VALPROIC ACID-INDUCED HEPATOCYTE-LIKE DIFFERENTIATION OF HUMAN AMNIOTIC EPITHELIAL STEM CELLS IN 2D MICROENVIRONMENT

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ABSTRACT: This experimental *in-vitro* study assessed the effectiveness of the 21-day differentiation of human amniotic epithelial cells (hAECs) into hepatic-like cells (HLC), displaying hepatocytes with functional features using culture media supplemented with Valproic Acid (VPA). hAECs were isolated from the human placenta which was taken from patients with full-term pregnancy after C-sections. The differentiated cells were analyzed for hepatic markers by qPCR, PAS staining for glycogen storage & Immunocytochemistry. The results demonstrated that the hAECs with large nuclei, sparse cytoplasm, and cobblestone-like structure morphology changed into single cells characterized by a polygonal shape with abundant cytoplasm, and having huge bright vacuoles resembling fat droplets after being treated with 2.5mM concentration of VPA for 21 days. These cells exhibited substantial upregulation of hepatic gene expression, CYP3A4, CYP27B1, Alpha-fetoprotein (AFP), Albumin, HNF1A, HNF3B, and hepatic protein, Albumin, signifying that hAECs possessed normal functioning hepatocyte characteristics. Analysis of H3 and H4 by immunofluorescence showed an increase in VPA-treated cells indicating alteration in the histone acetylation of the cells. Further analysis showed enhanced glycogen storage in the differentiated cells, a significant functional characteristic of hepatocytes. This research identified an appropriate substrate for the differentiation of hAECs into functional hepatocytes, their successful therapeutic application would represent an advancement towards the development of tissue-engineered liver tissue.

Keywords: Amniotic Membrane, Hepatocytes, Stem cells, Liver Diseases.

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INTRODUCTION

Around 56.8 million people globally are infected with chronic HCV, with around 51% concentrated in six countries, which include: India, the United States, Russia, Egypt, Pakistan, and China [1]. Worldwide, around 2 million deaths are related to liver diseases per year [2]. Orthotropic transplantation, being the only substantial treatment for liver end-stage diseases, is disadvantaged due to the extreme shortage of donors of liver organs, tissue/organ rejection, infection transmission, and complications from long-term immunosuppression usage [3]. Tissue engineering and cell-based therapies can be considered liver transplantation alternatives. For this purpose, researchers are pursuing the differentiation of hepatocytes from stem cells, which include induced pluripotent stem cells, embryonic stem cells, mesenchymal stem cells, and hepatic progenitor/stem cells [4]. However, researchers have recently shifted their focus to human placental stem cells due to the beneficial characteristics of their cells in this field, particularly human amniotic epithelial cells (hAECs) have received much attention lately, making them promising for clinical usage [5,6].

Primary hAECs are believed to have various attributes to be considered the most appropriate for cellular therapies. In addition to the already-known advantages of the placental stem cells, many other significant characteristics of hAECs have also added to their potential in the field of regenerative medicine, mainly their pluripotent capacity and expression of pluripotency surface markers, like TRA-1-81, TRA1-60, SSEA-3, and SSEA-4, similar to human embryonic stem cells [7]. They are also characterized by pluripotent cell-specific gene expressions, for instance, NANOG, SOX-2, OCT-4, REX-1, and TDGF-1 [8, 5]. hAECs can differentiate into several cell types *in-vivo* and *in-vitro*, such as alveolar type II cells, cardiomyocytes, neurons, and hepatocytes among others [9]. In some studies, authors have reported trilineage differentiation i.e. chondrogenic, osteogenic, adipogenic as well as hepatic differentiation [10-12]. The relative ease of hAECs' differentiation into functional and mature hepatocytes has specifically drawn interest along with their ability to colonize injured tissue following *in vivo* experiments. Therefore, various studies have been done to modify the current protocols or find new ones to obtain hepatocyte-

like cells (HLCs) using hAECs' potential for hepatic differentiation [13].

Several studies have reported successful induction of hAECs into HLCs with a multi-step method using hepatocyte growth factor, dexamethasone, insulin-like growth factor, and other various cytokines [14]; a hepatic differentiation-specific protocol [5] (Maymo et al, 2018); and a 4-step hepatic differentiation [15]. Particular interest is being given to Histone deacetylase inhibitors, like Valproic Acid (VPA), sodium butyrate, and trichostatin A, which through epigenetic modification of chromatin can initiate hepatic differentiation [16]. Particularly VPA, which is a short-chain fatty acid, is minimally cytotoxic and biologically relevant as an established drug being used in epilepsy treatment as well as a mood stabilizer [17]. There is less research done on VPA-induced differentiation in different stem cell types. Moreover, the ability of VPA to induce hepatic differentiation from hAECs and the molecular mechanism behind it is not well known and has been studied to a lesser extent. Therefore, this research aimed to evaluate the efficiency of the differentiation of human amniotic epithelial cells into cells displaying characteristics of functional hepatocytes using small molecules.

METHODOLOGY

This experimental invitro study was conducted at MDRL-1 Research Lab in Ziauddin University Clifton between May 2023 – June 2024 using a purposive sampling technique. The study included the Human Placenta (Amniotic membrane or Amnion) from pregnant females with full-term after C-section aged between 20-30 years pregnancy with any complication i.e., hypertension, maternal diabetes, placenta previa, thyroid abnormalities, placenta abruption, or any calcification. This study was conducted on 20-25 Human placenta-derived amniotic epithelial stem cells (AECs) and each experiment was done in triplicate.

Sample Collection: After obtaining approval from the Ziauddin University Ethical Research Committee, the human placenta was obtained from consenting mothers upon elective cesarean-section delivery, following normal term pregnancy (38 weeks gestation), under sterile conditions at the Dr. Ziauddin Hospital, Clifton. The placental amniotic membrane was stripped manually from the chorionic layer and then cut into 7 cm long pieces, rinsed with sterile PBS twice to remove the blood clots, transferred to a falcon containing 20 ml of PBS, 0.5% EDTA, and 100 µl Pen Strep, shifted to the cell culture laboratory for the isolation of hAECs.

hAECs Isolation: For the isolation of hAECs, after cutting the amniotic membrane into small pieces, it was washed with sterile PBS and digested with 10% Trypsin. The plates containing amniotic membrane with 10% trypsin were incubated for 50 minutes in 5% CO_2. With the help of two sterile forceps, the gel was removed, and αMEM culture medium was added. Centrifugation was done and then the pellet was incubated for cell proliferation in a CO_2 incubator at 37°C. After every three days, the medium was replaced till 70% cell confluency was achieved.

Sub Culturing of hAECs: Subculturing was done when cells became 70% confluent. The media was removed and sterile PBS was used to wash the cells. After that, 1.5 ml of 1X trypsin was added to each 25 cm^2 cell culture flask and incubated at 37^{o}C for 4-5 minutes, then cells were observed for detachment under a live cell imaging microscope. To stop trypsinization, 3 ml of αMEM was added and transferred into a falcon tube, followed by centrifugation at 1000 rpm for 8 minutes, the obtained pallet was then transferred to a sterile 25 cm^2 flask, each containing 3ml of αMEM.

Cell Viability Analysis: Cell viability analysis of hAECs with different concentrations of VPA was done *in-vitro* by taking a 30000 cells/ml suspension which was then added to a 24-well plate, and incubated for 24 hours for cell attachment to the plate surface. Media was removed when cells were attached, and replaced with different concentrations of VPA (1mM, 2.5mM, and 5mM). Cell viability of hAECs was calculated after 48 hrs.

Hepatic Differentiation Induction of hAECs *in-vitro:* Control cells were cultured in αMEM only, whereas, for hepatic differentiation induction, hAECs were first cultured in a serum-free medium for 24 hours. In the treatment group, the cells were treated with 5 mM VPA for 3 days after the incubation period. On the 4th day, 2.5mmol VPA was added, and media was replaced every third day for 3 weeks.

Analysis of Differentiated Cell:

Morphological Examination: The morphological changes of hAECs were analyzed after treatment with VPA differentiation protocol at 24 hrs., 7-, 14-, and 21 days under a live cell imaging microscope. The changes in morphology were observed in the treated hAECs and compared with the control cells.

Gene Expression Analysis:

RNA Isolation: RNA from the differentiated hepatocytes and undifferentiated (control) hAECs were isolated by using a Trizol reagent. Chloroform was added and the tube was vortexed followed by incubation for 15 mins at room temperature (RT). Phase separation was done through centrifugation and the aqueous phase was transferred into an Eppendorf tube. Chilled 1ml isopropanol was added for precipitation with incubation at RT followed by the addition of 1ml of 70% ethanol.

The RNA pellet obtained after removing the supernatant was air-dried and dissolved in 20-30µL of RNase-free water. The isolated RNA purity and concentration were analyzed using a multi-scan sky spectrophotometer.

Synthesis of cDNA: For the synthesis of cDNA, the Revert Aid First Strand cDNA synthesis kit was used accordingly. The sample amount to be utilized was in equivalence to 0.5µg of separated RNA. All procedures were performed on ice in sterile nuclease-free 200µL micro-centrifuge tubes. Primer designing was done online and was bought from Penicon.

Quantitative real-time PCR: The expression of liver-specific human gene makers panel, which included metabolizing enzymes (CYP3A, CYP27B1, and CYP24A1), and hepatocytes (AFP, ALB, G6PC, HNF1B, and HNF3B, HNF4B), were analyzed in the control and differentiated cells. GAPDH was used to normalize the expression. Initiation was done at 95^{o} C for 4mins, then denaturation was done at 95°C, annealing at 57-59°C, and extension at 72°C for 40 cycles for 30 secs.

Protein expression analysis by Immunocytochemistry: VPA-treated and control HAECs were cultured in a 24-well plate to perform immunocytochemistry. Cell fixation was carried out by using 4% paraformaldehyde after proper cell attachment, followed by incubation at RT for 20 mins, and permeabilized with Triton X-100 (0.1%). After washing with PBS, cells were incubated at RT for 1 hour with a blocking solution, and then overnight incubated at 4^{o}C with primary antibodies at 1:150 dilutions against H3, H4, and Albumin. After overnight incubation, the antibody solution was removed, washed with 1X PBS, and incubated at RT for 1 hour with secondary antibody Alexa Fluor 546 donkey anti-rabbit at a 1:100 dilution. Nuclei counterstaining was done with 0.5 g/mL 4', 6-Diamidino-2-phenylindole (DAPI). Lastly, cells were rinsed with PBS, mounted with a mounting medium, and observed under an inverted fluorescent microscope.

PAS Staining: The presence of glycogen was assessed in the treated cells after 21 days by PAS staining. Cells were fixed in PFA and permeabilized with 0.1% Triton X-100 in a 24-well plate for 10 mins. Cells were oxidized for 5 minutes with Periodic acid and then again rinsed with PBS. After this, cells were incubated for 5 minutes with Schiiff's Reagent and washed with PBS. Subsequently, counterstaining of cells with Hematoxylin was done for 1 minute and observed under the light microscope.

Statistical Analysis: The SPSS program version 25 was used for statistical analysis. Results were analyzed as mean ± SD. For comparison between groups, One-way ANOVA followed by Tukey's post hoc test was done. A p-value ≤ 0.05 is to be considered significant.

RESULTS

Cell Morphology of Cultured and Differentiated hAECs: The isolated hAECs from the amniotic membrane were round with a small diameter having a rounded nucleus. After 72 hrs. in the αMEM culture medium, some of the hAECs became spindle-shaped adherent cells. Subsequently, on 7- and 14 days, these cultured cells had a cobblestone morphology along with an enlarged nucleus and a sparse quantity of cytoplasm in a monolayer form (Figure 1).

In the treatment group supplemented with VPA, polygonal-shaped cells with enlarged nuclei and granulated cytoplasm morphology were observed along with the presence of fat droplet-like large vacuoles. (Figure 2)

Determination of Cell Viability of VPA-treated hAECs: Cell viability analysis showed that hAECs with 5 mM VPA-supplemented media were toxic having a significant (p-value <0.001) increase in the number of apoptotic cells. However, 1- and 2.5 mM VPA concentrations were not toxic to the cells (Figure 3). In our experiments, 2.5 mM VPA concentration was used in the treatment groups.

Gene Expression Analysis: After 21 days, mRNA expression of CYP3A4, CYP24A1, CYP27B1, AFP, ALB, HNF1A, HNF3B and HNF4A were assessed in control as well as treated hAECs. GADPH was taken as the housekeeping gene. The treatment group showed varied patterns of upregulation of all the genes in comparison with the control. AFP($p<0.01$), CYP27B1($p<0.01$), and HNF3B ($p<0.01$) showed highly significant upregulation, whereas mRNA expression of CYP24A1 and HNF4A showed no significant upregulation. ALB($p<0.05$), CYP3A4($p<0.05$), and HNF3B ($p<0.05$) expression were also significant in the treatment group as compared to control. (Figure 4)

Protein Expression by Immunofluorescence Analysis: Histone acetylation was accompanied by the VPA-mediated hepatic differentiation of hAECs as shown in Figure 5 with an increase in H3 and H4 expression in the cells.

After 21 days of hepatogenic differentiation, immunofluorescence analysis exhibited that the cells were positive for Albumin, indicating that hAECs possessed normal functioning hepatocyte characteristics. (Figure 6)

Functional Assessment of Differentiated Cells: Treated hAECs were evaluated further for their functionality by PAS to corroborate their hepatic differentiation. Differentiated cells showed a strong PAS positive signal at 21 days, indicating their glycogen-storing capability compared to the untreated control. (Figure 7).

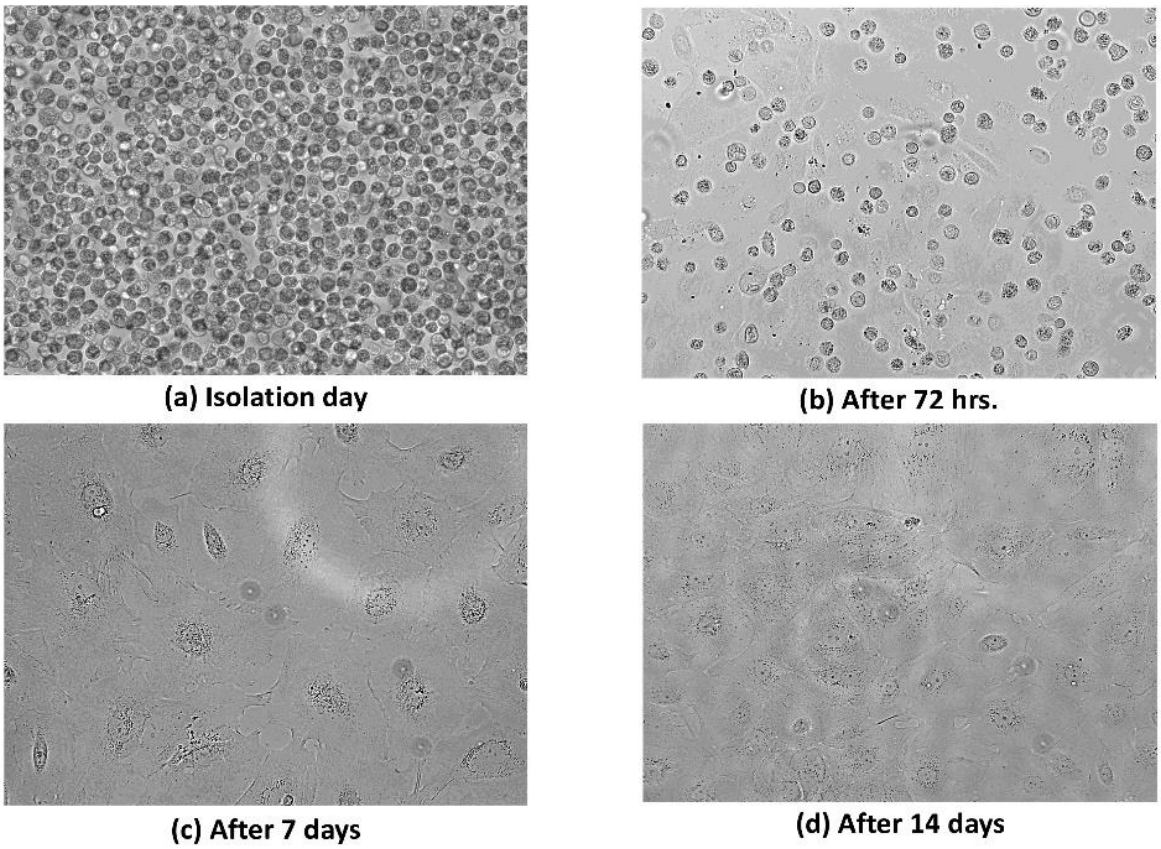

Figure 1: hAECs in the culture at passage 0 (P0) at (a) day 1 of isolation, (b) after 72 hours with few adherent cells, (c) after 7 days with cobblestone-shaped morphology, and (d) at 14 days with monolayer formation. Images were taken at 40× magnification under a live cell imaging microscope.

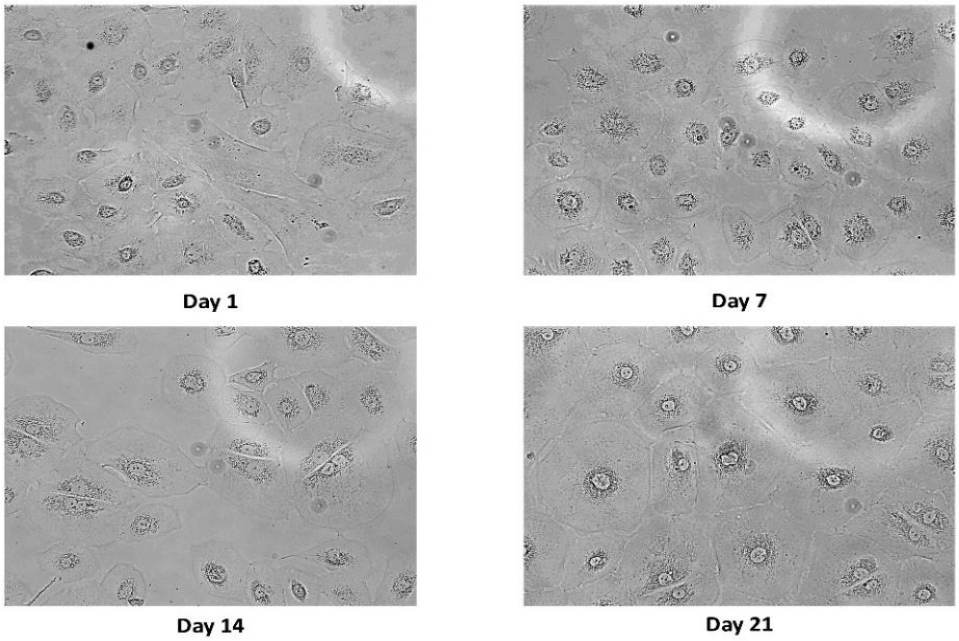

Figure 2: hAECs grown in the VPA-supplemented differentiation media for 21 days, changed from cobblestone morphology to polygonal-shaped cells with enlarged nuclei and granulated cytoplasm morphology.

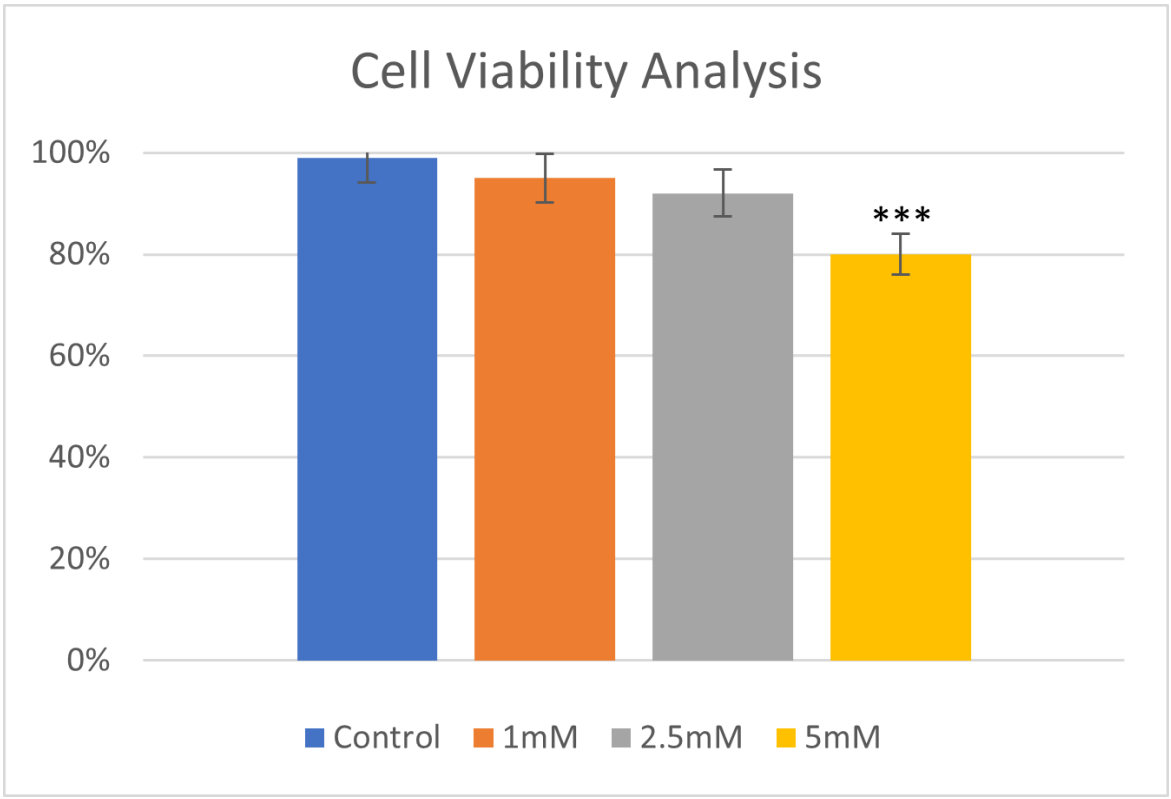

Figure 3: Measurement of cell viability showing the effect of various concentrations of valproic acid (VPA) on hAECs after 48 hrs. *P*-values < 0.05 were considered statistically significant, where ****P* < 0.001.

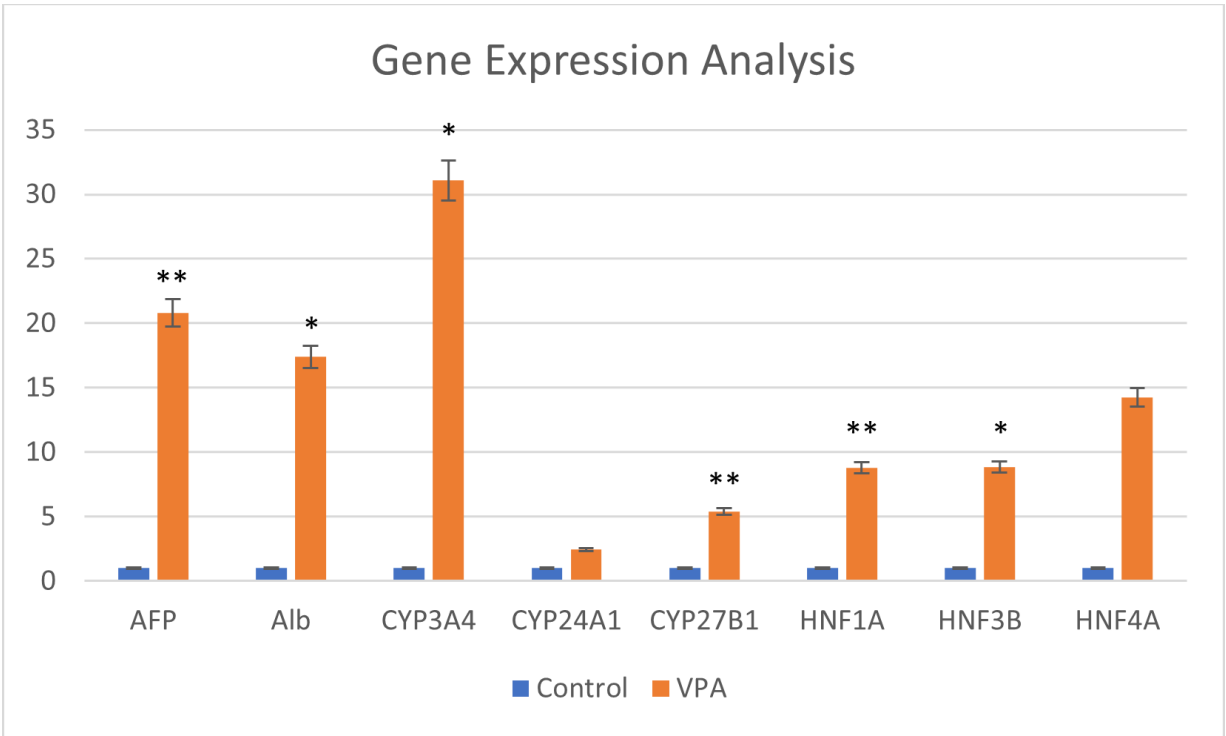

Figure 4: Bar diagram showing fold change analysis of hepatic gene expression by qPCR in VPA-treated hAECs in 2D culture. All data are presented as means ± standard error of means (SEM). *P* values < 0.05 were considered statistically significant, where **P* < 0.05, ***P* < 0.01.

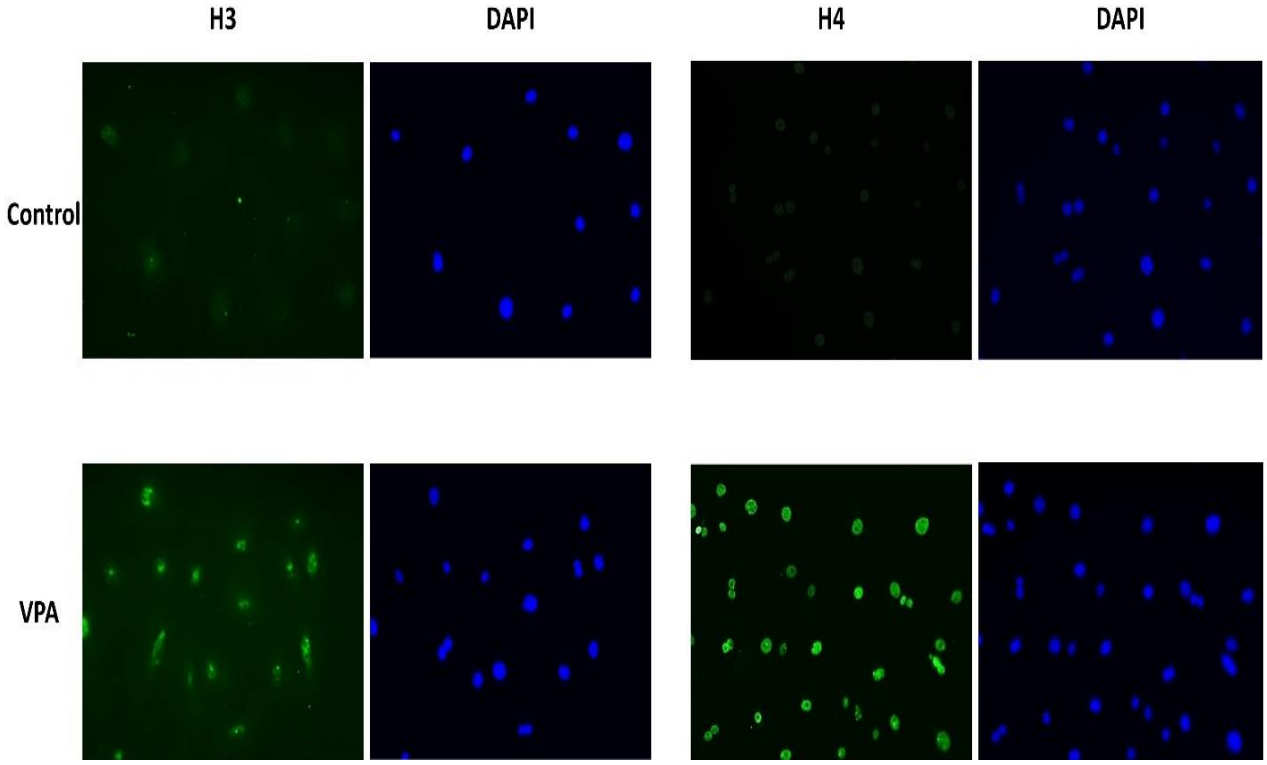

Figure 5: Immunofluorescence analysis of histones (H3 and H4) in VPA-treated and control hAECs. Alexa Fluor 546 donkey anti-rabbit secondary antibody was used for detection. Nuclei were stained with DAPI. Images were taken × 40 magnifications under the fluorescence microscope.

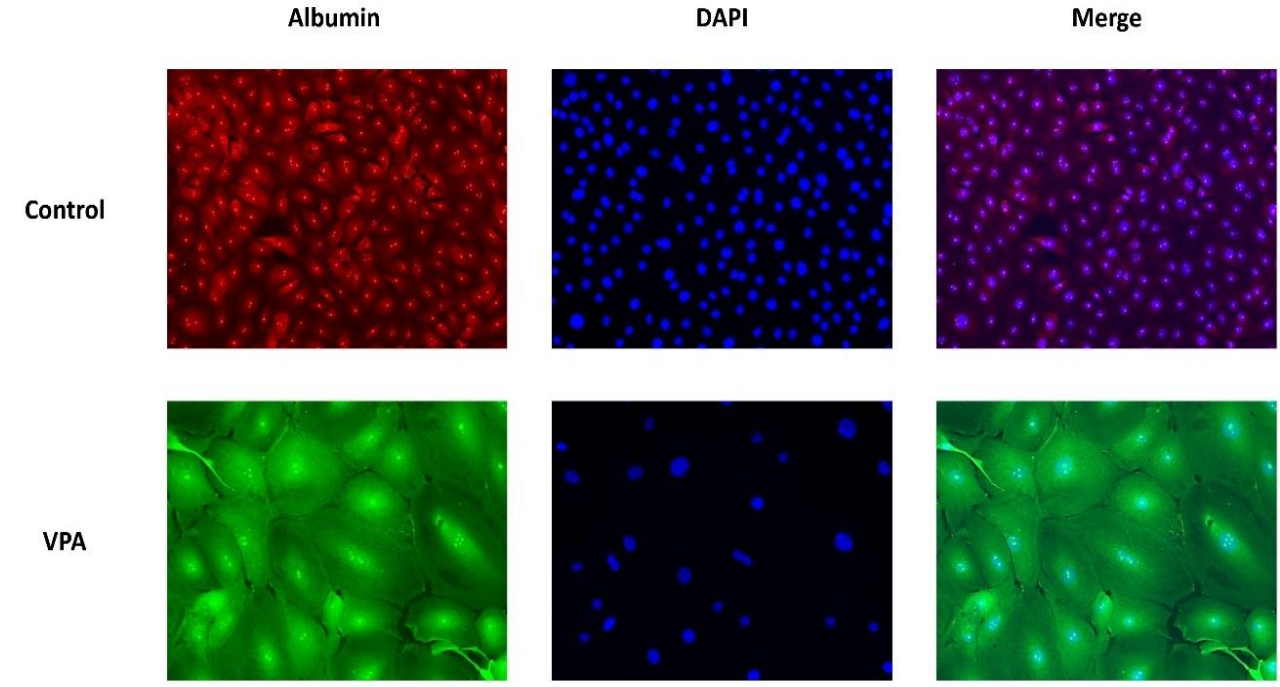

Figure 6: Immunocytochemistry analysis of hepatic protein, Albumin, in control and VPA-treated hAECs in 2D culture. Nuclei were stained with DAPI. Images were taken × 40 magnifications under the fluorescence microscope.

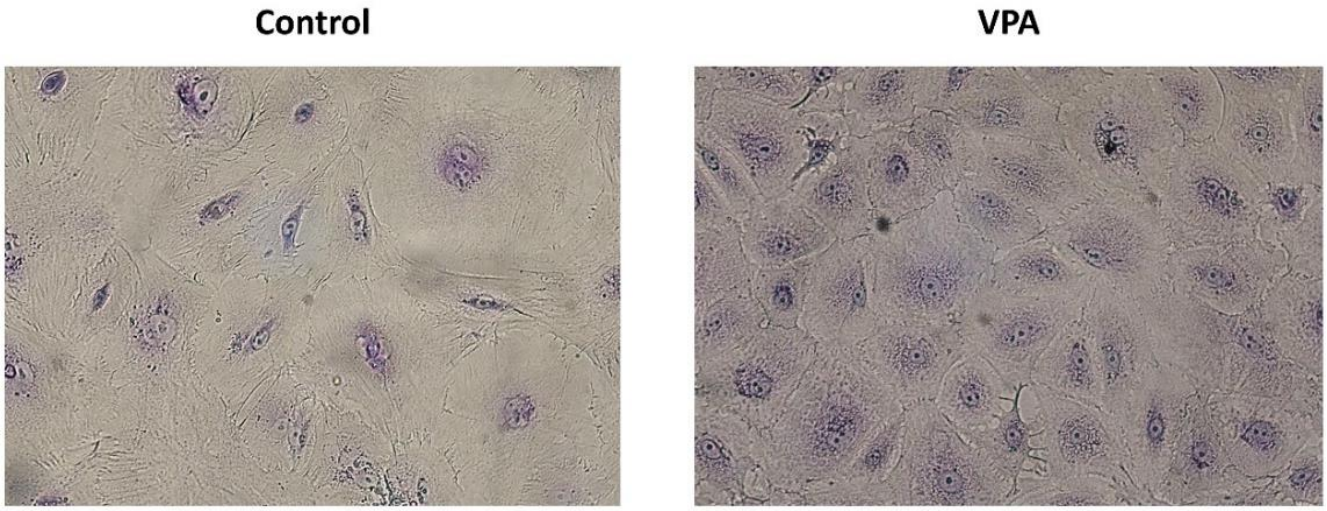

Figure 7: PAS staining of VPA-treated hAECs in 2D culture with their corresponding untreated controls. All images were taken in phase contrast at 40x magnification.

DISCUSSION

There is not enough knowledge regarding the molecular mechanism involved in the hepatic differentiation of hAECs and efforts are being made by researchers to find out the most appropriate and effective way to attain such a sort of differentiation [5]. Several researchers have used protocols that vary from a single step with hepatic stimulating and growth factors to multiple steps with various drugs to study the hepatic differentiation of hAECs. The hepatic progenitors' induction and maturation, rapid cellular proliferation, and HLC maintenance are the primary objectives. According to Miki et al., cultured hAECs exhibit both *in vitro* and *in vivo* expression of ALB and AFP. For 14 days, they employed a two-step procedure that promoted the hepatic maturation of hAECs. Also, they acquired HLCs that expressed C/EBP-α, albumin, alpha1-antitrypsin (α1-AT), and hepatocyte nuclear factor 4-alfa (HNF4-α) [12]. Whereas, Morangiu et al. applied a four-step, 21-day method for hepatic differentiation of hAECs. Cytochrome P450 genes, which are crucial for the metabolism of drugs in hepatocytes, were expressed by the differentiated cells [18]. In another study, Furuya et al confirmed that hAECs differentiated hepatocytes expressed distinctive liver markers using a two-step protocol of differentiation [19].

Using a straightforward one-step differentiation process with VPA, this study developed HLCs from hAECs successfully. Following 21 days of the differentiation process, these cells displayed hepatic properties such as glycogen storage, Hepatic Nuclear Factors, and CYP450 enzymatic induction and exhibited ALB protein which is a characteristic of hepatocytes.

When hAECs were first cultured in our study, they displayed small, spherical, cobblestone-shaped epithelial cells. They increased in number and eventually formed a monolayer. After day 21, they grew larger with abundant cytoplasm and started to form colonies. However, after being treated with VPA, amniotic cells rapidly multiplied and developed a monolayer by day 7. Their shape changed from epithelial to hepatocytic polygonal, with some multinucleated cells and cytoplasmic granules. By analyzing the expression of key liver markers after 21 days of VPA treatment, we were able to molecularly validate these findings. Hengstler et al. have examined the requirements needed to prove that hepatic-like cells and actual hepatocytes are identical. They emphasized the fact that some markers are easier to induce than others. Compared to albumin expression, cytochrome expression is more challenging to detect. They stressed how crucial it is to identify CYP3A4 expression [20]. Hepatocytes from the adult liver have significant amounts of albumin and CYP3A4 activity, while the primary serum protein expressed by the liver during fetal development is the AFP [21, 5]. Amnion cells that were cultured using the hepatic differentiation technique indicated significant levels of mRNA for the markers of fetal and adult hepatocytes, including CYP3A4, CYP27B1, HNF1A, HNF3B, ALB, and AFP. Moreover, following VPA therapy, albumin protein expression was elevated in our study.

The capacity of hAECs to differentiate into HLC is well known, and numerous studies employing various hepatic differentiation techniques have been published to determine the best procedure. The goal of liver tissue engineering and regenerative medicine is to promote the development of cell therapy based on hAECs for the treatment of liver diseases by obtaining definitively differentiated cells with functioning mature hepatocyte properties using both short-term and low-cost techniques.

Conclusion: In our study, we have demonstrated a simple, efficient, and cost-effective method using VPA that can successfully differentiate hAECs into hepatic-like cells (HLC) without the need for an exogenous supply of hepatic growth factors and suggests the possible application of these differentiated hepatocytes for liver tissue engineering.

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Authors Contributions: TR: Sample collection, experimentation, manuscript writing.
SU: Project designing, budgeting, experimentation, statistical inference, proofreading, critical analysis.
KH: Experimentation, supervision, proofreading, result analysis.
TM: Critical analysis, proofreading, supervision.
TU: Sample collection and isolation.

Conflict of Interest: There is no conflict of interest.

Ethics Approval: Ethical approval was obtained from the Ziauddin Ethical Review Board.

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