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Leech saliva extract increases mesenchymal stem cells proliferation and viability: *In vitro* study

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Medical leech therapy has been the subject of many scientific studies for years. However, the material basis of the leech in cell growth was not yet clear. First, this study proposes to evaluate the bioactivity of leech saliva extract (LSE) in mesenchymal stem cell culture. Effect of LSE was investigated on the viability and proliferation of bone marrow-derived mesenchymal stem cells *in vitro*. BM-MSCs were cultured and treated with 2 different doses of LSE. The proliferation rates and viability percentages of cells were calculated during 3 passages. The experiments were performed in three groups: BM-MSCs cultured with a high (1.00 mL) dose of LSE-Dose1 (LSE-D1); MSCs cultured with a low (0.2 mL) dose of LSE-Dose2 (LSE-D2); and MSC culture groups. LSE increased cell proliferation and viability in vitro (P<0.05). LSE-D2 showed the highest activity, increased cell proliferation, and cell viability in vitro (P<0.05). Cell culture can affect the cell population profile and change cell differentiation. These findings demonstrated that LSE-D2 interferes with MSCs' growth kinetics. Leech salvia extracts increased cell proliferation and viability in stem cell culture.

Keywords: Drug therapy, *Hirudo verbana*, Mesenchymal stem cell, Proliferation, Trypan blue, Viability

Stem cell therapy plays an important role in medicine and transplantation because it furthers the regeneration of tissue and organ abnormalities through the insertion of stem cells into tissues[1]. The stem cells' characteristics include the capacity to renew themselves and differentiate within an adequate microenvironment. However, a lack of necessary blood vessels, lymphatics, and neuronal innervation may reduce the proliferation and differentiation capacities of stem cell applications[2]. Bone marrow-derived mesenchymal stem cells (BM-MSCs) are predicted to have significant therapeutic potential as they enhance cell differentiation and maturation by releasing cytokines and growth factors that can enable cellular communication[3]. The fact that many drugs are still obtained from natural sources and the medical potential of various species is being investigated has increased the importance of traditional medicine[4]. The therapeutic use of leeches, which has become a popular method in traditional medicine, has recently been reconsidered due to its various medical benefits. Leech saliva contains substances that affect physiological circulation; such as thrombolytic, anticoagulant, anti-inflammatory and blood & lymph circulation-enhancing properties[5]. Leech saliva also has anticancer activity in prostate and breast cancer[6]. BMSCs are widely used in the treatment of various diseases thanks to their self-renewal, differentiation, and immunomodulatory properties. In vitro studies are being conducted to understand the mechanism, safety, and efficacy of these cells in clinical applications. However, standards are limited in terms of preparation, transport, and administration of BM-MSCs, which affects the process of therapy[7]. However, significant hurdles such as low proliferation, viability of MSCs, limited lifespan gradual decay of stem cell specificity during cell culture in therapeutic applications, and functional impairment of cells due to environmental problems remain obstacles in the clinical use of MSCs[8]. Leech extract (LE) has been used in various traditional and alternative medicine practices for its believed medicinal properties[9]. Leeches produce a substance called hirudin, which acts as a powerful anticoagulant. This feature is used in medicine to prevent blood clotting in conditions such as deep vein thrombosis and cardiovascular diseases[10]. Some studies indicate that leech extract has anti-inflammatory properties[11]. LSE is also used in skin care products due to its ability to improve skin texture and reduce wrinkles by promoting collagen production. It is believed to have anti-aging and skin-revitalizing effects[12]. LSE exhibits significant antitumor activity without any specific side effects. This can be attributed to its ability to inhibit cellular proliferation, induce apoptosis, and arrest the cell cycle through immune modulation, cell-cell adhesion, and inflammation[13]. A previous study reported that LSE (400–50 µg/mL) increased cell viability and a 50 µg/mL dose induced cell migration

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in the breast fibroblast cell line[14]. Therefore, optimization of MSC culture conditions and their protection against various environmental factors are very critical to ensuring the target quantity of productive MSCs. In this study, we investigated the effect of LSE on BM-MSCs during the cultivation process by applying LSE *in vitro*, considering that the effect of BM-MSCs in clinical applications can be enhanced. This study is expected to provide a valuable guide for the development of cell therapies in the field of health.

Materials and Methods

The experiment on animals was approved by the Committee of Animal Research Ethics of Kirsehir Ahi Evran University (Number: 68429034/09).

Mesenchymal stem cell culture

Mesenchymal stem cells were obtained from the bone marrow of male Wistar albino rats. Four rats were euthanized with sodium pentobarbital (100 mg/kg) before the dissection of the femur and tibia. They were then placed in a 50 mL centrifuge tube containing Dulbecco's Phosphate Buffered Saline (DPBS; Biological Industries) and antibiotics. After being placed in a petri dish containing Dulbecco's Modified Eagle's Media (DMEM; Capricorn Scientific) with antibiotics under a laminar hood, the metaphyseal regions of the bones were cut, and a needle was inserted into the medullary cavity to collect the bone marrow in a centrifuge tube using DMEM. The bone marrow was subsequently centrifuged at 1000 rpm for 5 min, and the cells |were seeded (1×10^6 cells/cm^2) in cell culture flasks (SPL Life Sciences) containing DMEM with 20% Fetal Bovine Serum (FBS; Sigma-Aldrich), 2 mM L-glutamine (Capricorn Scientific), 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Capricorn Scientific). The primary culture was washed with Dulbecco′s phosphate-buffered saline (PBS, Biological Industries) after 24-h of incubation at 37 °C in 5% CO_2. Non-adherent cells were removed and replaced with fresh medium, while adherent cells were expanded until they reached 80% confluence. Cell proliferation and viability rates were determined at each passage until the 3rd passage. For the passaging process, the cell medium is removed with a pipette. MSCs at the bottom of the flask are washed with PBS. To remove adherent cells from the surface, Trypsin-Ethylene diamine tetra acetic acid (Trypsin-EDTA; Capricorn Scientific) solution (5 mL) is added and kept in the incubator at 37 °C for 3 min. After 3 min, the cell cultures are taken from the incubator, and 10 mL of FBS is added after confirming that the cells have risen under the microscope[15,16] (Fig. 1).

Cell count-proliferation

The pellet obtained after centrifugation is diluted with DMEM. 10µL is dropped onto the Thoma slide and all cells are counted under microscopy[17] (Fig. 2).

Cell viability

The vitality of the cells is very important. Trypan blue is a colourimetric dye that stains dead cells blue, allowing cells to be easily observed under a light microscope. Because it stains rapidly, cells can be analyzed within minutes using a hemocytometer. Trypan blue staining is therefore a useful assay for quickly determining the overall vitality of cells in a culture before starting scientific experiments. This dye is negatively charged. If the cell membrane is undamaged and alive, the dye does not enter the cell. If the cell is not alive, it absorbs the dye and appears blue under the microscope. 500 µL of cell suspension is added to the Eppendorf tube. Then trypan blue solution (100 µL) was added to the suspension and kept for 5 min. The percentage of live cells is calculated by counting the cells on the thoma slide under the microscope[18,19]. Cell proliferation and viability were determined using the Trypan Blue solution. Using light microscopy, live cells were identified as round and shiny, while blue cells were considered nonviable. A cell count and calculation of percent viability were recorded (Fig. 3).

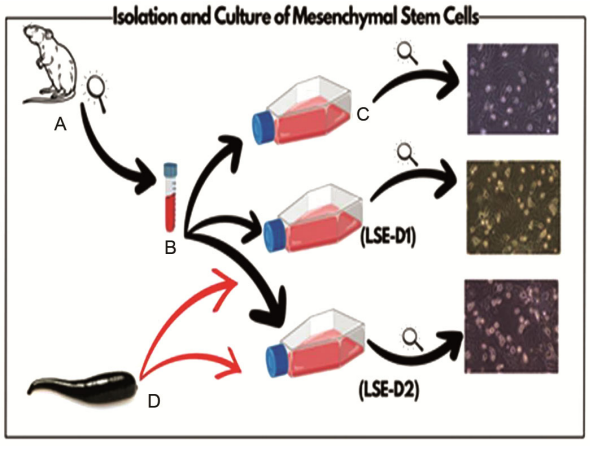

Fig. 1 — Study design showing the cultivation of 3 different cell cultures of bone marrow from subjects and the addition of LSE [A: Subject animal used in the study (male Wistar albino rat); B: Rat bone marrow; C: Control group cell culture; LSE-D1: high (1.00 mL) dose cell culture; LSE-D2: low (0.2 mL) dose cell culture;D: Hirudo verbana, leech used in the experiment].

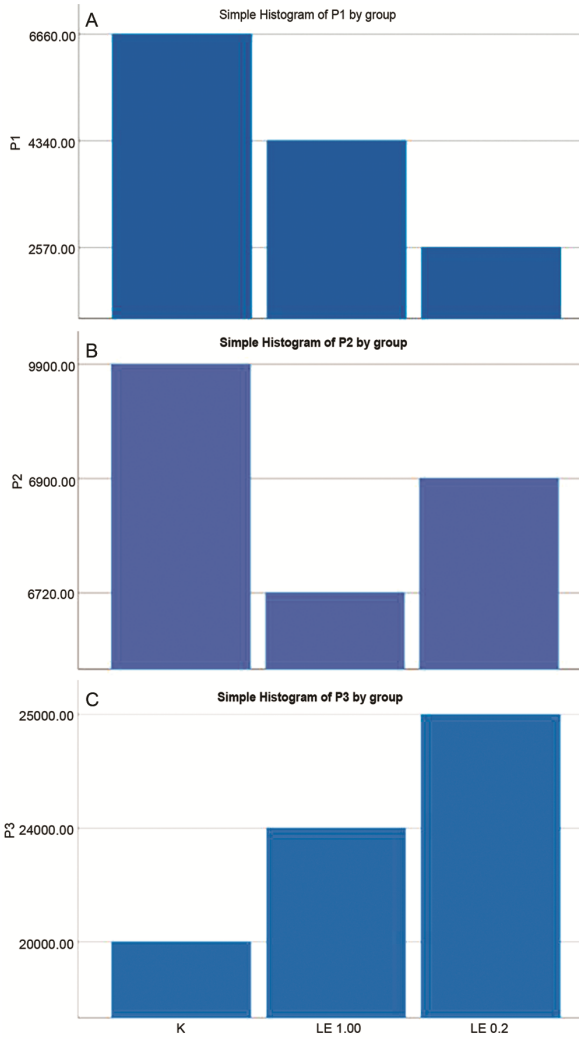

Fig. 2 — Cell Proliferation in Passage 1 (P1); Passage 2 (P2); Passage 3 (P3) [k: Control group, LE 1.00: LSE-D1group; LE 0.2: LSE-D2group].

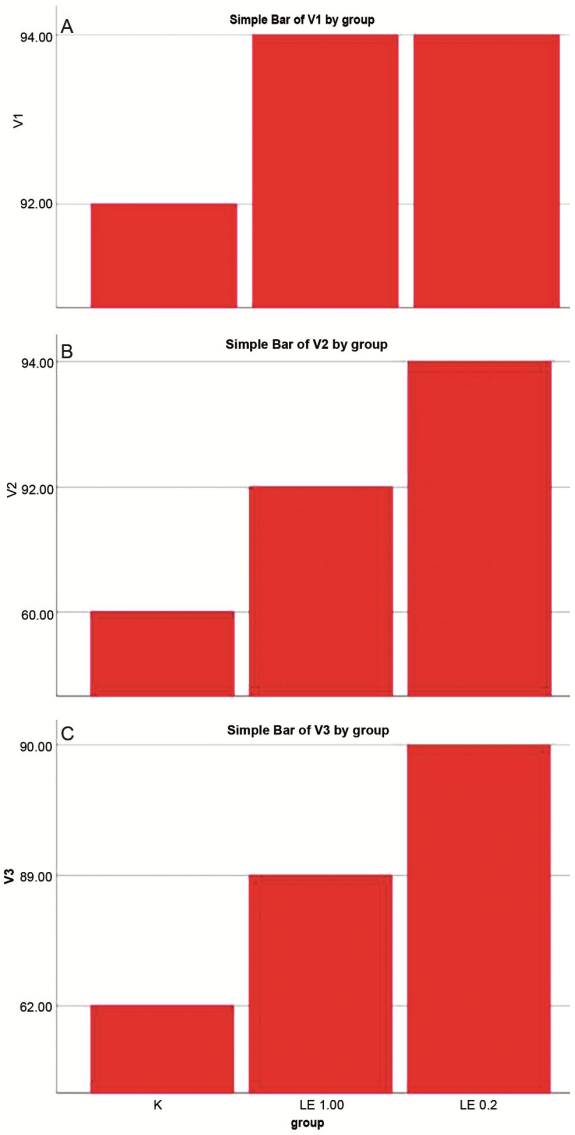

Fig. 3 — Cell Viability in Passage 1 (P1); Passage 2 (P2); Passage 3 (P3) [k: Control group; LE 1.00: LSE-D1group; LE 0.2: LSE-D2group; V1: Cell viability in P1; V2: Cell viability in P2; Cell viability in P3].

Leeches obtainment

Leeches produced at Kirsehir Ahi Evran University, Faculty of Medicine, Leech Application and Research Center will be used (Fig. 1D). Leeches kept hungry for the study will be fed with a solution of 0.001 M arginine in 0.15 M sodium chloride at the desired temperature of 37 °C. They will be wrapped in parafilm sheets with a glass funnel filled with the solution. The leeches will be brought close to the parafilm, and each leech will be allowed to hold onto the parafilm until it falls off [20].

Secretory extract obtainment

After the leeches separate from the parafilm, they will be immersed in an ice container, and their mouths will be closed. Leeches left on ice for 15 to 20 min will be temporarily paralyzed and will expel all the solution they have absorbed. The leech will then be squeezed from the rear end to the front to expel the remaining absorbed solution. Finally, the leeches will regain their activity by being kept in water at 37 °C for 15 to 30 min. Colourless liquids will be collected, passed through 0.45 µm filters, and centrifuged at 3000 rpm for 10 min. This collected raw leech secretion will be used[21].

Application of Leech saliva extract to cell Culture

Two different doses of leech extract, 1.0 mL and 0.2 mL, are added to the cell culture media at each medium renewal until the 4th passage. Control group

cells were cultured without LSE (Fig. 4A-C); the LSE-D1 group was cultured with 1.0 mL of LSE (Fig. 5A-C), while the LSE-D2 group received 0.2 mL of LSE (Fig. 6A-C). The medium was renewed every 4 days, and passaging was performed when cells covered 70-80% of the flask's surface area[14].

Statistical analysis

All analyses were performed using IBM SPSS STATISTICS 29. Differences between two groups were analyzed by a nonparametric Mann–Whitney test. ANOVA, Kruskal–Wallis test, were used for comparisons including three or more groups. Differences were considered statistically significant at $P<0.05$.

Results and Discussion

LSE increased cell proliferation

Results of dose dependent leech saliva extract in groups showed that significantly higher cell proliferation observed with LSE-D1 in passage 1 and with LSE-D2 in passage 2 and 3 compared to Control group ($P=0.008$).

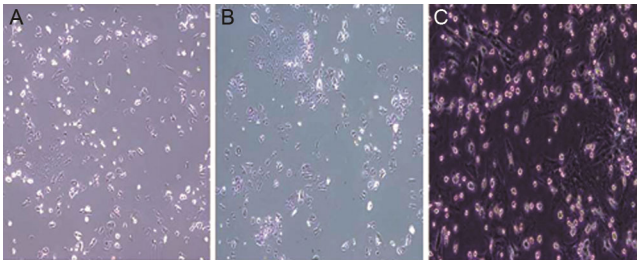

Fig. 4 — Microscopic images of Control group cells. [A: passage 1, B: passage 2 and C: passage 3].

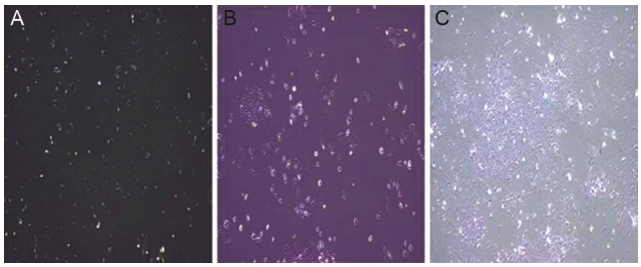

Fig. 5 — Microscopic images of LSE-D1 group cells. [A: passage 1, B: passage 2 and C: passage 3].

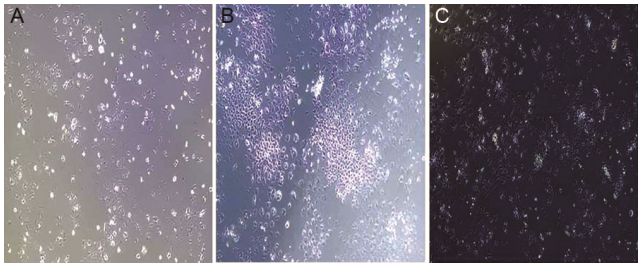

Fig. 6 — Microscopic images of LSE-D2 group cells. [A: passage 1, B: passage 2 and C: passage].

LSE increased cell viability

Findings of leech saliva extract on cell viability in dose dependent manner indicated 5% cell viability reduced in LSE-D1 group, 4% cell viability reduced in LSE-D2 compared to the Control group 32% and highly significant ($P<0.029$).

Leech therapy is one of the most widely used treatment methods in traditional medicine[22]. Leech extract contains many identified bioactive substances with various biological effects. Leech extract was discovered in the late 19th century and since then many pharmaceutical products have been produced using leech extract for various ailments. Ongoing studies are focused on developing pharmaceuticals that are suitable for health authorities to use as modern medicine[23]. In pharmacology, it is challenging to apply drug tests experimentally in organs, and sometimes this may not be possible. Therefore, stem cell cultures are of great importance for experimental studies. The most important cells for toxicity tests are neurons, cardiomyocytes, and hepatocytes. Since it is difficult to isolate them from organs, it is of great importance to obtain them from induced pluripotent stem cells. As a result, in stem cell culture studies, it is crucial that stem cells can proliferate, differentiate, and remain viable. Following MSC transplantation, cells are exposed to adverse conditions such as serum deficiency, oxygen free radicals and hypoxia triggering cell death. Therefore, it is essential to develop new strategies that enhance the efficiency of MSC transplantation and expand their application range. In this context, the viability and proliferation of MSCs were investigated by culturing under different LES densities in vitro. MSCs described by Friedenstein *et al* are cells first isolated from bone marrow tissue and later from different tissues[24]. The differentiation and homing abilities of MSCs, which are of great interest in cellular therapies, increase the regenerative capacity of tissues. Therefore, the clinical use of MSCs remains hampered by low proliferation, survival rates, and functional impairment of these cells due to oxidative stress factors during isolation[25]. Therefore, any strategy that will increase the survival and proliferative capacity of MSCs will be of great importance[26]. Mesenchymal stem cells within tissues' stem cell populations have the ability to differentiate into multiple lineages, including bone marrow stroma, osteoblasts, chondrocytes, and fat cells. These cells

have also been identified as important immune regulators. MSCs can contribute to tissue repair with their ability to migrate to the damaged area and differentiate[27]. In addition, the proliferation, viability, migration and anti-apoptotic capacities of MSCs need to be improved to increase the efficacy of MSC transplantation. Exendin-4 (Ex-4), isolated from the salivary glands of the Gila monster has a beneficial effect on MSC survival, proliferation and migration[28]. Stem cell therapy has been limited by the decreased survival of MSCs following *in vivo* injection. A strategy to increase the cells' efficiency, as recommended in preconditioning studies, is necessary, but further studies are required to determine its safety and mechanisms. Preconditioning with low concentrations of H_2O_2 and FBS deprivation effectively enhanced the proliferation of MSCs[29]. *In vitro* studies have shown that calcium phosphates support the inflammatory, immunomodulatory and osteogenic responses of MSCs by inducing the expression of some proinflammatory cytokines through activation of the Mitogen-Activated Protein Kinase (MAPK-dependent), Nuclear Factor kappa B (NF-κB) pathway[30]. Several studies also demonstrate that herbal extracts hold great promise for the proliferation, differentiation, and neurogenesis of MSCs, with a foundation in traditional Chinese medicine. However, the side effects of these natural products make it difficult to apply them in stem cell therapy; more research is needed to provide a more reliable understanding of the mechanisms of action and effective ways[31]. For example, ZD-I stimulated cell proliferation of human mesenchymal stem cells (hMSCs), decreased bone mineral content, and reduced cell viability at concentrations higher than 100 μg/mL. ZD-I is a traditional Chinese medicine (TCM) formula extract; a water-soluble fraction of a formula consisting of seven herbs[32]. However, one of the main neglected difficulties in the use of herbal medicine is plant toxicity. In addition, plant mixtures have simultaneous effects on physiological systems. hMSCs and their derivatives offer a cost-effective, rapid, and effective method for investigating the potential efficacy and toxicity of tested extracts. Therefore, these studies need to be increased in the pharmacological field[33]. U.S. Food and Drug Administration (FDA) approved medical leech therapy, which is an integrative and complementary treating technique also used in plastic surgery patients[34,35]. The effects of medical leech therapy or hirudotherapy have been investigated by many researchers on various diseases such as inflammatory diseases. Many studies have determined that leech secretions contain various bioactive molecules. Among these antistasins, hirudin, guamerin, eglins, saratin, bdellins and carboxypeptidase inhibitors were identified. Their spectrum of action may be expanded through further studies, with the discovery of their analgesic, anti-inflammatory, platelet-inhibiting, anticoagulant, and thrombin-regulatory functions[8]. The anticoagulation, antithrombin and antiplatelet aggregation effects and mechanisms of leech saliva extract have been previously investigated. Although it has been found to have apoptosis-inducing effects on cancer cells, there is a lack of studies on its impact on the proliferation and viability of MSCs. The main components of leeches are protein and peptide macromolecules. According to their pharmacological effects, they are divided into two categories such as hirudin, heparin and histamine, which directly target the coagulation system, and decorsin and hementin, which have protease inhibitor effects[36]. Hirudin, considered a natural thrombin inhibitor, is secreted by the salivary glands of *Hirudo medicinalis* and it is capable of inhibiting fluid phase and clot-bound thrombin[37,38]. Hirudin induced the proliferation and differentiation of hMSCs by activating the cGMP signaling pathway[39]. It is thought that the increase in MSC proliferation seen in this study may also be due to hirudin found in LSE.

Stem cells studies in the pharmacological field are increasing day by day. MSCs hold promise for drug delivery strategies due to their unique properties. MSCs offer advantages, making them ideal for targeted drug delivery systems. The self-renewal and differentiation capabilities of MSCs make them a unique cell type for regenerative medicine and tissue engineering applications. Continuing research into regulating the self-renewal and differentiation of MSCs will improve their full therapeutic potential[40]. Long-term cell culture led to the accumulation of chromosomal abnormalities. Cell culture microenvironment can affect the cell profile, cell differentiation, and protein expression, and may later lead to negative effects as well as positive results in the future. Therefore, cell culture studies with LSE application should be increased, and its effect should be investigated by conducting application studies. LSE application shows positive improvements in many diseases. It is thought that the results obtained with the application of LSE in *in vitro* studies will show positive improvements in many diseases. To confirm the potential therapeutic

benefits of leech treatment, further studies are needed in more extensive areas with more precise methodologies. Some therapeutic agents, such as leech extract and leech extract-based drugs, have been withdrawn, while others remain on the market. Ongoing studies focus on developing pharmaceuticals to be used as modern medicine. The data obtained from this study will also significantly contribute to addressing this issue. Clinical developments regarding leech extract have led us to think that stem cell potential can be increased. Therefore, the effects of different concentrations of leech extract on the proliferation and viability of mesenchymal stem cells obtained from bone marrow were investigated in this study. In further studies, their differentiation levels can also be investigated. Thus, perhaps new applications at the pharmaceutical level can be developed.

Since this study was conducted as a preliminary study, the characterization of cells and differentiation levels was not examined in each passage. In other studies, it can be investigated which substance in leech secretion extract is effective in cell development.

Conclusion

In this study, we investigated the effects of medicinal LSE on bone marrow mesenchymal stem cells *in vitro*. The results obtained from LSE can be interpreted as indicating a potential new agent in cell therapies, due to its ability to stimulate the release of specific proteins and growth factors that contribute to cell proliferation and survival, depending on the applied dose.

Low-dose leech extract increased cell viability. This situation increases the safety and effectiveness of LSE's use in the pharmacological field. It was concluded that the compound affecting cell development in LSE should be investigated in the continuation of this study.

Ethical statement

All procedures performed in studies involving animal study were reviewed and approved by the Committee of Animal Research Ethical of Kirsehir Ahi Evran University (Number:68429034/09).

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Conflict of interest

The authors declare that there is no conflicts of interest regarding the publication of this article.

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