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Effect of a mixture of ethyl esters of polyunsaturated fatty acids from flax, blackcurrant seed, borage, and evening primrose on animal body weight, as well as on the proliferation potential and morphology of mesenchymal stem cells

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Summary

Obesity is an increasingly common problem in animals, and researchers are seeking new methods for weight control. Supplements based on oils and their derivatives are gaining supporters. Owing to the fact that many oils cause weight gain, researchers have begun to pay attention to their derivatives: ethyl esters of polyunsaturated fatty acids. In our study, we tested the influence of ethyl esters from flax, blackcurrant, borage, and evening primrose on changes in body weight and the proliferation potential of a population of mesenchymal stem cells (MSCs). Over a 5-week period, a group of mice was fed with esters. After euthanasia, a population of MSCs was isolated. In the experiment, there was no statistically significant decrease in body weight, but a significant effect was observed on the morphology and proliferation rate of mesenchymal stem cells. We also demonstrated a negative effect of the supplement on cultures of bone marrow stem cells, whereas in the case of adipose-derived stem cells the supplement decreased the population-doubling time and increased the proliferation rate.

Keywords: borage, flax, black currant, evening primrose, ethyl esters, oils, mesenchymal stem cells

Obesity is becoming an increasingly common problem in animals, which leads to heart diseases, type-2 diabetes, cancer, and other age-related chronic diseases. The underlying cause of obesity is a long-lasting imbalance between energy input and energy output, resulting in the accumulation of body lipids. This phenomenon may have a variety of genetic, physiological, epigenetic, or environmental causes (23). Naturally and spontaneously occurring obesity may be caused by genetic problems, or it may be related to aging or to the interaction of a gene with another gene or with the environment. The most well-known form is dietary/nutritional obesity. The root cause of this kind of obesity is a high-fat diet and high-fat/high-density tasting foods. Another cause is feeding at an excessive speed. Rarest factors influencing obesity are neuroendocrine disorders (3). Currently, researchers and doctors are searching for a new method to control the body weight of animals. Studies on animal models of obesity, such as mice, provide an enormous amount of information about the influence of diet, environment, and other factors on body weight, which makes it possible to implement new therapies.

Supplementing the diet with naturally derived oils seems to be a new approach to weight loss. It has been demonstrated that many different oils may have appli-

cations not only in cooking, but also in cosmetology, regeneration pharmacology, and even in nutrigenomics. In addition, it has been shown that oils containing polyunsaturated fatty acids may play a crucial role in many processes occurring in the organism. Flax seeds are rich in omega Ω-3 (especially α-linolenic acid, ALA) and Ω-6 (especially γ-linolenic acid, GLA), and it has been demonstrated that these acids have the potential to decrease the risk of cancer and cardiovascular diseases. Moreover, these acids have anti-inflammatory and laxative activities and can mitigate menopausal symptoms and osteoporosis (8). This oil participates in relieving autoimmune and neurological diseases, and has anti-arrhythmic properties (1, 7, 22). Evening primrose oil, blackcurrant, and borage contain large amounts of polyunsaturated fatty acids (7-25% GLA). They also contain high quantities of ALA and stearidonic acid (SDA) (5, 6, 21). GLA is currently used to treat diabetes, heart diseases, multiple sclerosis, and atopic dermatitis. It takes part in lipid metabolism and many other processes in the organism (26). A correct content of linolenic acid (LA) in human organism is responsible for maintaining a proper water permeability barrier of the skin.

It was shown that a high-fat diet can induce obesity. Mice fed on a diet including soybean oil, palm oil, lard, rapeseed oil, safflower oil, and perilla oil showed an increase in body weight (10). The bioavailability of polyunsaturated fatty acids was increased by transesterification to ethyl ester. The solubility of oxygen in ethyl ester is less than in oils, and it reduces the peroxidation of unsaturated, oxidizable fatty acids. Esters have a simple, unbranched molecular structure. Characterized by faster kinetics of the release of fatty acids, they can be digested more quickly. The use of esters limits the production of toxic compounds from the cyanohydrin family.

In recent years, there has been a markedly increased interest in mesenchymal stem cells (MSCs). MSCs are used in tissue engineering and regenerative therapies (13, 18, 19). Owing to MSCs' phenotypic plasticity and proliferative potential, they can act as an indicator of changes in cell culture conditions. Changes in MSC morphology and immobilization provide information on biological processes (14). Supplementing the diet with diverse compounds, such as oils, can affect the metabolism not only of the organism as a whole, but also of individual cells (12). For instance, it has been shown (4) that ethyl esters of borage seed have a different, dose-dependent influence on the morphology and proliferation rate of Wharton Jelly mesenchymal stem cells. The addition of 2% borage ethyl esters had a positive effect on proliferative activity and no effect on cell morphology. Higher concentrations of the supplement (4% and 6%) resulted in degenerative changes in morphology. In our study, we isolated a population of adipose-derived stem cells (ASCs) to compare the effects of *in vivo* supplementation with ethyl esters.

The first purpose of this experiment was to determine the effect of supplementation with a mixture of ethyl esters of polyunsaturated fatty acids obtained from flax oil, blackcurrant seed oil, borage seed oil, and evening primrose oil on the body weight of mice. The second aim was to determine the proliferation factor, population-doubling time, and morphology of the isolated MSC population.

Material and methods

The study was conducted with the approval of the Second Local Bioethical Commission at the Department of Biology and Animal Breeding, Wroclaw University of Environmental and Life Sciences, Wrocław, Chełmońskiego 38C, Poland (Dec. number 177/2010 of 11.15.2010).

Eight-week-old mice (C57L/6J, both sexes) were obtained from the Animal Vivarium Wroclaw Medical School (Poland). The mice were randomly divided into two groups: control (n = 6) and experimental (n = 10). The animals were kept at a constant temperature of 22°C with an artificial light cycle (12 hours of light and 12 hours of darkness). They had *ad libitum* access to water and feed, consisting of 67% carbohydrates, 25% fat, and 8% protein (Labofeed B standard, Wytwórnia Pasz "Morawski," Poland). A 2% supplement of ethyl esters from a mixture of polyunsaturated fatty acids was administered to the experimental group in water. A half of the esters mixture consisted of ethyl esters of fatty acids from flax, and the remaining 50% comprised equal quantities of ethyl esters of fatty acids from blackcurrant, borage, and evening primrose. The water with the supplement was changed every three days throughout the 5-week experiment.

The ethyl esters of unsaturated fatty acids were prepared by the esterification of oils (Skotan S.A) with ethanol (POCH) in the presence of KOH (Sigma Aldrich) under nitrogen (Messer). A 16-fold molar excess of ethanol was used in order to obtain an efficiency close to 100%. The reaction space was saturated with nitrogen atmosphere because of the susceptibility of polyunsaturated fatty acids to oxidation. The oil was also saturated with nitrogen. The esterification time was 1 hour, and the temperature was maintained between 33 and 49°C. The efficiency after esterification was not checked because of the excess of ethanol applied. The oils used include the following contents of individual fatty acids: flax oil – > 50% linolenic acid, 15% linoleic acid, 15% oleic acid; blackcurrant seed oil – more than 80% of polyunsaturated fatty acids, including linoleic, linolenic, and stearidonic acids; borage oil – 18-25% γ-linoleic acids, 38% linolenic acids, 4% eicosanoic acid, and 3% docosanoic acid; primrose oil – 73.5-81.9% linoleic acid, 6.8-9.4% linolenic acids, and 4.7-10.7% oleic acid.

Animals were euthanized by carbon dioxide inhalation and then weighed on a laboratory scale (Radwag, Poland). Adipose-derived mesenchymal stem cells were isolated from the subcutaneous adipose tissue. Adipose tissue was collected immediately after euthanasia and placed in a sterile Hanks' balanced salt solution (HBSS, Sigma Aldrich). ASCs were isolated by cutting adipose tissue into small

pieces and digesting it with collagenase type I (Sigma Aldrich) at a concentration of 1 mg/ml for 40 minutes in an incubator at 37°C and 5% CO_2 (9). The tissue was centrifuged at 300 × g for 4 minutes (IEC CL31R, Thermo Scientific), the pellets were re-suspended in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich) containing Ham's F-12 nutrient mixture supplemented with 10% of fetal bovine serum (FBS, Sigma Aldrich) and a 1.5% Antibiotic Antimycotic Solution (Sigma Aldrich) and then transferred to a culture flask. Additionally, we isolated bone marrow mesenchymal stem cells from the femurs. BMSCs were isolated by flushing with a syringe in HBSS. The cell suspension was transferred into a falcon tube and centrifuged at 300 × g for 4 minutes. The pellets were re-suspended in the medium and transferred to a culture flask.

The proliferation factor of adipose-derived mesenchymal stem cells was determined with a resazurin-based In Vitro Toxicology Assay Kit (Sigma Aldrich) after the first passage of culture. For assays, cells were inoculated into 24-well plates. The initial concentration of cells was 2.5×10^4 per well (0.5 ml medium). The dye was added to the culture medium in a final concentration of 10%, and the cells were incubated for 2 hours at 37°C in 5% CO_2. The absorbance of the cell culture supernatant was measured at a wavelength of 600 nm and a reference length of 690 nm (9, 25). The metabolic activity indicates the amount of NADPH (Nicotinamide adenine dinucleotide phosphate) formed and NADH (Nicotinamide adenine dinucleotide), which reduces the dye used for the assay (resazurin) to resorufin. The degree of reduction is shown by the change in the dye color. The analysis was performed at hours 24, 96, 120, and 168 of the experiment.

The population doubling time was calculated on the basis of the estimated values of the proliferation coefficient. The number of cells was calculated for each measurement point, and then processed by the "Doubling Time Online Calculator" (4, 25) software. The calculator used two points of measurement: 0 hours for 15 000 cells, and 120 hours for a value corresponding to the sample.

The morphology of the cells was compared on day 7 (BMSCs) and day 21 (ASCs) post isolation. The cells were fixed in 4% paraformaldehyde for 45 min at room temperature, then washed with HBSS, and permeabilized in 0.2% Tween 20 in order to increase the permeability of the membranes. The cell nuclei were stained with DAPI fluorochrome, whereas the cytoskeleton was visualized by staining the actin filaments with phalloidin (15, 17). The cultures were documented under an inverted fluorescence microscope (Axio Observer.A1, Zeiss). Photographs were taken with a Power Shot Camera (Cannon) at 100-fold magnification.

The analysis of changes in morphology and cytophysiology was performed with a scanning electron microscope (Zeiss EVO LS15) on day 7 (BMSCs) and day 21 (ASCs) post isolation (11, 16). The cultures were fixed in 4% paraformaldehyde (45 minutes, room temperature), and then dehydrated in a series of increasing concentrations of alcohol, from 50% to 100%. The samples were sprayed with gold for 250 seconds in a coater (Edwards, Scancoat six). An electron microscopic SE1 detector was used in this study with 2000-fold and then 5000-fold magnification.

Results and discussion

The body weight of mice before supplementation was 22.018 ± 0.807 g in the control group and 22.084 ± 0.979 g in the experimental group. The average weight loss in the experimental group after 5 weeks of supplementation was 2.8% (Fig. 1). The data are not statistically significant. The weight of the control group ranged from 21.27 g to 22.86 g. The body weight of experimental mice was more varied; there were individuals weighing less than 20 g. Body weight ranged from 19.52 g to 22.54 g.

Stem cells isolated from adipose tissue also differed between the experimental group and the control group. Cells from the experimental group were characterized by a strong, extensive cytoskeleton and faster proliferation. Culture growth allowed the proliferation factor and population-doubling time for the cell population to be assessed. The proliferation factor for these cells was determined on days 1, 4, and 7 of culture after the first passage. On day 1, the proliferation rate was lower than in the control group, but by day 7 it had reached a level higher by about 20.5%. This factor was characterized by an upward trend (Fig. 2). These results indicate that cells obtained from the experimental group supplemented with a mixture of ethyl esters took longer time to accommodate to environmental conditions. The population-doubling time of the cells in the experimental group was reduced by 13.4% (Fig. 3). These results provide evidence of an increased metabolic activity and proliferation of cells isolated from stimulated animals. The above observations were confirmed by micrographs obtained by fluorescence microscopy. The cell culture from mice stimulated by the mixture of ethyl esters was characterized by a more extensive cytoskeleton and similarity in the cell morphotype (Fig. 4B). The cells grew in large clusters, and centrally-placed nuclei were observed (Fig. 4D). All cells were of a similar appearance, and the culture appeared identical. In contrast, cells isolated from the adipose tissue of the control group showed a greater variation in cell morphology. We noticed cells similar to those from the experimental group as well as cells with a less developed body. The architecture of the culture was not uniform throughout the image of the control culture (Fig. 4A). Images were obtained with a scanning electron microscope and showed a similar picture: well-development connections between cells in both groups. In addition, the presence of microvesicles was observed. This may suggest cytophysiologic activity in cells. Microvesicles were found on the body of cells, especially in marginal regions (Fig. 4E, F).

Additionally, we tested the influence of the esters on bone marrow stem cells. Cells after stimulation showed different properties compared to the control group. The experimental group adhered less firmly to the surface of the culture vessel. Furthermore, epithelial cells were found more frequently in the experimental group than

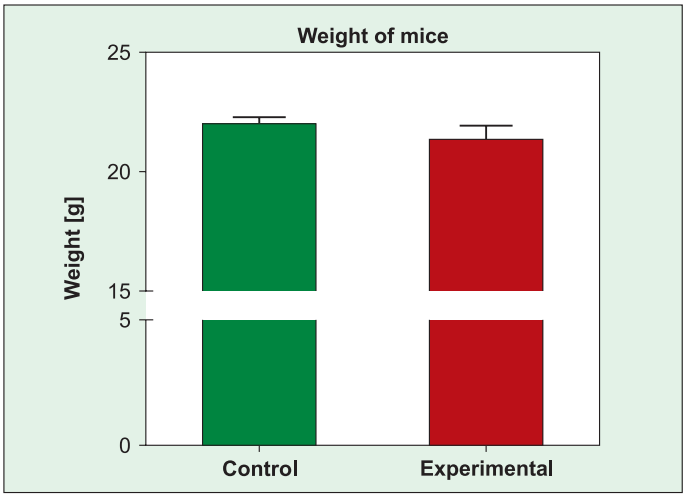

Fig. 1. Comparison of the body weight of mice supplemented with a mixture of esters and the body weight of the control group. The weight was 21.98 g in the control group and 21.37 g in the experimental group.

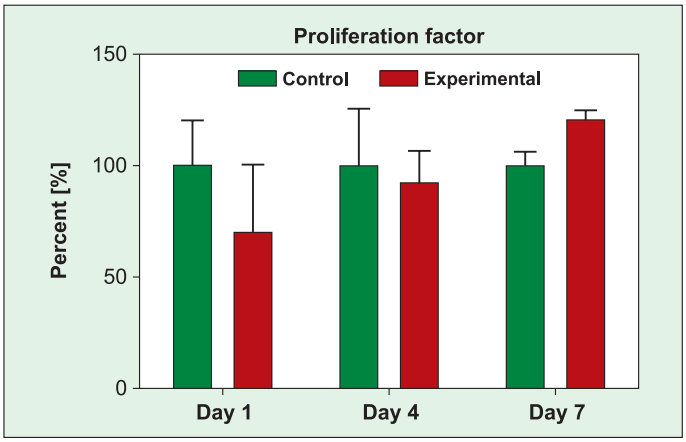

Fig. 2. The proliferation rate for adipose-derived stem cells stimulated with the addition of esters compared to the control culture. In the experimental group, the proliferation rate reached 69.67% on day 1 of culture, 92.25% on day 4, and over 120.5% on day 7.

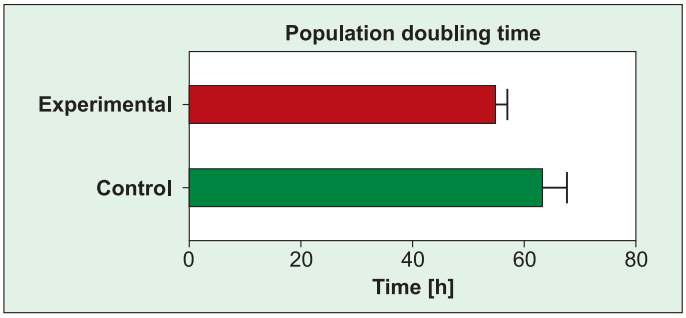

Fig. 3. The population-doubling time of adipose-derived stem cells after the addition of esters and in the control group. The time was 54.8 h for the experimental population and 63.25 h for the control.

in the control group. On day 5 of culture, degenerative changes were revealed, as the cells disconnected from the bottom. Owing to degenerative processes, the culture was stopped on day 7 of primary culture.

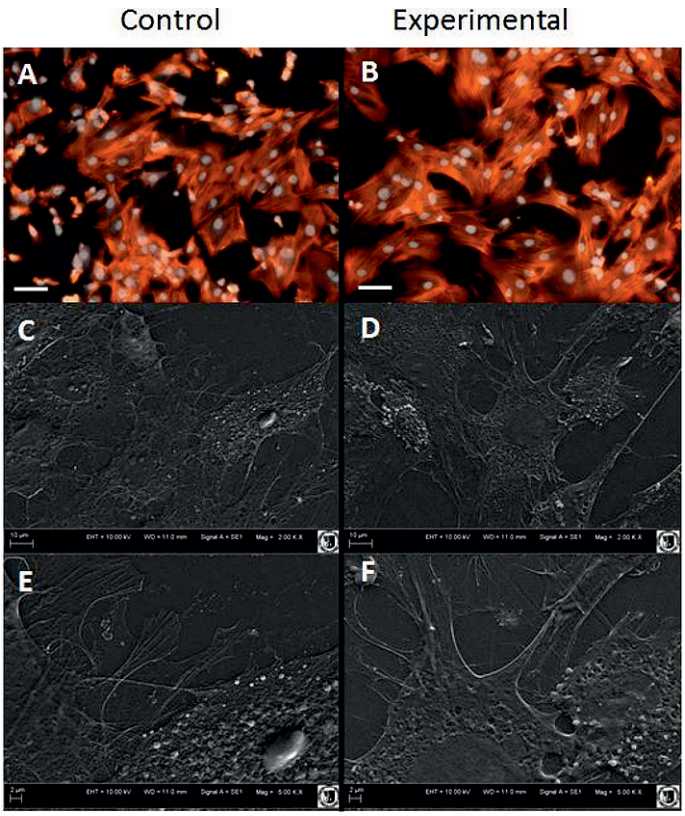

Fig. 4. Micrographs of adipose-derived stem cells from mice on day 21 of culture. Documentation was performed with a fluorescence inverted microscope (A, B – magnification – 100 ×, scale bar – 125 µm) and a scanning electron microscope (magnification – C, D – 2000 ×, E, F – 5000 ×). The experimental group was characterized by normal, extensive cell bodies and uniform morphology (B, D), whereas the control group had a more varied morphology (A, C). Both groups had well development filopodias and microvesicles located on the surface (E, F).

There was no measurement of proliferation potential. The low number of MSCs was identified by means of a fluorescence microscope (Fig. 5B). These cells grew only in clusters and had poorly developed, shrunken cell bodies. The nuclei of MSCs were arranged peripherally and did not have a bipolar or multipolar appearance (Fig. 5D). Cells isolated from the bone marrow of the control group were characterized by a smaller number of epithelial cells compared to those from the experimental group. There were no signs of degenerative culture. The cells showed a normal morphotype with an extensive cytoskeleton and were bipolar (Fig. 5C). They grew evenly in culture wells (Fig. 5A). The analysis of the culture isolated from the experimental group, carried out with a scanning electron microscope, confirmed the degeneration of the cell culture. The culture contained cells with a shrunken cell body. Very weak connections between cells were also observed – filopodia occurred only locally, without contact with other cells (Fig. 5F), whereas the control group cells showed properly developed connections and bodies (Fig. 5E). On cell surface, microvesicles were observed. Supplementation with a mixture of

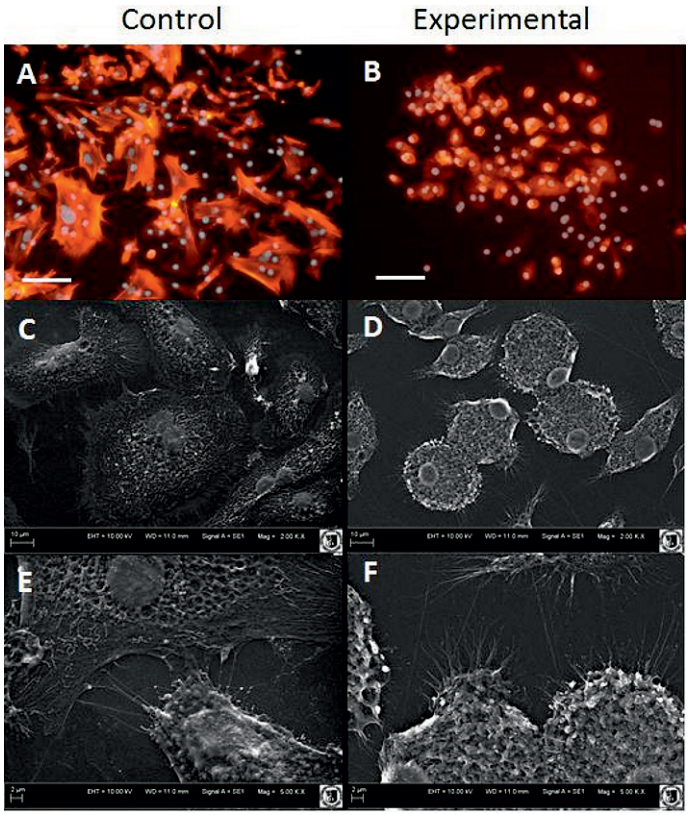

Fig. 5. Micrographs of mouse MSCs isolated from bone marrow on day 7 of culture. Documentation was performed with a fluorescence inverted microscope (A, B – magnification – 100 ×, scale bar – 250 µm) and a scanning electron microscope (magnification – C, D – 2000 ×, E, F – 5000 ×). MSCs in the experimental group grew in clusters (B), had shrunken bodies with a peripherally placed nucleus (D) and less developed connections between cells (F). The control culture had well development bodies (A, C) and connections between cells (E).

esters adversely affected the cells' ability to renew the population and the immobilization of mesenchymal stem cells isolated from bone marrow.

Individual differences in body weight may have been due to the short time of supplementation. Ikemoto et al. (1996) tested the effects of oils on mice during a 16-week *in vivo* administration of additive factors (10). Long-lasting supplementation with a mixture of esters may significantly change the results obtained, by normalizing them. It is worth pointing out that the hydrolysis of ethyl esters in alcohol is likely to affect metabolism and body weight. Diets including a variety of oils, such as soybean oil, palm oil, lard, rapeseed oil, safflower oil, and perilla oil, cause an increase in body weight (10). Supplementation with esters of fatty acids had no significant effect on body weight. An *in vivo* study with an extended administration of a mixture of esters is advisable.

In this study, it was shown that a population of adipose-derived mesenchymal stem cells responded to ethyl ester supplementation. The esters used in this study had a positive effect on MSCs isolated from adipose tissue. It has been shown that the addition of esters has a positive influence on ASCs (4), which confirms ours conclusions. On the other hand, we found that the ester mixture had a cytotoxic effect on cells isolated from bone marrow. This suggests that bone marrow cells are less adaptable to culture conditions. For instance, it was shown that equine mesenchymal stem cells isolated from bone marrow needed more time for adhesion. It was also demonstrated that the achievement of the same level of confluence of both populations takes longer in bone marrow stem cells than in adipose-derived stem cells (2). Other researchers showed (20) that the population-doubling time of bone marrow stem cells isolated from rats increased by 35.4% compared with adipose-derived stem cells. Furthermore, for mice, both the adhesion and proliferation rates were slower in a BMSC culture (24). This may be due to the fact that bone marrow mesenchymal cells are less adaptable to environmental changes. Changes caused by the administration of esters resulted in a loss of their self-renewal capacity. In contrast, mesenchymal stem cells isolated from adipose tissue were able to adapt to environmental changes. The addition of the supplement increased the proliferative potential of cells, without causing degenerative changes in their morphology. The proper ratio of Ω-3 and Ω-6 fatty acids intensified stem cell metabolism, increasing their capacity for self-renewal. It has been shown that the effect of a supplement on a population of cells depends on its concentration (4).

To summarize, we confirm that a mixture of ethyl esters improves the proliferative potential of adipose-derived stem cells without affecting their morphology. We also showed that bone marrow stem cells are more susceptible to external factors and more demanding in terms of culture conditions compared to adipose-derived stem cells. In this study, we found that adipose-derived and bone marrow stem cells isolated from the experimental group required much more time to adapt to environmental conditions. The degeneration of the BMSC culture was a result of cells sensitivity and the long period to adapt to culture conditions. In the future, a reduction in the concentration of esters may have a positive impact on the properties of a population derived from bone marrow. The concentration used in this experiment seems to have been appropriate for the stimulation of adipose-derived stem cells. Ethyl esters may find wide application as carriers or solvents for drugs in various therapies, such as chemotherapy. They may also have significant applications in cosmetology, nutrigenomics, and regenerative pharmacology.

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