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## EFFECT OF CONDITIONED MEDIUM FROM 3D SPHEROIDS OF HUMAN UMBILICAL CORD MESENCHYMAL STEM CELLS ON ENDOTHELIAL CELL MIGRATION IN AN IN VITRO WOUND-HEALING MODEL

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### ABSTRACT

Three-dimensional (3D) spheroid culture may modulate the paracrine activity of mesenchymal stem cells. This study compared the effects of conditioned media from two-dimensional (2D) cultures and 3D spheroids of human umbilical cord mesenchymal stem cells (hUC-MSCs) on endothelial wound closure *in vitro*. Passage-3 hUC-MSCs were cultured as adherent monolayers or assembled into spheroids using MicroTissues® agarose micromolds. Spheroid morphology and diameter were assessed at 24, 48, and 72 h. Immunofluorescence analysis included CD73, CD90, CD105, and CD45 in 2D cultures and CD90 and CD105 in intact 3D spheroids. Human umbilical vein endothelial cells (HUVECs) were exposed to 2D-conditioned medium (2D-CM), 3D-conditioned medium (3D-CM), or endothelial growth medium (control). Scratch-assay images were acquired at 0 and 24 h, and wound closure was calculated relative to the initial cell-free area. Spheroids formed in all 81 assessed microwells. Mean diameter increased from  $310 \pm 20 \mu\text{m}$  at 24 h to  $420 \pm 25 \mu\text{m}$  at 48 h and  $500 \pm 30 \mu\text{m}$  at 72 h (mean  $\pm$  SD). CD90 and CD105 remained detectable following spheroid formation. At 24 h, wound closure estimated from individually selected images (not from independent experiments) was approximately 100%, 66.0%, and 72.7% for 3D-CM, 2D-CM, and control, respectively. These preliminary descriptive findings suggest greater endothelial wound closure with 3D-CM; confirmation requires matched-field analysis across independent experiments with statistical analysis.

**Key words:** Mesenchymal stem cells, 3D spheroid culture, conditioned medium, endothelial cell migration, scratch wound-healing assay, angiogenesis, paracrine signaling, regenerative medicine

### INTRODUCTION

Human mesenchymal stem cells (MSCs) have attracted considerable interest in regenerative medicine because their biological effects extend beyond direct cellular replacement. Increasing evidence indicates that soluble mediators and other extracellular products released by MSCs contribute substantially to their interactions with surrounding cells and tissues [1,2]. Consequently, culture conditions that modify MSC organization and paracrine signaling may influence their functional activity.

Conventional two-dimensional (2D) culture provides a practical system for MSC expansion but imposes an artificial planar environment with limited cell–cell contact. Three-dimensional aggregation restores extensive intercellular interactions and creates local gradients of nutrients, oxygen and signaling molecules that are absent or less pronounced in monolayer culture. These changes can influence cellular organization, extracellular matrix interactions and the composition of products released into the surrounding medium [3,4]. Comparative studies of 2D and 3D MSC cultures have reported differences in cellular metabolism [5], phenotypic homogeneity and immunomodulatory activity [6], cell size and lifespan [7], and stem-cell characteristics [8]. Spheroid culture has therefore emerged as a useful approach for investigating how spatial organization affects MSC function.

The biological properties of MSC spheroids depend strongly on the method of aggregation and the resulting aggregate dimensions. Spheroid size has been associated with differences in cellular state and the angiogenic activity of

MSC-derived soluble factors and extracellular vesicles [9]. Accordingly, reproducible control of aggregate formation is important when comparing the functional properties of 2D and 3D cultures. Non-adherent micromolding offers a relatively simple means of generating multiple spheroids under geometrically defined conditions without introducing an additional scaffold material into the cellular aggregate, unlike hydrogel-based 3D systems [10,11].

The paracrine consequences of spheroid formation are particularly relevant to vascular and regenerative applications [12]. MSC-derived conditioned media contain complex mixtures of biologically active molecules capable of influencing endothelial behavior [2,12], including extracellular vesicles whose potency differs between 2D and 3D culture conditions [13]. Previous studies have reported wound-related and pro-angiogenic activities of conditioned media or secreted products obtained from 3D MSC cultures [14–16]. However, these effects depend on cell source, spheroid configuration, conditioning protocol and recipient-cell model, making direct functional assessment essential for each culture system.

Human umbilical vein endothelial cells (HUVECs) provide a useful recipient-cell model for assessing endothelial responses to MSC-derived products. Scratch-based wound-closure assays can capture the integrated effects of conditioned media on endothelial migration, proliferation and cell spreading. Comparison of conditioned media generated under 2D and 3D conditions can therefore provide functional information on whether spheroid organization is associated with altered MSC paracrine activity.

Based on this body of evidence, the aim of the present

study was to investigate the effect of conditioned media derived from 3D hUC-MSC spheroids on in vitro wound healing, in comparison with conditioned media derived from conventional 2D-cultured hUC-MSCs, using a HUVEC scratch/wound-healing assay as a model of endothelial regeneration.

## MATERIALS AND METHODS

**Isolation and expansion of hUC-MSCs.** Human umbilical cord mesenchymal stem cells (hUC-MSCs) were isolated from umbilical cord tissue provided by Medicell (Astana, Kazakhstan). The tissue was obtained from a healthy full-term pregnancy with written informed consent, under institutional ethical oversight at the National Center for Biotechnology.

Cells were isolated using the explant (tissue-outgrowth) method, following the previously described protocol for Wharton's jelly-derived MSCs [17]. Briefly, umbilical cord segments were transported to the laboratory in phosphate-buffered saline (PBS; Gibco) supplemented with 1% antibiotic-antimycotic solution (Anti-Anti; Gibco), rinsed to remove residual blood, and cut into fragments of approximately 5 cm<sup>2</sup>. The umbilical vessels were carefully dissected and removed to minimize contamination with endothelial and perivascular cells. The remaining Wharton's jelly tissue was placed directly onto the surface of T25 culture flasks and maintained in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM; Gibco) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

Explants were left undisturbed for the first 5–7 days to facilitate attachment and initial cell outgrowth, after which the medium was replaced every 3–4 days. Spindle-shaped, fibroblast-like cells migrated from the explants and adhered to the culture surface within 10–14 days. After 2–3 weeks, the tissue fragments were removed once sufficient cellular outgrowth had developed. The primary adherent population (passage 0) was cultured to approximately 80–90% confluence before the first passage.

For subsequent monolayer expansion, cells were seeded at  $2.8 \times 10^4$  cells/cm<sup>2</sup> in the same complete medium, which was replaced every 2 days. At approximately 80% confluence, cells were detached using 0.25% trypsin-EDTA for 5 min at 37 °C. Following enzyme neutralization, cell suspensions were centrifuged at 1,000 rpm for 5 min, resuspended in complete medium, and assessed for cell number and viability using an automated cell counter with trypan-blue exclusion. Cells were expanded to passage 3 and used for subsequent comparisons between 2D and 3D culture conditions.

**Generation and morphological characterization of hUC-MSC spheroids using agarose micromolds.** Three-dimensional hUC-MSC spheroids were generated using a scaffold-free, non-adherent agarose micromold system (MicroTissues® 3D Petri Dish®, 12-81 format) comprising a 9 × 9 array of 81 recesses, with nominal dimensions of 800  $\mu$ m in diameter and 800  $\mu$ m in depth. Agarose inserts were prepared by dispensing 500  $\mu$ L of sterile 2% (w/v) agarose dissolved in 0.9% (w/v) sterile saline into each autoclaved casting mold. Following gelation at room temperature, the agarose inserts were carefully removed, transferred to 12-well tissue culture plates, and equilibrated in complete culture medium through two consecutive 15-min incubations.

Passage-3 hUC-MSCs were harvested by trypsinization, counted, and resuspended in complete culture medium. The seeding density was adjusted empirically to obtain spheroids within the intended diameter range of approximately 400–500  $\mu$ m, using approximately  $1.5\text{--}2.0 \times 10^4$  cells per recess, corresponding to approximately  $1.2\text{--}1.6 \times 10^6$  cells per agarose insert. A 190- $\mu$ L single-cell suspension was carefully dispensed into the central seeding chamber and left undisturbed for 1 h to allow gravitational settling into the microwells. Subsequently, 2.5 mL of complete culture medium was gently added to the surrounding well without disturbing the seeding chamber. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. The reservoir medium was replaced every 2–3 days while leaving the agarose inserts undisturbed.

Spheroid formation and morphology were examined at 24, 48, and 72 h using an inverted phase-contrast microscope. Quantitative morphometric analysis was performed on calibrated brightfield images using ImageJ (NIH, USA). The projected area (A) of each spheroid was delineated, and the equivalent circular diameter (d) was calculated as  $d = 2\sqrt{(A/\pi)}$ , providing a standardized linear measure of spheroid size [18]. At each time point, all 81 spheroids within a single agarose insert were evaluated, and diameters were expressed as mean  $\pm$  standard deviation (SD). These measurements characterized within-insert size variation rather than variability across independent biological replicates. Spheroid compactness, contour regularity, and the presence of loose aggregates or fragmentation were additionally assessed qualitatively using the same image set.

**Immunocytochemistry.** Immunocytochemical staining was performed to evaluate the expression of the mesenchymal stem cell-associated markers CD73 (Abcam, ab317462), CD90 (Abcam, ab181469), and CD105 (Abcam, ab231774), with CD45 (Abcam, ab10558) included as a hematopoietic-lineage marker. Staining was conducted on coverslip-adherent 2D cultures and intact 3D spheroids to examine marker expression in both culture formats.

For 2D cultures, hUC-MSCs were seeded onto sterile coverslips and allowed to attach overnight. The culture medium was subsequently removed, and the cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. Following fixation, the specimens were washed with PBS and subjected to blocking to reduce nonspecific antibody binding.

For 3D cultures, intact spheroids were recovered from the agarose micromolds before fixation. The micromolds were centrifuged at 10,000 rpm for 10 min and carefully inverted onto a sterile plastic surface to collect the released spheroids. The recovered spheroids were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, and subjected to the same blocking procedure as the 2D specimens. Spheroids were processed as intact aggregates without enzymatic dissociation.

Following blocking, 2D cultures and intact spheroids were incubated with the respective primary antibodies against CD73, CD90, CD105, or CD45, each diluted 1:100. After primary antibody incubation, the specimens were washed with PBS to remove unbound antibodies and incubated with species-appropriate secondary antibodies diluted 1:500: goat an-

ti-rabbit IgG H&L conjugated to Alexa Fluor 488 (Abcam, ab150081) or goat anti-mouse IgG H&L conjugated to Alexa Fluor 555 (Abcam, ab150118). Following secondary antibody incubation, the specimens were washed with PBS, and nuclei were counterstained with DAPI. Subsequent handling was performed with protection from light to minimize photobleaching.

Fluorescence images were acquired using a Zeiss fluorescence microscope with filter sets appropriate for DAPI, Alexa Fluor 488, and Alexa Fluor 555. Marker-specific fluorescence was assessed qualitatively in 2D cultures and intact spheroids to evaluate the presence and apparent distribution of the investigated antigens.

**HUVEC scratch assay and wound-closure analysis.** Human umbilical vein endothelial cells (HUVECs; ATCC) were cultured in Vascular Cell Basal Medium (ATCC PCS-100-030) supplemented with the Endothelial Cell Growth Kit-VEGF (ATCC PCS-100-041) at the manufacturer-recommended concentrations. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> and used at passage ≤5.

For the scratch assay, HUVECs were seeded in six-well plates at  $1.0 \times 10^5$  cells/well and cultured to approximately 75–90% confluence. A linear scratch was generated using a sterile 200-μL pipette tip held perpendicular to the culture surface. The medium was removed, and the cells were gently washed twice with sterile phosphate-buffered saline (PBS) to eliminate detached cells and debris.

Following scratching, HUVECs were exposed to conditioned medium from 2D hUC-MSC monolayer cultures (2D-CM), conditioned medium from 3D hUC-MSC spheroids (3D-CM), or fresh complete endothelial growth medium (EGM; control). Cultures were incubated at 37 °C and 5% CO<sub>2</sub>, and phase-contrast images were acquired using an inverted microscope immediately after scratching (0 h) and after 24 h.

The cell-free wound area was measured in ImageJ using calibrated images. Wound margins were delineated, and percentage wound closure was calculated according to the following equation [19]:

Wound closure (%) =  $100 \times (A_0 - A_{24})/A_0$ , where  $A_0$  is the cell-free area immediately after scratching and  $A_{24}$  is the remaining cell-free area after 24 h. Wound closure was interpreted as a combined measure of endothelial migration, proliferation, and spreading rather than migration alone. Fresh

EGM served as a reference control rather than a matched cell-free conditioning-medium control.

**Statistical analysis.** Spheroid diameters are expressed as mean ± standard deviation (SD), based on measurements of 81 spheroids per time point (24, 48, and 72 h). Individual spheroids within the same agarose insert were treated as observational units rather than independent biological replicates; therefore, these measurements describe within-insert morphometric variability. HUVEC wound closure at 24 h is reported descriptively as percentage closure relative to the initial wound area, calculated from individually selected images for each group rather than from multiple independent experiments. Accordingly, no measures of dispersion or statistical tests were applied, and the comparison among groups is descriptive only.

## RESULTS

Human umbilical cord mesenchymal stem cells (hUC-MSCs) maintained under conventional 2D culture conditions exhibited the fibroblast-like morphology characteristic of MSCs. Cells adhered to the culture surface and displayed an elongated, spindle-shaped appearance. During culture, cell density progressively increased, resulting in the formation of a relatively uniform adherent monolayer (Figure 1A).

Transfer to the non-adherent microwells of MicroTissues® micromolds was accompanied by a distinct change in cellular organization. Instead of spreading across the culture surface, hUC-MSCs assembled into small, loosely packed aggregates. These aggregates subsequently became more compact and developed increasingly rounded outlines, forming discrete multicellular spheroids (Figure 1B). The observed transition from elongated, surface-adherent cells to compact three-dimensional structures demonstrated successful spheroid formation using the micromold platform.

### Spheroid formation and size

Following morphological confirmation of spheroid formation, the consistency of aggregate development was assessed across the micromold field. Spheroids were observed in all 81 evaluated microwells, corresponding to a formation efficiency of 100% within the assessed field. Thus, spheroid formation occurred throughout the evaluated array rather than being restricted to individual microwells.

Morphometric analysis revealed a progressive increase in spheroid diameter during the observation period. Mean diam-

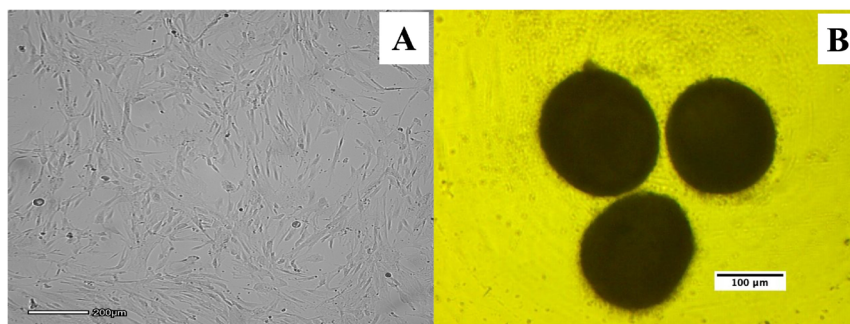


**Figure 1.** Morphological characteristics of hUC-MSCs cultured under 2D and 3D conditions.

A. hUC-MSCs cultured in 2D monolayer. B. hUC-MSC spheroids formed in 3D MicroTissues® micromolds.

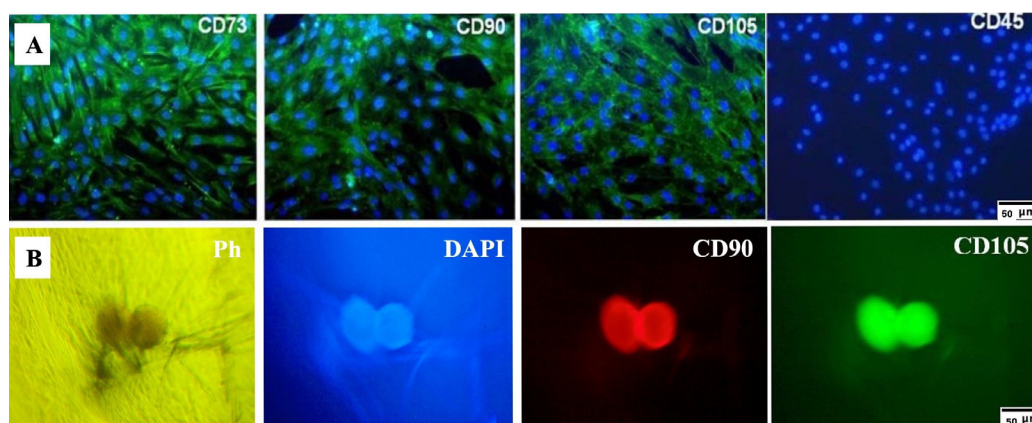


**Figure 2.** Immunocytochemistry of hUC-MSCs.

A. 2D-expanded hUC-MSCs. B. 3D spheroid-derived hUC-MSCs.

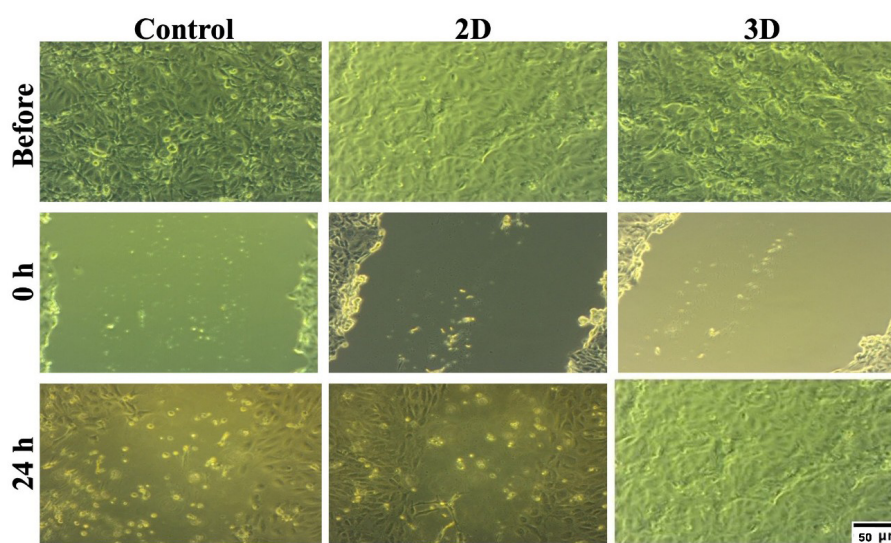


**Figure 3.** Representative images of HUVEC migration in response to conditioned media derived from 2D- and 3D-cultured hUC-MSCs.

eter increased from  $310 \pm 20 \mu\text{m}$  at 24 h to  $420 \pm 25 \mu\text{m}$  at 48 h and  $500 \pm 30 \mu\text{m}$  at 72 h (mean  $\pm$  SD;  $n = 81$  spheroids per time point). These measurements documented a time-dependent change in spheroid size over 72 h, accompanying the establishment of a compact, rounded multicellular morphology.

The reported measurements describe the distribution of spheroid diameters at each observation time within the evaluated micromold population. Individual spheroids formed within the same micromold were not considered independent biological replicates; therefore, these findings characterize within-field variation and temporal changes in spheroid size rather than an effect established across independent experiments.

#### *MSC-associated marker expression*

Immunofluorescence staining was performed to assess the expression of MSC-associated markers under 2D culture conditions and following spheroid formation. In 2D-expanded hUC-MSCs, positive staining was observed for CD73, CD90, and CD105, whereas CD45 fluorescence was not detected (Figure 2A). This staining pattern was consistent with the MSC-associated immunophenotype of the adherent cell population.

Intact 3D spheroids exhibited clear fluorescence signals for CD90 and CD105, demonstrating retention of these markers after the transition to three-dimensional culture. Nuclear counterstaining with DAPI enabled visualization of nuclei within the multicellular structures (Figure 2B). These observations supported the preservation of the two assessed MSC-associated markers following cellular aggregation and spheroid formation.

The immunofluorescence findings were evaluated qualitatively using representative fields acquired by widefield microscopy of intact, non-sectioned spheroids. Accordingly, the results establish the presence of CD90 and CD105 in the spheroids but do not provide quantitative comparisons of marker expression between 2D and 3D cultures. Similarly, the images were not used to determine the distribution of marker expression throughout the full depth of the spheroids.

#### *HUVEC wound closure*

The effects of conditioned media from 2D- and 3D-cultured hUC-MSCs on endothelial wound closure were examined using a HUVEC scratch assay. Representative phase-contrast images obtained during the 24-h observation period showed repopulation of the initially cell-free wound area in all experimental groups (Figure 3). The extent and apparent

temporal pattern of closure differed among HUVECs exposed to conditioned medium from 3D spheroids (3D-CM), conditioned medium from 2D cultures (2D-CM), and fresh endothelial growth medium (EGM; control).

The most extensive apparent wound closure was observed in the 3D-CM group. By 18–24 h, the initially cell-free region was nearly completely occupied by cells. In comparison, the 2D-CM and control groups exhibited less extensive closure at 24 h and less consistent changes across the representative fields examined at successive time points.

Quantification of individually selected images yielded estimated wound closure values of approximately 100% for 3D-CM, 66.0% for 2D-CM, and 72.7% for the EGM control at 24 h (Figure 4). These percentages were derived from individual images rather than from several independent experiments and therefore have no associated estimate of variability; the difference between 3D-CM (approximately 100%) and the other groups (66.0–72.7%) is accordingly presented as a preliminary observation. These estimates were consistent with the greater apparent repopulation observed in the representative images of the 3D-CM group. At this endpoint, the estimated closure in the 2D-CM group was lower than that in the EGM control.

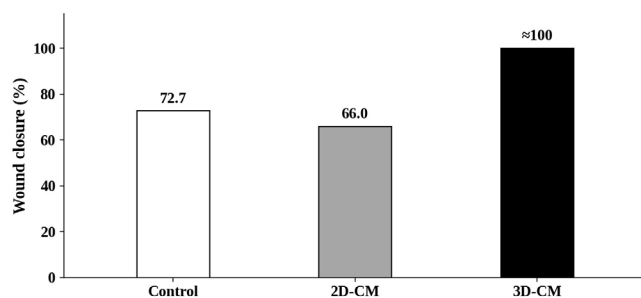


**Figure 4.** Quantitative analysis of HUVEC wound closure following treatment with conditioned media derived from 2D- and 3D-cultured hUC-MSCs. Wound closure at 24 h was estimated from individually selected images, not from independent experiments; each bar shows a single estimate, without error bars or statistical testing.

Across the assessed post-scratch time points, 3D-CM produced the highest image-derived estimates of wound closure. Near-complete apparent closure was recorded as early as 3 h. However, the corresponding image contained numerous rounded cells within the wound region, which may have reduced the area classified as cell-free and thereby inflated the estimated closure value. This early observation therefore requires cautious interpretation.

## DISCUSSION

The present study examined whether three-dimensional spheroid culture modifies the capacity of human umbilical cord mesenchymal stem cell-conditioned medium to support endothelial wound closure. The principal observation was that medium conditioned by micromold-generated hUC-MSC spheroids was associated with more extensive repopulation of the HUVEC scratch area than either 2D-conditioned medium or the endothelial growth medium control. This re-

sponse accompanied spheroid formation and the retention of the MSC-associated markers examined. Together, these observations support the hypothesis that spatial organization of hUC-MSCs can influence the functional activity of their conditioned medium. However, because wound closure was estimated from individually selected images rather than from independent experiments, the findings establish a preliminary association rather than a statistically confirmed enhancement of endothelial repair.

Agarose micromolding provided a geometrically defined environment for hUC-MSC aggregation, with spheroids forming in all 81 evaluated microwells. Mean diameter increased from  $310 \pm 20 \mu\text{m}$  at 24 h to  $500 \pm 30 \mu\text{m}$  at 72 h. These measurements demonstrate consistent aggregate formation within the assessed mold field, although reproducibility across independent preparations remains to be established. The increase in diameter should not be interpreted as evidence of increased proliferation: changes in aggregate dimensions can reflect several processes, including cellular rearrangement, altered packing density and extracellular matrix accumulation. The present morphometric analysis does not distinguish among these possibilities.

Spheroid dimensions are relevant to the interpretation of conditioned-medium activity because aggregate size can influence both cellular state and secretory function. Rovere et al. demonstrated that MSC spheroid size affected senescence-associated characteristics and the angiogenic activity of soluble and extracellular vesicle fractions, with smaller spheroids exhibiting greater activity in their experimental system [9]. Thus, increasing spheroid size cannot be assumed to improve biological potency. In the present study, the relationship between aggregate diameter and endothelial response was not directly tested. Comparing conditioned media collected from spheroids of defined sizes, together with spatial assessment of viability and hypoxia, would help establish whether the observed dimensions support a favorable balance between cellular organization and mass transport.

Immunocytochemical analysis provided evidence that spheroid formation was compatible with retention of selected MSC-associated markers. CD73, CD90 and CD105 were detected in 2D cultures, whereas CD90 and CD105 were demonstrated in intact spheroids; CD45 staining was not apparent in either condition. These observations support phenotypic continuity during short-term aggregation but do not establish equivalence of the complete immunophenotype between culture formats. In particular, qualitative imaging of intact spheroids cannot resolve differences in marker abundance or distribution throughout the aggregate. The coexistence of retained marker expression and an altered recipient-cell response nevertheless highlights the value of functional assays alongside phenotypic characterization when evaluating MSC culture systems.

The HUVEC assay addressed the main functional objective of the study. At 24 h, estimated wound closure reached approximately 100% with 3D-CM, compared with 66.0% with 2D-CM and 72.7% with endothelial growth medium. The difference between approximately 100% and 66.0–72.7% is of interest, but it remains preliminary: each percentage was derived from individually selected images rather than from independent experiments, so neither the size nor the reproduc-

ibility of the difference can be estimated. The most defensible interpretation is that 3D-CM was associated with greater endothelial coverage of the scratched region under the conditions examined. The lower estimate for 2D-CM relative to the control does not establish an inhibitory effect, particularly given the absence of replicate-level analysis and the different composition of the control medium. Instead, this comparison emphasizes that conditioned-medium activity must be evaluated against an appropriate medium background.

The direction of the observed response is consistent with experimental evidence linking three-dimensional MSC culture to wound-related and vascular activities. Gangadaran et al. reported improved burn-wound healing following treatment with secretome from three-dimensionally cultured bone marrow MSCs [14]. More directly relevant to the cell source used here, Rajendran et al. associated umbilical cord MSC spheroid culture with enrichment of proangiogenic microRNAs and enhanced angiogenic activity [16]. These studies provide biological context for the present observations, although differences in cell source, aggregation method, conditioning protocol and recipient model preclude direct comparison of effect magnitude. Moreover, the current scratch assay does not establish angiogenesis or tissue regeneration *in vivo*.

A spheroid-dependent change in the extracellular milieu is a plausible explanation for the endothelial response, but its molecular basis remains unresolved. Increased intercellular contact and local microenvironmental gradients could alter the products released during conditioning. Nevertheless, conditioned medium reflects the combined consequences of secretion, nutrient consumption and modification of pre-existing medium components. Without matched conditioning conditions and molecular characterization, the observed activity cannot be attributed exclusively to increased secretion or to any specific mediator. Comparisons using equivalent viable-cell input, collection volume and conditioning duration, together with time-matched cell-free medium controls, would strengthen attribution of the response to culture dimensionality. Characterization of the extracellular-vesicle and soluble fractions of the conditioned media in line with current minimal-information guidelines [20] would be a natural step toward identifying the active components.

Wound closure integrates endothelial migration, proliferation and spreading; therefore, the present measurements cannot isolate a migration-specific effect. In addition, the use of individually selected rather than positionally matched fields likely contributed to the non-monotonic estimates in the 2D-CM and control groups. Rounded cells within the early 3D-CM wound area also complicate interpretation of apparent closure at 3 h. The near-complete coverage observed at 18–24 h is consequently more informative than the early estimate, although it still requires confirmation through longitudinal imaging and analysis across independent biological replicates.

The next experimental priority is to determine whether the apparent advantage of 3D-CM persists under standardized collection and recipient-cell conditions. Matched-field imaging, parallel assessment of endothelial proliferation and replicate-based statistical analysis would establish the magnitude and reproducibility of the wound-closure response. Complementary migration, sprouting and tube-formation assays could then clarify its vascular relevance, while secretome profiling

and targeted functional perturbation could identify contributing mediators. The present findings provide a focused rationale for these studies by linking micromold-generated hUC-MSC spheroids to a potentially favorable endothelial response without assigning an unverified mechanism. Should the activity be confirmed, delivery of conditioned-medium-derived factors in polymer-based carrier systems, developed for regenerative applications such as bone tissue repair [21], could also be considered, although this lies beyond the scope of the present data.

## CONCLUSIONS

Agarose micromolding supported consistent hUC-MSC spheroid formation within the evaluated mold field and retention of selected MSC-associated markers. Conditioned medium from these spheroids was associated with the greatest HUVEC wound closure, reaching approximately complete closure at 24 h in individually selected images, compared with 66.0% for 2D-conditioned medium and 72.7% for the endothelial growth medium control. These estimates, derived from individually selected images rather than from independent experiments, provide only preliminary evidence that three-dimensional organization may enhance the capacity of hUC-MSC-conditioned medium to support endothelial monolayer repair; confirmation in independent biological replicates with statistical analysis is required. The results support further investigation of micromold-generated hUC-MSC spheroids as a platform for producing conditioned medium with wound-healing-relevant activity.

## AUTHOR CONTRIBUTIONS

Conceptualization, V.O. and S.M.; methodology, S.M., M.I., and A.N.; investigation, S.M., M.I., A.N., N.A., M.A., and A.O.; formal analysis, S.M., M.I., and V.O.; data curation, S.M., N.A., M.A., and A.O.; visualization, S.M. and A.N.; writing-original draft preparation, V.O. and S.M.; writing-review and editing, all authors; supervision, V.O. All authors have read and agreed to the published version of the manuscript.

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**ВЛИЯНИЕ КОНДИЦИОНИРОВАННОЙ СРЕДЫ 3D-СФЕРОИДОВ МЕЗЕНХИМАЛЬНЫХ СТЕВЛОВЫХ КЛЕТОК ПУПОВИНЫ ЧЕЛОВЕКА НА МИГРАЦИЮ ЭНДОТЕЛИАЛЬНЫХ КЛЕТОК В МОДЕЛИ ЗАЖИВЛЕНИЯ РАНЫ IN VITRO****Ш.Е. Мухлис<sup>1,2</sup>, М. Иманбекова<sup>1</sup>, А. Нурғалиева<sup>1</sup>, Н. Альтаева<sup>2</sup>, М. Ашиқбаева<sup>2</sup>, А. Оспанова<sup>3</sup>, В.Б. Оғай<sup>1\*</sup>**<sup>1</sup>Лаборатория ствловых клеток, Национальный центр биотехнологии, 010000, Астана, Казахстан<sup>2</sup>Медицинский университет Астана, 010000, Астана, Казахстан<sup>3</sup>Евразийский национальный университет имени Л.Н. Гумилева, 010000, Астана, Казахстан

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**АННОТАЦИЯ**

Трёхмерное (3D) культивирование может усилить паракринную активность мезенхимальных ствловых клеток. Цель исследования - сравнить влияние кондиционированных сред двумерных (2D) культур и 3D-сфероидов мезенхимальных ствловых клеток пуповины человека (hUC-MSCs) на закрытие дефекта эндотелиального монослоя *in vitro*. hUC-MSCs третьего пассажа культивировали в виде адгезивного монослоя или сфероидов, сформированных с использованием агарозных микромолдов MicroTissues®. Морфологию и диаметр сфероидов оценивали через 24, 48 и 72 ч. Методом иммунофлуоресценции исследовали CD73, CD90, CD105 и CD45 в 2D-культурах, а также CD90 и CD105 в интактных 3D-сфероидах. Эндотелиальные клетки пупочной вены человека (HUVECs) культивировали в присутствии кондиционированных сред 2D-культур (2D-CM), 3D-сфероидов (3D-CM) или эндотелиальной ростовой среды (контроль). Изображения в scratch-тесте получали через 0 и 24 ч; закрытие дефекта рассчитывали относительно исходной площади, свободной от клеток. Сфероиды сформировались во всех 81 исследованной микролунке. Средний диаметр увеличивался с  $310 \pm 20$  мкм через 24 ч до  $420 \pm 25$  мкм через 48 ч и  $500 \pm 30$  мкм через 72 ч (среднее  $\pm$  SD). После формирования сфероидов сохранялось выявляемое окрашивание на CD90 и CD105. Через 24 ч закрытие дефекта, оценённое по отдельным выбранным изображениям (без независимых экспериментов), составляло приблизительно 100%, 66,0% и 72,7% в группах 3D-CM, 2D-CM и контроля соответственно. Эти предварительные описательные результаты указывают на более выраженное закрытие дефекта в присутствии 3D-CM; их подтверждение требует анализа одних и тех же полей зрения в динамике в независимых экспериментах со статистической обработкой данных.

**Ключевые слова:** мезенхимальные ствловые клетки, 3D-сфероиды, кондиционированная среда, миграция эндотелиальных клеток, скретч-тест заживления раны, ангиогенез, паракринная активность, регенеративная медицина

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**АДАМ КІНДІК БАУЫНЫҢ МЕЗЕНХИМАЛЫҚ БАҒАНАЛЫ ЖАСУШАЛАРЫНЫҢ 3D-СФЕРОИДТАРЫНАН АЛЫНҒАН КОНДИЦИЯЛАНҒАН ОРТАНЫҢ IN VITRO ЖАРАНЫҢ ЖАЗЫЛУ МОДЕЛІНДЕГІ ЭНДОТЕЛИЙ ЖАСУШАЛАРЫНЫҢ МИГРАЦИЯСЫНА ӘСЕРІ****Ш.Е. Мухлис<sup>1,2</sup>, М. Иманбекова<sup>1</sup>, А. Нурғалиева<sup>1</sup>, Н. Альтаева<sup>2</sup>, М. Ашиқбаева<sup>2</sup>, А. Оспанова<sup>3</sup>, В.Б. Оғай<sup>1\*</sup>**<sup>1</sup>Бағаналы жасушалар зертханасы, Ұлттық биотехнология орталығы, 010000, Астана, Қазақстан<sup>2</sup>Астана медицина университеті, 010000, Астана, Қазақстан<sup>3</sup>Л.Н. Гумилев атындағы Еуразия ұлттық университеті, 010000, Астана, Қазақстан

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**АНДАТПА**

Үшөлшемді (3D) сферидтық культивирлеу мезенхималық бағаналы жасушалардың паракриндік белсенділігін күшейтуі мүмкін. Зерттеудің мақсаты — адам кіндік бауының мезенхималық бағаналы жасушаларының (hUC-MSCs) екіөлшемді (2D) культивирлеу мен 3D-сферидтарынан алынған кондицияланған орталардың эндотелий монокабатындағы жара ақауының *in vitro* жабылуына әсерін салыстыру. Үшінші пассаждағы hUC-MSCs адгезиялық монокабат түрінде немесе MicroTissues® агарозалық микрокалыптарында түзілген сферидтар түрінде дақылданды. Сферидтардың морфологиясы мен диаметрі 24, 48 және 72 сағаттан кейін бағаланды. Иммунофлуоресценция әдісімен 2D-дақылдарда CD73, CD90, CD105 және CD45, ал тұтас 3D-сферидтарда CD90 және CD105 маркерлері зерттелді. Адам кіндік венасының эндотелий жасушалары (HUVECs) 2D-дақылдардың кондицияланған ортасында (2D-CM), 3D-сферидтардың кондицияланған ортасында (3D-CM) немесе эндотелий жасушаларына арналған өсу ортасында (бақылау) дақылданды. Скретч-тестте кескіндер 0 және 24 сағатта алынды; жара ақауының жабылуы бастапқы жасушасыз ауданға қатысты есептелді. Сферидтар зерттелген 81 микроұяшықтың барлығында түзілді. Орташа диаметрі 24 сағатта  $310 \pm 20$  мкм, 48 сағатта  $420 \pm 25$  мкм және 72 сағатта  $500 \pm 30$  мкм болды (орташа мән  $\pm$  SD). Сферидтар түзілгеннен кейін CD90 және CD105 маркерлерінің анықталатын сигналы сақталды. Жеке тандалған кескіндер бойынша (тәуелсіз эксперименттерсіз) бағаланған жара ақауының жабылуы 24 сағатта 3D-CM, 2D-CM және бақылау топтарында тиісінше шамамен 100%, 66,0% және 72,7% құрады. Бұл алдын ала сипаттамалық нәтижелер 3D-

СМ қатысында жара ақауының айқынырақ жабылуын көрсетеді; оларды растау үшін статистикалық өңдеумен тәуелсіз эксперименттерде бірдей көру өрістерін уақыт бойынша талдау қажет.

**Кілт сөздер:** мезенхималық бағаналы жасушалар, 3D-сфероидтар, кондицияланған орта, эндотелий жасушаларының миграциясы, жараның жазылуын скретч-тестпен бағалау, ангиогенез, паракриндік белсенділік, регенеративті медицина