed that whey protein derivatives applied to MCF-7 cells are cytotoxic and may be useful in breast cancer treatment.

**Keywords:** Whey proteins, Oleic acid, Cell survival, Apoptosis, Cell cycle

## 1. INTRODUCTION

The remaining liquid after precipitation of casein in cheese production is called whey or milk serum. This by-product contains soluble proteins that are grouped into major and minor fractions [1,2]. Major whey proteins are  $\alpha$ -lactalbumin ( $\alpha$ -La, 20%),  $\beta$ -lactoglobulin ( $\beta$ -Lg, 50%), serum albumin (10%), immunoglobulins (10%) and proteose-peptones (10%). Among minor proteins are lactoperoxidase, lactoferrin, insulin-like growth factor and  $\gamma$ -globulins [3,4]. Whey proteins are important in terms of both their biological value and their high content of sulfur-containing amino acids [5,6].

The human  $\alpha$ -La and oleic acid (OA) complex called human alpha-lactalbumin made lethal to tumor cells (HAMLET) exhibits remarkable apoptotic activity [7-9]. A similar complex BAMLET, which is made with bovine  $\alpha$ -La and oleic acid, can

induce cell death in transformed cells [10, 11] and it has been used for the treatment of skin papilloma [12] and bladder cancer [13]. It was also reported that the lactoferrin-oleic acid complex showed similar apoptotic activity on cancer cells [14].

HAMLET-like cytotoxic complexes can be formed in different ways, in which  $\alpha$ -La is assumed to be in partially unfolded conformation and able to bind OA at various stoichiometries [15]. OA has an important role in these complexes whereas the protein component acts as a vehicle for transporting toxic OA into cells and keeping OA in solution. Although, the protein/OA molar ratio was initially reported to be 1:1, recent data have shown that the OA complex is delivered by an oligomeric protein capable of binding a large number of OA molecules per protein monomer [16]. In general, the number of HAMLET-like

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complexes with proteinaceous component is not a prerequisite for their effectiveness. The cytotoxicity of these complexes is mostly because of OA [17].

Oleic acid is present in both hazelnut oil (HO) and olive oil (OO) in high amounts (70-80%). In addition to fatty acids, OO contains many phenolic compounds, i.e. hydroxytyrosol, tyrosol, oleuropein, oleocanthal, luteolin, apigenin, caffeic acid and ferulic acid [18]. The antioxidant, anti-inflammatory, immunomodulatory and antineoplastic activities of OO's phenolic components, as well as the effects of fatty acids, are of great biological importance [19,20]. These compounds show anticancer properties by promoting apoptosis, modulating epigenetic patterns, blocking the cell cycle, and reducing cell migration and angiogenesis [21–23]. Among the phenolic components oleocanthal suppresses STAT3 activation and prevents malignant cell migration, and luteolin causes apoptosis with suppression in the G2/M phase [24,25]. In HO, there are main components such as waxes, sterols, methyl-sterols, aliphatic and terpenic alcohols, tocopherols, tocotrienols and hydrocarbons, as well as triacylglycerides and fatty acids. The lipid profile of HO appears to be very similar to OO [26].

In this study, HO and OO were used as the source of OA, which has an important role in the structure of HAMLET. Both oils contain various long-chain fatty acids and additional bioactive molecules. It was important to investigate the effect of these different molecules in forming possible protein derivatives with whey proteins and to observe their effects on viability of MCF-7 cells. We therefore aimed to obtain cytotoxic protein complexes of whey proteins, HO and OO, and determined their possible effects on apoptosis, wound healing, reactive oxygen species (ROS) production, colony formation, cell cycle phase distribution and cell cycle related protein expressions.

## 2. MATERIALS and METHODS

Whey powder with high protein content (11%) was used for the stock solution of whey and was supplied from Malkara Birlik (Malkara Birlik A.Ş., Malkara-Turkey). HO and OO were from Çotanak (Altaş, Ordu-Turkey) and Komili (Bunge Gıda A.Ş., İstanbul-Turkey), respectively. A stock solution of whey (6%) containing 116 µM α-La was prepared using 0.01 M phosphate-buffered saline (PBS) and whey powder [27]. Stock solutions of HO and OO containing 1160 µM OA were prepared using Dulbecco's modified Eagle's medium (DMEM) containing dimethyl sulfoxide (DMSO). Final concentration of DMSO was kept below 0.1%. These two solutions were mixed at a ratio of 1:1 and incubated at 60°C for 120 minutes. In this way cytotoxic whey protein complexes (WP:HO or WP:OO) containing 58 µM α-La and 580 µM OA with a protein:fatty acid ratio of 1:10 was prepared [28].

### Determination of cell viability

Human breast cancer cells (MCF-7) and human foreskin fibroblast cells (HFF) obtained from American Type Culture Collection (ATCC, USA) were used. Cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma,

USA) containing 1% penicillin and streptomycin in a 5% CO<sub>2</sub> incubator at 37°C. The effect of whey protein derivatives on cell viability was determined by MTT Cell Viability Assay (Roche, Mannheim, Germany). Whey protein complexes (WP:HO or WP:OO) were applied to 96 well-plates, each well containing 1x10<sup>4</sup> cells, and incubated at 37°C for 24 h. After incubation, MTT assay was applied to determine cell viability. Cell viability of each group was calculated from absorbance at 560 nm measured in a microplate reader. Measurements were repeated 3 times.

### Determination of apoptotic and necrotic cell death

The effect of whey protein complexes on cell death was evaluated by flow cytometry using the FITC Annexin V Apoptosis kit (BioLegend). For this purpose, 1x10<sup>5</sup> cells/well were seeded into a 6-well plate. Six mL of each sample was then added to each well and incubated for 24 h. Cells were harvested, washed with PBS, and stained with Annexin-V / PI according to the user instructions. Annexin-V-negative and PI-negative cells represented the healthy cells, while Annexin-V-positive and PI-negative cells corresponded to early apoptotic cells, Annexin-V-positive and PI-positive cells corresponded to late apoptotic cells and Annexin-V-negative and PI-positive cells corresponded to necrotic cells. Stained cells were detected by FACS Calibur flow cytometer, and the results were calculated with Cell Search software (BD Biosciences, USA).

### Measurement of ROS

Reactive oxygen species were detected using 5-(and-6)-chloromethyl-20,70-dichlorodihydrofluorescein diacetate (CM-H<sub>2</sub>DCFDA) staining. Cells were seeded into 6-well plates (1x10<sup>5</sup> cells/well) and treated with whey protein derivatives for 24 h. After treatment cells were washed and incubated with phenol red-free DMEM medium containing 10 µM CM-H<sub>2</sub>DCFDA for 30 min at 37°C, in the dark. Stained cells were analyzed in FACS Calibur flow cytometer. The analysis was performed by comparing the stained and unstained cells.

### Wound healing assay

To determine the effect of whey protein derivatives on cell migration, a wound healing assay was performed [29]. MCF-7 cells were grown in 6-well plates until 70–80% confluency was reached. At this time a 100 µL pipette tip was used to create a scratch/wound with clear edges across the width of a well. Wells were treated either with vehicle control (DMSO) or whey protein derivatives and photomicrographs were taken at 24 and 48 h. A Nikon TS100 inverted microscope was used to measure and photograph cell migration from the wound/scratch edge. Experiments were performed in triplicate.

### Colony formation

Colony formation was performed to determine the efficiency in inhibiting colony formation of the cells [29]. MCF-7 cells were seeded (1x10<sup>5</sup>cells/well) in 6 well plates containing 2 mL of medium and waited for cell adhesion. Following incubation of

cells for 24 h, the colonies were stained with 0.5% crystal violet and counted.

### Cell cycle analysis

Cells were seeded at ( $1 \times 10^5$  cells/well) in 6-well plates containing 2 mL of medium. Analysis was performed 24 h after the application of whey protein derivatives. The collected cells were fixed with 70% ethanol and DNA extraction was performed in phosphate-citric acid buffer (0.2M, pH 7.8). The extracted DNA were stained with PI and incubated for 15 minutes at room temperature. Cell cycle phase distribution was analyzed using FACS Calibur flow cytometer.

### Western blot analysis

Expression of p21(Waf1/Cip1) and AKT was analyzed by Western blot analysis. Cells were seeded in 10 cm culture dishes ( $1 \times 10^6$  cells). Protein was extracted from cells with lysis buffer (50 mM Tris-HCl, pH 6.8, 15 mM EDTA, 15 mM MgCl<sub>2</sub>, 50 mM Glycerol, 150 mg/mL digitonin containing 1 mM dithiothreitol, and 100 mM PMSF). Samples were kept on ice for 15 min and the supernatant was collected after centrifugation at 18,000 x g for 10 min. Protein concentration was determined with BCA assay (Pierce Chemical, USA). Forty µg of total protein was loaded into each well. After SDS-PAGE, proteins were transferred to nitrocellulose membranes by wet-transfer. Membranes were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween 20 and immunoblotted overnight at 4°C with corresponding primary antibodies (GAPDH, p21(Waf1/Cip1), AKT) followed by horseradish peroxidase-linked secondary antibody. Detection was performed using the West Pico chemiluminescent substrate kit (Thermo Scientific, USA) and the ChemiDoc MP System (Bio-Rad, USA).

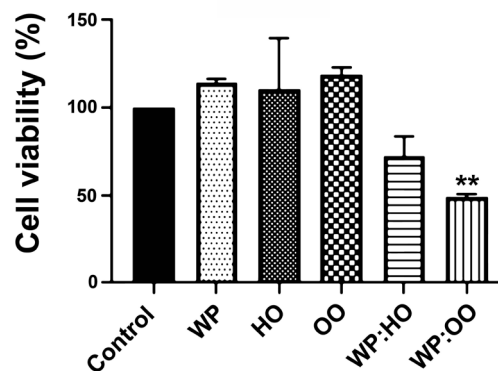
Ethical approval for the study was obtained from the Clinical Research Ethical Committee of Altınbaş University (Approval number: 121/27.5.2022).

### Statistical Analysis

Results were presented as mean ± standard deviation and statistical analysis was performed using Graphpad Prism 8.0.1 software. The limit of significance was  $p < 0.05$  using the ANOVA test. The significance level between groups was evaluated by the Tukey test.

## 3. RESULTS

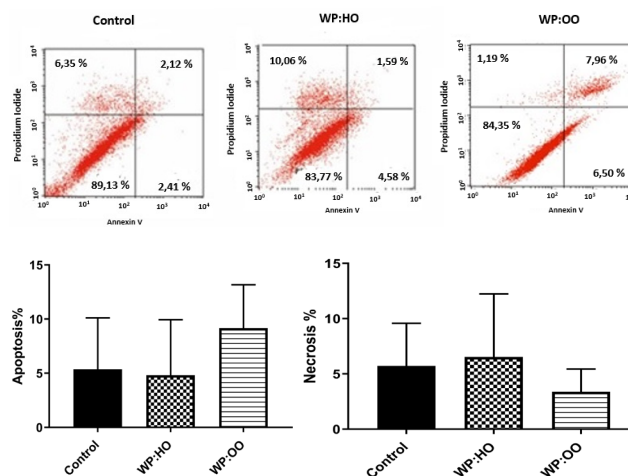
The effect of whey protein derivatives (WP:HO and WP:OO) on viability of MCF-7 cells is shown in Figure 1. Cell viability was decreased after 24 h of incubation with WP:OO and WP:HO, but the effect was significant only for WP:OO. We then investigated the effects of WP:HO and WP:OO on apoptotic and/or necrotic cell death by flow cytometry. The percentage of apoptotic or necrotic cells varied between 5-10% and although there was a slight difference between WP:HO and WP:OO no statistically significant effect was observed (Figure 2).



**Figure 1.** Viability of MCF-7 cells after treatment with WP (58 µM α-LA), HO (580 µM oleic acid), OO (580 µM oleic acid), WP:HO (58 µM α-LA+580 µM oleic acid) and WP:OO (58 µM α-LA+580 µM oleic acid) for 24 h.

Control group (Control) received no treatment. Cell viability was measured by MTT assay. Data presented as the mean ± SD of three independent experiments.

\*\* $p < 0.001$  compared with control



**Figure 2.** Flow cytometric analysis for apoptosis and necrosis in MCF-7 cells after treatment with whey protein derivatives.

Control group received no treatment. Percentage of viable cells (Annexin-V negative/PI negative), early apoptotic cells (Annexin-V positive/PI negative), late apoptotic cells (Annexin-V positive/PI positive) and necrotic cells (Annexin-V negative/PI positive) were calculated.

Data presented as the mean of at least three independent experiments.  $p < 0.05$  compared with control.

The effect of whey protein derivatives on the production of ROS was investigated by CM-H<sub>2</sub>DCFDA staining in MCF-7 cells (Figure 3). No statistically significant difference was found between controls and WP:HO or WP:OO groups. We have also performed wound healing assay to determine whether whey protein derivatives

decrease cell viability through their effect on cell migration (Figure 4). Treatment of cells with WP:HO and WP:OO for 24 h decreased cell migration compared to the control group. Here again the decrease was significant only for WP:OO. Next we determined colony formation which is also related to cell proliferation. However, there was no significant change in colony formation when the protein mixtures were applied for 24 h (Figure 5).

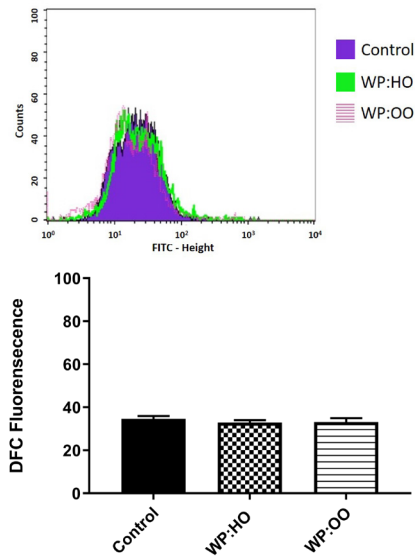


Figure 3. Effect of whey protein derivatives on ROS production in MCF-7 cells.

ROS production was investigated by flow cytometry. Data presented as the mean of at least three independent experiments.  $p < 0.05$  compared with control.

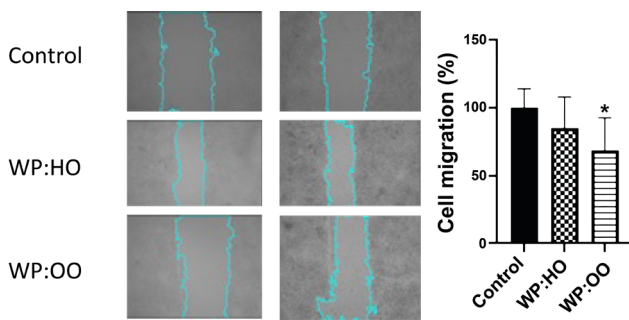


Figure 4. Effect of whey protein derivatives on migration in MCF-7 cells.

Cell migration was investigated by wound healing assay. Data presented as the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$  compared with control.

Finally, we have investigated the effect of whey protein derivatives on cell cycle phase distribution. It was determined that G2/M phase was significantly suppressed in WP:OO group compared to control group (Figure 6). Western blot analysis for the expression of cell cycle related proteins Akt and p21(Waf1/Cip1)

showed that WP:HO mixture decreased the expression of AKT and increased the expression of p21(Waf1/Cip1) significantly. Additionally, it was observed that WP:OO increased expression of p21(Waf1/Cip1) was more than that of WP:HO (Figure 7).

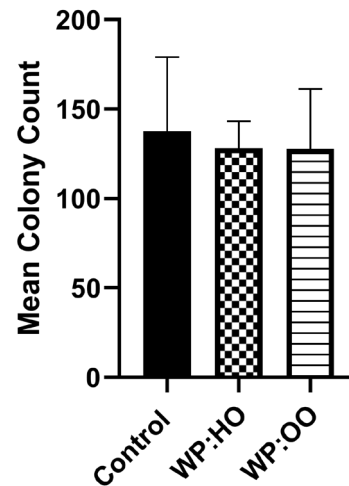


Figure 5. Effect of whey protein derivatives on colony formation in MCF-7 cells.

Results were obtained by colony formation assay as described in the methods.

Data presented as the mean  $\pm$  SD of three independent experiments.  $p < 0.05$  compared with control.

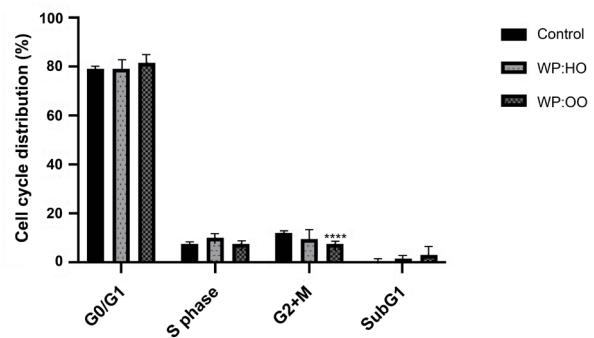
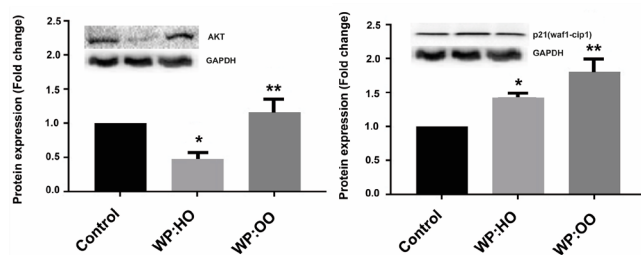


Figure 6. Effect of whey protein derivatives on cell cycle distribution of MCF-7 cells.

Cell cycle progression was measured by flow cytometry. Histogram shows representative distribution of G0/G1, S, G2/M and apoptotic phases after treatment with whey protein derivatives.

Data presented as the mean  $\pm$  SD of at least three independent experiments.

\*\*\*\* $p < 0.0001$  compared with control



**Figure 7.** Effect of whey protein derivatives on cell cycle related protein expressions.

MCF-7 cells were treated with WP:HO and WP:OO for 24 h. Control group received no treatment. Protein lysates were analyzed by Western blotting as described in materials and methods. GAPDH was used as loading control.

Protein expression levels are given as fold changes of each protein compared to loading control.

Data presented as the mean  $\pm$  SD of at least three independent experiments.

\*\* $p < 0.001$  compared with control

#### 4. DISCUSSION

Several different approaches to obtain cytotoxic complexes of whey proteins and OA exist in the literature [15]. In this study, we have used high protein whey powder and HO or OO to prepare whey protein derivatives. The polyunsaturated fatty acid contents of HO and OO were 15% and 11%, respectively. The ratio of whey proteins to the OA sources was 1:10 and whey protein derivatives were obtained by applying heat treatment [15]. We have determined that the effects of WP:OO on cell viability, cell migration ability, G2/M cell cycle phase and expression of cell cycle-related proteins AKT and p21(Waf1/Cip1) was significantly more and different compared to those of WP:HO.

Zhang et al., reported that both OA and linoleic acid induced apo-B/La intermediate to form amorphous aggregates at pH 4.0-4.5 [30]. The aggregates formed were structurally similar to HAMLET and BAMLET, a complex of partially unfolded  $\alpha$ -La with OA. Their cell viability assays showed that apo-B/La aggregates induced by OA and linoleic acid showed significant dose-dependent cytotoxicity to human lung tumor cells. A simple 2-step method for purification of calcium free  $\alpha$ -La was developed and the purified protein was used in another study to generate a complex with OA using 3 different cell lines and 2 types of cell viability assays [31]. It was determined that both bovine and human  $\alpha$ -La showed comparable cytotoxic activity.

Cell adhesion is tightly regulated by certain molecular interactions. Dissociation from extracellular matrix affects cellular activities such as proliferation and survival. HAMLET is a protein-lipid complex that also triggers tumor cell detachment, both in vitro and in vivo [15, 30, 31]. A549 lung cancer cell membrane extracts treated with HAMLET binds  $\alpha$ -actinins and adherent tumor cells were rolled and separated in response to HAMLET [32]. As a result, the cytoskeletal organization was disrupted and decreased cell migration observed in wound

healing assay might be related to this change. Another factor might be the presence of various phenolic components in OO [18].

Increasing evidence has shown that natural ingredients, including lactoferrin, OA, docosahexaenoic acid (DHA), and linolenic acid have anti-inflammatory and anti-tumor activities. Yao et al., examined the viability, migration, invasion and apoptosis of HT29 cells to select appropriate doses of these components [33]. BALB/c nude mice bearing colorectal tumors were used to investigate the role of the chosen combination in inhibiting tumor growth. Their results showed that lactoferrin at 6.25 mM, OA at 0.18 mM, DHA at 0.18 mM and linolenic acid at 0.15 mM significantly inhibited the viability of HT29 cells. The combination of lactoferrin + linolenic acid exhibited the strongest activity in inhibiting migration and invasion of HT29 cells. Moreover, the combination of lactoferrin + linolenic acid activates p-AMPK and p-JNK and apoptosis of HT29 cells. The study was the first to demonstrate that the combination of lactoferrin+linolenic acid inhibits HT29 tumorigenesis by activating the AMPK/JNK-related pathway. It is possible that similar compounds may exist in our WP:OO mixture.

Interaction of OA with albumins isolated from human, bovine and camel milk results in the formation of complexes with high antitumor activity against Caco-2, HepG-2, PC-3 and MCF-7 tumor cells [34]. The antitumor effect of these complexes was mostly due to OA. The viability of tumor cells as assessed by the MTT method was inhibited in a dose-dependent manner by albumin-OA complexes. The strong induction of apoptosis in tumor cells after administration of the complexes caused morphological change in MCF-7 cells, showed an apoptotic effect around 50% and suppressed the G2/M cell cycle phase. Trullson et al., showed that HAMLET binds to  $\alpha$ -actin, which is a cytoskeletal protein, and disrupts the integrin-dependent cell adhesion signal, and as a result the integrity of tumor cells is impaired [32]. In our study, although, we had a lower apoptotic effect there were similar changes in MCF-7 cells and suppression in the G2/M phase. As stated in the introduction, among of the phenolic components of OO, oleochemical prevents malignant cell migration, and luteolin causes apoptosis with suppression in the G2/M phase. This may explain our cell cycle and cell migration results.

Bovine  $\beta$ -Lg was shown to gain cytotoxicity against tumor cells upon binding to OA and increasing the molar ratio also increased the cytotoxicity of the complex [35]. In another study in which  $\alpha$ -La and OA complexes were prepared, cytotoxic effects against both cancer cells and non-cancer primary cells were observed [36]. Flow cytometry was used to observe the cell killing mechanisms, apoptotic and/or necrotic effects of the complex and OA alone. Jurkat cells derived from T-cell leukemia mainly underwent apoptosis-like cell death, whereas THP1 cells derived from monocytic leukemia adopted a more necrotic-like cell death.

Considering all of the above-mentioned studies by various groups that are consistent with our results, we conclude that OA complexes of whey protein are indeed cytotoxic to cancer cells. Different OA protein complexes seem to be responsible for the

observed changes in viability, migration and cell cycle phases of MCF-7 cells. However, we must admit that the role of other ingredients present in OO cannot be excluded and additional studies are required to elucidate the structures of the specific components involved in the observed changes.

### Compliance with the Ethical Standards

**Ethical Approval:** Ethical approval for the study was obtained from the Clinical Research Ethical Committee of Altınbaşı University (Approval number: 121/27.5.2022).

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**Conflict of interest:** The authors have no potential conflicts to declare.

**Authors' Contributions:** FTA and ASY: Generated the initial idea and experimental design, FTA, GB and AMY: Performed the experiments and analyzed data, FTA and ASY: Wrote the manuscript and all authors contributed to the critical revision and gave final approval to the submitted version.

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