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Preconditioning of Human Umbilical Cord Mesenchymal Stem Cells with a Histone Deacetylase Inhibitor: Valproic Acid

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Background: Mesenchymal stem cells (MSCs) play a key role in regenerative medicine due to their capacity to differentiate into multiple cell lines, regulate the immune system, and exert paracrine effects. The therapeutic impact of MSCs is primarily mediated through their secretome. The secretory and therapeutic potential of MSCs can be improved through preconditioning, which entails the application of hypoxic environments, 3-dimensional cell cultures, and pharmacological agents. Valproic acid (VPA) is a histone deacetylase inhibitor that is employed in medical practice for treating epilepsy and bipolar disorder. Hence, preconditioning MSCs with VPA is expected to induce histone acetylation, enhance gene expression, and beneficially modify the cells' secretomes.

Aims: To assess the effectiveness of VPA in enhancing and regulating the therapeutic potential of cells as well as its impact on MSC secretome profiles and ultrastructural morphologies.

Study Design: Expiremental study.

Methods: Human umbilical cord MSCs were preconditioned with 2 mM VPA for 24 and 48 hours; untreated MSCs served as controls. The secretome secreted by the cells was assessed for its total protein content. Subsequently, interferon-gamma (IFN-γ), interleukin-17 (IL-17), IL-10, vascular endothelial growth factor, nerve growth factor (NGF), glial cell line-derived neurotrophic factor, and brain-derived neurotrophic factor (BDNF) levels in the secretome were analyzed using the ELISA method. The ultrastructural properties of the cells were studied under transmission electron microscopy.

Results: Ultrastructural examinations revealed that the chromatin content of VPA-treated cells was reduced. VPA-preconditioned cells exhibited a higher density of rough endoplasmic reticulum, autophagic vesicles, and myelin figures on cytoplasmic structure analysis, which was indicative of increased secretion. Protein secretion was elevated in those cells, with notable increases in NGF and BDNF levels. Furthermore, the cytoskeletal rearrangement and elevated autophagic activity observed in the 48-hour preconditioned cells could indicate the initiation of neuronal differentiation. IL-10, IL-17, and IFN-γ were not detected in the secretome.

Conclusion: This study indicate that preconditioning with VPA enhances MSC activity and subsequently modifies the secretome content.

INTRODUCTION

Mesenchymal stem cells (MSCs) hold significant therapeutic relevance in regenerative medicine due to their proliferative capabilities, multilineage differentiation potential, immunomodulatory characteristics, and beneficial paracrine effects.[1] MSCs secrete soluble and insoluble factors into the extracellular milieu. Increasing evidence indicates that the therapeutic effects of the cells are primarily mediated through the secretome they generate, which acts in a paracrine manner.[2] The soluble factors secreted by MSCs include growth factors, cytokines, chemokines, and a variety of immunomodulatory molecules, while the insoluble factors comprise exosomes and microvesicles.[3] These secreted factors confer on MSCs angiogenic, anti-apoptotic, antioxidant, regenerative, immunomodulatory, and immunosuppressive properties.[2,3]

MSCs possess an inherent capacity for secreting specific factors; however, it has been demonstrated that their secretory potential can be enhanced or altered through specific inductions. These inductions involve the establishment of a hypoxic environment, employing three-dimensional (3D) cell culture settings, exposing them to inflammatory conditions, and utilizing various pharmacological agents. This collective approach is frequently referred to as the "preconditioning" of these cells.[4] For instance, it has been observed

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that subjecting MSCs to hypoxic conditions during preconditioning enhances their proliferative and migratory capabilities, primarily through a positive influence on their paracrine secretion profiles. Furthermore, the hypoxic environment can elevate the expression of genes that promote cell survival and diverse trophic factors within MSCs.[5,6] Our previous study demonstrated that the secretion patterns of MSCs were altered when preconditioned with hypoxia mimetic agents. This was particularly evident from their significantly increased capacity to secrete vascular endothelial growth factor (VEGF).[7,8] A 3D microenvironment, in contrast to traditional two-dimensional (2D) cell culture, is an alternative method for enhancing the MSC secretome. This methodology creates the requisite conditions for complex biological functions, including interactions with the extracellular matrix, migration, and transcriptional regulation. In addition, this technique can alter the cell secretome by facilitating cell-cell interaction and providing adequate space for MSC proliferation.[9,10] Kim et al.[11] demonstrated that the secretion of fibroblast growth factor (FGF), transforming growth factor beta-1, and interleukin-6 (IL-6) by MSCs cultivated within a 3D environment exhibited a significant increase. These data indicate that targeted modification can enhance the therapeutic potential of MSC.

Valproic acid (VPA) is a short-chain fatty acid that is widely recognized for its function as a histone deacetylase (HDAC) inhibitor.[12] It is employed in medical practice for managing epilepsy and bipolar disorder.[13] The neuronal,[12] osteogenic[14] and hepatic differentiation[15] capacities of VPA-induced MSCs have been found to increase in the limited studies that have examined the impact of VPA on MSC differentiation. Santos et al.[12] demonstrated that exposing adult MSCs to VPA-induced neural differentiation within 24 hours by upregulating the expression of suppressor of cytokine signaling 5 (SOCS5) and FGF21 without increasing the potential death rate of the cells. Salami et al.[16] discovered that VPA exhibits varying effects on differentiation depending on the conditions. They determined that the application of VPA at 0.5 mM concentration to embryonic stem cells in a normal culture medium resulted in an increase in pluripotency markers, whereas its application at 2 mM concentration in a neuronal differentiation medium led to a decrease in these markers.[16] Another study by Rashid et al.[15] demonstrated that 5 mM VPA-treated MSCs differentiated into hepatocyte-like cells within 24 h. In addition to the differentiation, Linares et al.'s research demonstrated that MSCs treated with VPA exhibited improved therapeutic effects in a mouse model of Huntington's disease. The altered gene profile in preconditioned MSCs is responsible for the enhanced therapeutic effect, as evidenced by a substantial increase in the expression of genes involved in trophic effects, antioxidant activity, anti-apoptosis, cytokine/chemokine receptors, migration, mitochondrial energy metabolism, and stress response signaling pathways. In addition, it has been reported that VPA preconditioning augments the immunosuppressive effects of MSCs through its influence on the T-cell mechanism.[17] Upon a literature review, it is evident that VPA exhibits highly variable effects depending on dose and duration. The cytotoxic effects of these doses were analyzed, and it was found that 20 mM VPA is toxic to MSCs, while no cytotoxic effect was observed at doses below 10 mM.[15]

This study hypothesized that since VPA serves as a HDAC inhibitor, preconditioning MSCs with VPA can trigger histone acetylation, activating transcription, and thereby enhancing gene expression. Subsequently, this would induce favorable modifications in the cell secretome. To test this hypothesis, we evaluated the changes in the secretome profile and MSC ultrastructural morphologies caused by VPA preconditioning, selecting a dose that would effectively activate histone acetylation.[13] Given the current clinical application of VPA, demonstrating its ability to enhance the therapeutic efficacy of MSCs and their secretome would be significant, as it could lead to additional novel clinical applications for VPA.

MATERIALS AND METHODS

Isolation of mesenchymal stem cells

The umbilical cord was procured from a patient who had undergone a cesarean section at the İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Department of Gynecology and Obstetrics after obtaining approval from the Ethics Committee (approval number: E-83045809-604.01.01-380308, date: 10.05.2022). The patient provided written, informed consent. The tissue explant method was employed to isolate MSCs from umbilical cord tissue, as described in our previous study.[18] Briefly, the umbilical cord was placed in leibovitz L-15 medium with 1% amphotericin B for transportation. After washing the cord tissue with phosphate-buffered saline (PBS) to remove the blood clots as well as the two arteries and one vein, the tissue was cut into 1-2 mm^3 pieces. These pieces were subsequently cultured in Dulbecco's modified Eagle's medium (F12:1/1) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2.5 µg/ml amphotericin B for three hours at 37 °C in a 5% CO_2 humidified environment. Subsequently, 6 ml of culture medium was added to the tissue fragments, and the medium in the Petri dishes was replenished three times a week. An inverted microscope (Olympus IX71) was employed to monitor the isolation process. The tissue pieces were removed, and the cells were passaged once they attained a sufficient level of confluency.

Characterization of mesenchymal stem cells

Immunophenotypic and differentiation analyses verified the MSC characteristics of the isolated cells. Flow cytometry was employed to identify the presence of characteristic MSC markers, CD44-PE (BD Biosciences: 550989) and CD90-APC (BD Biosciences: 559869), as well as the absence of negative markers, CD34-FITC (BD Biosciences: 555801) and CD45-APC-Cy (BD Biosciences: 557833). For this purpose, the cells at the third passage were initially harvested through trypsinization, which was subsequently followed by centrifugation at 1,500 rpm for five minutes. Trypan blue staining was employed to ascertain the number of viable cells. Subsequently, 10^6 cells were filtered through a 70 µm filter and prepared for flow cytometry analysis. The cells were then washed twice in staining buffer (2% FBS in PBS), incubated in a mixture containing primary and isotype antibodies for 30 minutes in the dark, and analyzed using the BD FACS Calibur system.[7]

The differentiation characteristics of the cells were demonstrated in the third passage by inducing adipogenic and osteogenic differentiation. Commercially obtained differentiation media were

employed for this purpose (sigma: 417D-250, sigma: 811D-250, respectively) in accordance with the manufacturer's guidelines. Upon completion of the procedure, the presence of adipogenic differentiation was confirmed through Oil Red O staining, while osteogenic differentiation was verified by Alizarin red staining.[18] The results were analyzed using a light microscope, the Olympus BX61.

Preconditioning of mesenchymal stem cells with valproic acid and preparation of conditioned medium

The MSCs were seeded in Petri dishes at a density of 3 x 10^5 cells per 10 ml of medium. The culture medium was replaced with a 10 ml serum-free medium after the cells reached 70% confluence. Subsequently, the medium was supplemented with VPA at a concentration of 2 mM for the preconditioning step.[13] The cells were incubated in the serum-free medium containing VPA for 24 and 48 hours. The control group consisted of untreated MSCs. Following the incubation, the conditioned medium (CM) was harvested for secretome analysis, subjected to centrifugation to eliminate cellular debris, and subsequently stored at -80 °C.

Content analysis of the conditioned medium

The bicinchoninic acid (BCA) analysis was employed to quantify the total protein content of the CMs (23225, ThermoFisher). The growth factors and cytokines were analyzed using the following commercial "ELISA" assays, in accordance with the manufacturer's instructions: EK0373 (Boster) for interferon-gamma (IFN-γ); EK0430 (Boster) for IL-17; EK0416 (Boster) for IL-10; KET6033 (Abbkine) for VEGF-alpha (α); EK0469 (Boster) for nerve growth factor (NGF); EK0362 (Boster) for glial cell line-derived neurotrophic factor (GDNF); and EK0307 (Boster) for brain-derived neurotrophic factor (BDNF). All the measurements were conducted using a microplate reader (Allsheng AMR-100 Microplate Reader AS-16050-00). The sample concentrations were determined in ug/ml or pg/ml based on the results.

Ultrastructural analysis

Ultrastructural evaluation was performed on MSCs that had been pre-treated with VPA for 24 and 48 hours, as well as untreated MSCs that served as the control group. For this purpose, the cells were initially fixed with glutaraldehyde and subsequently with osmium tetroxide. The cells were meticulously embedded in 2% liquid agar after fixation, and upon solidification, the agar was cut into 1-2 mm^3 pieces. These agar-embedded cells were dehydrated through a graded alcohol series and embedded in araldite. After designating the location for examination in 0.5 µm semi-thin sections, 40-50 nm thin sections were collected and placed on copper grids. The grids were contrasted with uranyl acetate and lead citrate and examined with transmission electron microscopy (Jeol Jem-1011).[8] The analysis focused on heterochromatin, mitochondria, rough endoplasmic reticulum (RER), autophagic vesicles, and myelin figures, with an average of 15 cells examined. The average values per cell were determined by analyzing the RER density, autophagic vesicle, and myelin figure structures within the cells.

Statistical analysis

For statistical analysis, the SPSS version 21.0 statistical program was used. The data are expressed as the mean ± standard error of the mean. The normality of the data was evaluated with the Shapiro-Wilk test. The non-parametric Kruskal-Wallis test was employed to assess the significance of the differences between groups, as the data were not normally distributed. A p value < 0.05 was considered statistically significant.

RESULTS

Isolation and characterization of mesenchymal stem cells

The isolated cells adhered to plastic surfaces, exhibited a spindle-shaped morphology, and demonstrated a high proliferation capacity (Figure 1). CD44 and CD90 were expressed at a significant level of 98.75% and 97.57%, respectively, during the immunophenotypic assessment, which verified the mesenchymal characteristics of the MSCs. However, MSC-negative markers exhibited only limited expression, with 0.34% for endothelial marker CD34 and 2.7% for hematopoietic marker CD45 (Figure 1). Additionally, the lipid droplets formed during adipogenic differentiation induction were visualized using Oil Red O staining, while the extracellular calcium deposits formed due to osteogenic differentiation were highlighted using Alizarin red staining (Figure 1). These results demonstrate that the isolated cells exhibited typical MSC characteristics.

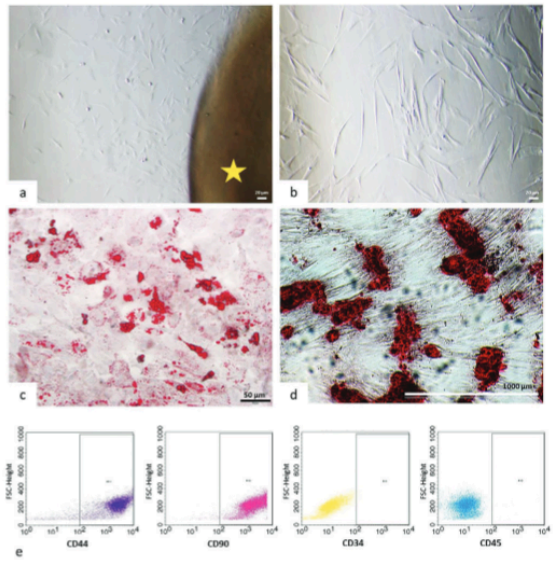

FIG. 1. (a-d) Isolation and characterization analyses of mesenchymal stem cells. Representative inverted microscope images (a, b) asterisk: umbilical cord tissue, representative adipogenic differentiation image, Oil Red O staining (c), representative osteogenic differentiation image, Alizarin red staining (d), flow cytometric analysis of CD34, CD45, CD44, and CD90 cell surface markers (e).

Content analysis of the conditioned medium

Based on the BCA analysis of the CMs at the different predefined time intervals, the total protein concentrations were determined to be 855.76, 810.74, 1026.75, and 1017.73 µg/ml in the 24 h-N-CM, 48 h-N-CM, 24 h-VPA-CM, and 48 h-VPA-CM, respectively. No measurable levels of IFN-γ, IL-10, IL-17, or GDNF were detected in the CMs. While the amount of NGF was found to be 109.82 pg/ml in 24 h-N-CM, it was 79.58 pg/ml in 48 h-N-CM, 588.91 pg/ml in 24 h-VPA-CM, and 92.55 pg/ml in 48 h-VPA-CM. The BDNF levels were detectable in 48 h-N-CM (42.5 pg/ml) and were significantly elevated within 48 h of VPA induction (300.83 pg/ml). Subsequently, the evaluation of VEGF levels revealed concentrations of 104.44 pg/ml in 24 h-N-CM, 51.85 pg/ml in 24 h-VPA-CM, 418.52 pg/ml in 48 h-N-CM, and 30.00 pg/ml in 48 h-VPA-CM. The data are presented in Figure 2.

Transmission electron microscopic evaluation

The ultrastructural examination of MSCs treated with VPA, a HDAC inhibitor, for 24 and 48 hours revealed that all groups exhibited euchromatic nuclei. MSCs typically possess euchromatic nuclei with minimal heterochromatic material. In mesenchymal stem cells without any treatment (N-MSCs), heterochromatin was located immediately beneath the nuclear membrane. Nevertheless, the nuclei of 24 h VPA-MSCs exhibit an even greater reduction in heterochromatin levels. In 48 h VPA-MSCs, the amount of heterochromatin was higher compared to 24 h VPA-MSCs. In comparison to N-MSCs, the nucleus of both 24 h and 48 h cells was deficient in heterochromatin (Figure 3). The cytoplasmic contents of the cells were semi-quantitatively quantified in terms of RER, autophagic vesicles, and myelin figures. Table 1 illustrates the results. Consequently, it was observed that the number of RER ($p < 0.001$) and autophagic vesicles ($p < 0.001$) in 24 h-VPA-MSCs and 48 h-VPA-MSCs increased. Additionally, the increase in myelin figure structures in 48 h VPA-MSCs was statistically significant ($p < 0.05$). In general, cells treated with VPA exhibited branched and lengthy mitochondrion structures. Representative micrographs of the groups are displayed in Figure 4. Furthermore, peripherally organized cytoskeletal filaments were observed in 48 h VPA-MSCs (Figure 5).

DISCUSSION

This study aimed to investigate cellular ultrastructural changes and the content of specific factors in the secretome produced following the preconditioning of MSCs with VPA, a HDAC inhibitor commonly used to treat conditions such as epilepsy and bipolar disorder. The findings were then evaluated in a correlative manner. The inhibition

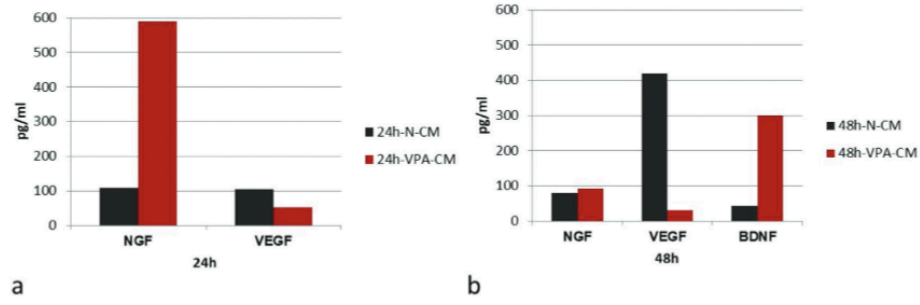

FIG. 2. (a, b) Growth factor and cytokine analysis. (a) 24 h-N-CM and 24 h-VPA-CM, (b) 48 h-N-CM and 48 h-VPA-CM.
NGF, nerve growth factor; VEGF, vascular endothelial growth factor; BDNF, brain-derived neurotrophic factor; h, hours; VPA, valproic acid; CM, conditioned medium.

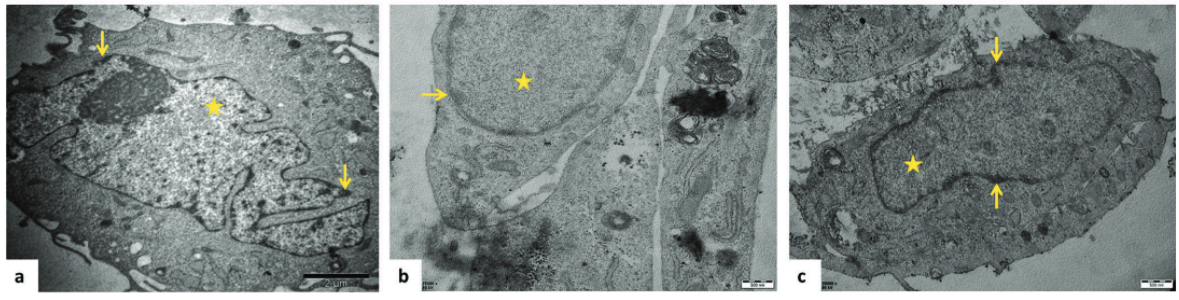

FIG. 3. (a-c) Transmission electron microscopy-based evaluation of nuclear chromatin. Stars: nucleus, arrows: chromatin. N-MSC (a), 24 h-VPA-MSC (b), 48 h-VPA-MSC (c).
N-MSC, mesenchymal stem cells without any treatment; h, hours; VPA, valproic acid.

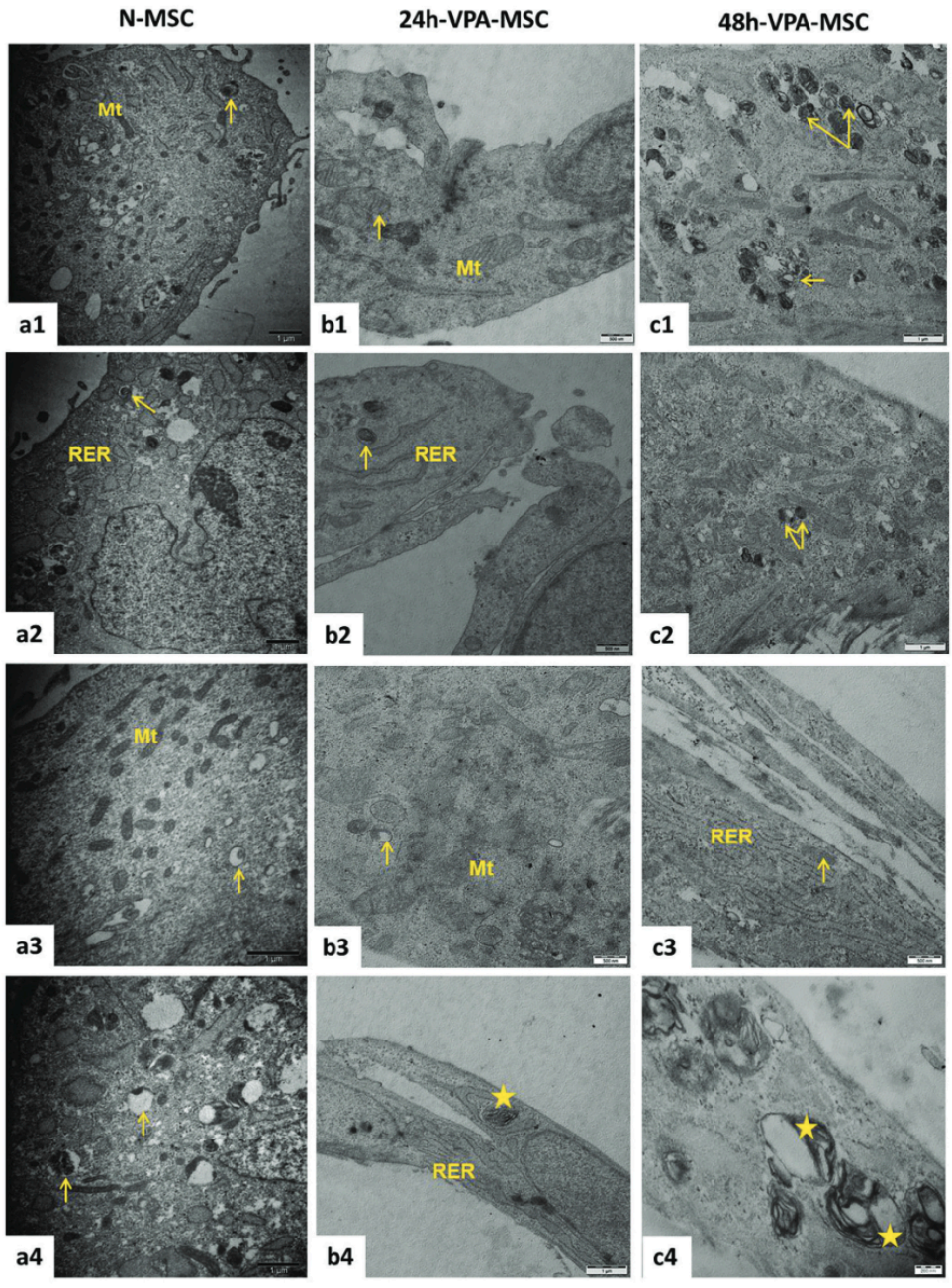

FIG. 4. (a-c) Ultrastructural analysis of cellular cytoplasmic content.
RER, rough endoplasmic reticulum; Mt, mitochondria, stars: myelin figures, arrows: autophagic vesicles. N-MSC (a1-a4), 24 h-VPA-MSC (b1-b4), 48 h-VPA-MSC (c1-c4).
N-MSC, mesenchymal stem cells without any treatment; h, hours; VPA, valproic acid.

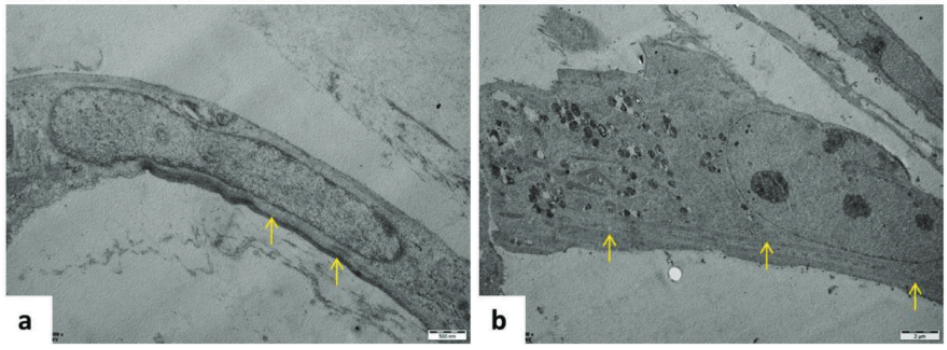

FIG. 5. (a, b) Transmission electron microscopy-based evaluation of 48 h-VPA-MSCs. Arrows: peripherally organized cytoskeletal filaments.
h, hours; VPA, valproic acid; MSC, mesenchymal stem cells.

TABLE 1. Data Analysis of Cellular Ultrastructural Components Included the RER Density Per Cell, the Number of Autophagic Vesicles, and the Number of Myelin Figures.

	RER	Autophagic vesicles	Myelin figures
N-MSC	1.2 ± 0.13	1.45 ± 0.28	0.5 ± 0.22
24 h VPA-MSC	1.87 ± 0.19[a]	2 ± 0.24	1.06 ± 0.46
48 h VPA-MSC	2.5 ± 0.13[b,c]	4.05 ± 0.56[d,e]	3.25 ± 0.71[f,g]

N-MSC, mesenchymal stem cells without any treatment, 24 h-VPA-MSC, mesenchymal stem cells preconditioned with valproic acid for 24 hours, 48 h-VPA-MSC, mesenchymal stem cells preconditioned with valproic acid for 48 hours, RER, rough endoplasmic reticulum. [a]$p < 0.05$ vs. N-MSC, [b]$p < 0.001$ vs. N-MSC, [c]$p < 0.05$ vs. 24 h-VPA-MSC, [d]$p < 0.01$ vs. N-MSC, [e]$p < 0.01$ vs. 24 h-VPA-MSC, [f]$p < 0.01$ vs. N-MSC, [g]$p < 0.05$ vs. 24 h-VPA-MSC.

of HDAC in cells leads to the loosening of chromatin structures, a reduction in condensed chromatin, and, consequently, elevated gene expression.[13,19] Baumann et al.[20] have examined embryonic stem cell differentiation in response to HDAC inhibitors and detected alterations in the cellular chromatin structure. This VPA effect can be attributed to the decreased heterochromatin observed in MSC nuclei preconditioned with VPA in this study, and therefore a corresponding increase in cellular activity would be expected.

At the cellular level, ultrastructural analysis of MSCs preconditioned with VPA revealed an increase in the RER after 24 and 48 h of preconditioning. The semi-quantitative assessment showed a gradual rise in RER correlating with the duration of exposure. Previous studies have demonstrated that this ultrastructural alteration indicates increased protein synthesis.[21] In this context, examining the secretome secretion capacity of the cell by considering the total protein concentration in CM revealed that the concentrations in VPA-24 h-CM and VPA-48 h-CM were higher than those secreted by N-MSCs. Similar increases in protein secretion were observed when the cells were preconditioned for 24 and 48 h. These findings suggest that preconditioning with VPA enhances cellular activity. The increase in cellular activity and protein secretion in VPA-treated MSCs can be attributed to VPA-induced histone (H3)[13] and (H4)[15] hyperacetylation. It has been demonstrated that the dose of VPA used determines the occurrence of H3 hyperacetylation. Our study's dosages and durations were effective in increasing the secretome content, as this effect is known to be dose- and time-dependent. Furthermore, prior research has indicated that VPA upregulates the G-protein-coupled chemokine receptor (CXCR4) transcript, which is associated with histone hyperacetylation in the *CXCR4* gene promoter region.[13,22] Consequently, increased CXCR4 expression indicates chromatin remodeling. Our results, when combined with the electron microscopy findings, indicate that VPA exhibits these effects through chromatin organization and histone hyperacetylation, which is consistent with the existing literature.

After analyzing studies aimed at improving the therapeutic potency of VPA in cells, it appears that VPA treatment of MSCs leads to increased expression of various genes related to trophic effects, antioxidant activity, anti-apoptosis, cytokine/chemokine receptors, migration, stress response, and mitochondrial energy metabolism signaling pathways, as reported by Linares et al.[23] In experimental therapies for Huntington's disease, the beneficial effects of enhanced expression of these genes have been demonstrated. Another study revealed that VPA application at a concentration of 1 mM to human adipose tissue-derived MSCs upregulated cellular somatostatin expression. Therefore, it can be predicted that VPA may stimulate pancreatic endocrine lineage differentiation in cells, which could be applied to diabetes treatment.[24] Consistent with the effects reported in the literature, our findings, particularly concerning the secretome, revealed that cells preconditioned with VPA possessed potential therapeutic properties. The study findings pertaining to the increase in secretome significantly contribute to the existing literature.

Additionally, preconditioning MSCs with VPA increased the number of autophagic vesicles and myelin figures in the cell cytoplasm. The increase in autophagic vesicles and the corresponding myelin figures,[25] which is particularly evident in cells treated with VPA for 48 hours, suggests that the autophagic pathway is activated as a survival mechanism in response to the cellular stress initiated by VPA application.[26] The observed branched and long mitochondrial structures may be associated with VPA's capacity to enhance the expression of regulators that promote mitochondrial biogenesis.[27] Given that mitochondrial biogenesis is a crucial mechanism for

maintaining energy homeostasis and regulating cellular stress, it is essential to observe this process in VPA-treated MSCs. Autophagy has been previously reported to be induced by VPA in various tissues.[28,29] However, to our knowledge, our study is the first to demonstrate VPA-induced autophagy in MSCs.

Electron microscopic analysis of the cytoplasm in both VPA-preconditioned and non-preconditioned cells revealed that actin filament-like structures began to condense at the periphery of 48 h-VPA-treated cells. Ultrastructurally, the reorganization and increase of cytoskeletal components at the cell periphery have been associated with neuronal differentiation.[30] Furthermore, an increase in autophagy in human MSCs has been demonstrated to promote neural differentiation, with autophagy being triggered in differentiating cells.[31] It has been reported that VPA induces early neuronalal differentiation in adult MSCs by altering protein signaling pathways.[12] The application of 0.2 mM VPA to adult MSCs induces neuronal differentiation within 24 h by upregulating the expression of SOCS5 and FGF21. This process downregulates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway and activates the mitogen-activated protein kinase (MAPK) cascade. Bioinformatics analyses revealed the expression of several neuro-specific proteins, and various functional and structural proteins are involved in neuronal formation and development. In accordance with the observed ultrastructural differentiation and the existing literature, we examined the levels of NGF, BDNF, and GDNF, which are neuronal growth factors, as well as VEGF in the MSC secretome. In MSCs that were preconditioned for 24 hours, we observed a substantial increase in NGF levels, while BDNF levels were observed to increase in those MSCs that were preconditioned for 48 hours. None of the four cell groups exhibited measurable levels of GDNF. Additionally, while VEGF levels were initially high in normal MSCs, they gradually decreased in cells preconditioned with VPA for 24 and 48 hours. After evaluating the secretome content and electron microscopic observations, it was evident that the VPA-preconditioned cells may have been in the early phases of neuronal differentiation. These findings hold significance for studies concerning the MSC secretome and the induction of neuronal differentiation in MSCs. We can infer that VPA could serve as a significant priming agent in MSC-based treatments for neurodegenerative diseases, given the advancements in targeted modification of secretomes.

It has been reported that VPA may positively impact the immunomodulatory properties of MSCs.[17] To evaluate this effect through secretome content, IL-10, IL-17, and IFN-γ levels were analyzed. However, they were not detectable at a measurable level in normal MSCs or in the secretome of VPA-preconditioned MSCs. Although the immunomodulatory properties of MSCs have been well documented, recent literature indicates that their cytokine secretion capacity is limited; however, they can be enhanced under certain conditions.[32,33] This study illustrated that VPA does not influence the release of specific cytokines. However, given the multifaceted nature of cellular immunomodulatory effects, there is a need for more comprehensive research into this subject.

In conclusion, this study displayed the ultrastructural impact of preconditioning MSCs with VPA and assessed the extent to which these structural modifications affect cellular function. Consequently, VPA functioned as a HDAC inhibitor, activating elevated cell secretome production. Even though the levels of secreted proteins were comparable between the 24 h and 48 h applications, it is evident that their protein compositions differ. Prolonged exposure to VPA facilitated cellular differentiation. Furthermore, the observed early neuronal differentiation and corresponding alterations in growth factor levels illustrate the potential for targeted modification of the MSC secretome. Our study contributes fresh data to the literature and offers insights into potential routes for future investigation, particularly regarding the potential of HDAC inhibitors in modulating the secretome of cells and enhancing their paracrine effects. However, it is evident that standardized and detailed studies are required to comprehensively understand the secretome of MSCs and their diverse responses to external stimuli.

Ethics Committee Approval: The requisite ethical approvals were provided by İstanbul University-Cerrahpasa, Cerrahpasa Faculty of Medicine (approval number: E-83045809-604.01.01-380308, date: 10.05.2022).

Informed Consent: The patient provided written, informed consent.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authorship Contributions: Concept- B.I., S.Ö., M.K.; Design- B.I., S.Ö., M.K.; Supervision- M.K.; Materials- B.I., S.Ö.; Data Collection or Processing- B.I., S.Ö.; Analysis and/or Interpretation- B.I., S.Ö., M.K.; Literature Search- B.I., S.Ö., M.K.; Writing- B.I., S.Ö.; Critical Review- B.I., S.Ö., M.K.

Conflict of Interest: The authors declare that they have no conflict of interest.

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