Angiogenic Response to Atorvastatin and Human Umbilical Cord Blood-Mesenchymal Stem Cell-Derived Exosomes in the IN-OVO Chorioallantoic Membrane Model

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ABSTRACT:

Exosomes derived from human umbilical cord blood–mesenchymal stem cells (hUCB-MSCs) contain various proangiogenic mediators, whereas atorvastatin exerts pleiotropic endothelial effects through the PI3K/Akt/eNOS pathway and enhanced nitric oxide bioavailability. However, the angiogenic responses to atorvastatin, hUCB-MSC-derived exosomes, and their combination have not been fully elucidated.. To compare the angiogenic effects of atorvastatin, hUCB-MSC-derived exosomes, and their combination in an in ovo chorioallantoic membrane (CAM) model. Four groups were included: control, atorvastatin, hUCB-MSC-derived exosome, and exosome plus atorvastatin. Seven embryonated chicken eggs per group were assessed at D0, D1, and D2, with vascular density as the primary outcome. A significant time-by-group interaction was observed for vascular density, $F(6,48) = 23.278$, $p < 0.001$, partial $\eta^2 = 0.744$. Cumulative vascular density change from D0 to D2 differed significantly among the control, atorvastatin, exosome, and combination groups (0.671 ± 0.647, 3.196 ± 0.734, 4.786 ± 0.828, and 2.642 ± 0.683 percentage points, respectively; $F(3,24) = 38.302$, $p < 0.001$, $\eta^2 = 0.827$). Exosomes produced greater increases than control ($p < 0.001$), atorvastatin ($p = 0.002$), and combination treatment ($p < 0.001$). Atorvastatin and combination treatment also exceeded control (both $p < 0.001$), whereas they did not differ from each other ($p = 0.495$). Atorvastatin and hUCB-MSC-derived exosomes induced significant proangiogenic responses in the in ovo CAM model. hUCB-MSC-derived exosomes produced the strongest angiogenic response. hUCB-MSC-derived exosomes produced the strongest proangiogenic response, while atorvastatin also increased vascular density. Adding atorvastatin to exosomes provided no additional angiogenic benefit at the doses evaluated.

Keywords: Angiogenesis, Atorvastatin, Exosome, Human Umbilical Cord Blood-Mesenchymal Stem Cells (hUCB-MSCs), Chorioallantoic Membrane

1. Introduction

An essential step in tissue adaptation and repair is angiogenesis, the creation of new blood vessels from pre-existing vasculature (Babaei et al., 2021). Restoring a sufficient microvascular network is crucial for enhancing tissue perfusion and promoting tissue healing in ischemic circumstances. The development of medicinal approaches that can promote neovascularization is justified, tho, since the endogenous angiogenic response might not be enough to overcome persistent ischemia. Endothelial proliferation, migration, vascular sprouting, extracellular matrix remodeling, and eventual vascular maturation all interact intricately to control the angiogenic response. In this process, angiopoietin-1/Tie-2 signaling aids in vascular maturation and stabilization, while vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are significant promoters of vascular sprouting. Under hypoxic conditions, the angiogenic response is further enhanced via signaling mediated by hypoxia-inducible factor (HIF) (Li et al., 2025).

The complexity of angiogenesis has sparked interest in medicinal treatments that can boost endothelial activity while also promoting the formation of a more structured vascular network. In this context, cell-free regeneration therapies based on mesenchymal stem cell (MSC)-derived extracellular vesicles have emerged as a promising technique, while pharmaceutical drugs with pleiotropic vascular effects may serve as an alternate or complementary approach (Piotrowska et al., 2025; Heusch, 2024; Jin et al., 2013; Hori et al., 2024; Oktaviono et al., 2021).

Human umbilical cord blood-derived mesenchymal stem cell (hUCB-MSC) exosomes are a promising cell-free therapeutic platform for stimulating angiogenesis. Rather of introducing viable stem cells, exosome-based therapy employs extracellular vesicles carrying biologically active proteins, lipids, messenger RNA, and microRNA that can transmit paracrine signals to target cells. These bioactive cargos have been linked to endothelial proliferation, migration, tube formation, and the control of proangiogenic signaling pathways such PI3K/Akt and eNOS/NO. Exosomes may also influence inflammatory responses and extracellular matrix remodeling, perhaps facilitating the formation of a more stable vascular network (Piotrowska et al., 2025; Heusch, 2023; Jin et al., 2013; Hori et al., 2024; Oktaviono et al., 2021).

The presence of angiogenesis-related mediators such as VEGF, hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), and angiopoietin-1 in hUCB-MSC-derived exosomes further supports their proangiogenic potential. These mediators can stimulate endothelial proliferation, migration, vascular sprouting, and vascular maturation by acting on complimentary processes. Exosomal microRNAs and matrix-remodeling factors may also help to provide a favorable milieu for vascular growth. These properties make hUCB-MSC-derived exosomes a promising cell-free method to angiogenic treatment. However, the extent of their angiogenic impact and potential combination with existing pharmacological treatments remains unknown, particularly in controlled in vivo experimental models (Chen et al., 2021; Mabotuwana et al., 2022).

Atorvastatin is most known for its ability to reduce lipid levels by inhibiting HMG-CoA reductase. Aside from its lipid-lowering function, atorvastatin has pleiotropic effects on the vascular system that may be related to angiogenesis. Previous research, presented in the current paper, suggests that atorvastatin can stimulate endothelial proliferation and migration while also enhancing angiogenic signaling via processes including VEGF, PI3K/Akt/eNOS signaling, nitric oxide bioavailability and endothelial function. Atorvastatin has also been linked to enhanced activity of endothelial progenitor cells, suggesting another possible pathway for vascular regeneration (Elewa et al., 2010; Khalighfard et al., 2021; Dimmeler et al., 2001; Oesterle et al., 2017; Xu et al., 2013).

The proangiogenic effects of atorvastatin may be especially important in a growing vascular environment where hypoxia and HIF signaling

promote VEGF production. Atorvastatin can enhance VEGF-related angiogenic signaling via influencing the PHD2/HIF-1α axis. Additionally, its antioxidant and anti-inflammatory qualities may improve endothelial function. The convergence of atorvastatin-mediated endothelial signaling and the multifactorial paracrine action of MSC-derived exosomes provides a biological basis for examining these therapies concurrently. Exosomes may deliver proangiogenic biological signals, whereas atorvastatin may affect the endothelium environment and intracellular signaling pathways that promote vascular development (Piotrowska et al., 2025; Heusch, 2024).

The chorioallantoic membrane (CAM) assay is a useful in-vivo platform for assessing angiogenic responses to biological and pharmacological interventions. The CAM is highly vascularized, has rapid vascular development during embryogenesis, and allows for direct imaging and quantification of vascular alterations after exposure to experimental substances. These properties have made the CAM model helpful for preclinical testing of proangiogenic and antiangiogenic drugs, stem-cell-derived products, and biomaterials before moving on to more complicated mammalian models. The CAM approach also provides a significant methodological advantage for longitudinal assessments. Because vascular growth is rapid during the crucial embryonic period, vascular density can be measured multiple times inside the same experimental unit. In the current study, vascularization was measured relative to each CAM's own baseline, which reduced the potential impact of inter-egg variations in starting vascular density. Image-based quantification with ImageJ allows for objective assessment of vascular density within a standardized region of interest (Shekatkar et al., 2024; Zhang et al., 2015).

Although both hUCB-MSC-derived exosomes and atorvastatin have been shown to have potential proangiogenic capabilities, their interaction has yet to be fully defined. There is limited experimental evidence that atorvastatin can augment, sustain, or change the angiogenic response induced by hUCB-MSC-derived exosomes. The study gap is particularly significant because the two therapies operate via biologically distinct but potentially convergent routes. The present study protocol indicated the lack of comprehensive evaluation of the combination of atorvastatin and hUCB-MSC-derived exosomes in an in ovo CAM model as a significant unanswered question. Therefore, this study is designed to experimentally examine the angiogenic response of atorvastatin and HUCB-MSC exosomes in the in-ovo CAM model, with the expectation of providing a scientific foundation for the future development of a combined pharmacological and cell-free exosome-based therapeutic strategy for ischemic cardiovascular diseases. This study uti-lized a survey to address the following research questions:

(1) What is the angiogenic response, assessed by vascular density, in the CAM membrane treated with hUCB-MSC exosomes?

(2) What is the angiogenic response, assessed by vascular density, in the CAM membrane treated with atorvastatin?

(3) How does angiogenesis, assessed by vascular density, differ in the CAM membrane treated with the combination of hUCB-MSC exosomes and atorvastatin compared to those treated with atorvastatin and hUCB-MSC exosomes alone?

2. Methodology

Study Design

The angiogenic effects of atorvastatin, human umbilical cord blood-derived mesenchymal stem cell (hUCB-MSC) exosomes, and their combination were assessed in this controlled experimental study employing an in ovo chorioallantoic membrane (CAM) model. Because of its highly vascularized and quickly growing vascular network, which enables immediate evaluation of angiogenic responses after pharmacological or biological manipulations, the CAM model was chosen.

Twenty-eight embryonated chicken eggs were allocated equally into four experimental groups:

control, atorvastatin, hUCB-MSC-derived exosome, and hUCB-MSC-derived exosome plus atorvastatin, with seven eggs per group. Each egg was followed longitudinally, with vascular density assessed at baseline (D0), 24 hours (D1), and 48 hours (D2) after intervention. The primary outcome was vascular density, expressed as the percentage of the standardized CAM region of interest occupied by blood vessels. The longitudinal design enabled assessment of both the absolute vascular density and the change in vascular density relative to baseline.

Sample

The experimental samples consisted of twenty eight embryonated chicken eggs weighing 40–50 g. Eggs were obtained from a single farm and derived from the same incubation batch to minimize variation related to egg source and incubation conditions. Embryonic viability and the presence of CAM vascularization were assessed by candling at embryonic days 3 and 7 (Wan, 2017).

Intervention was performed at embryonic day 8 (ED-8), corresponding to D0. Subsequent observations were performed at ED-9 (D1) and ED-10 (D2). The experimental groups received a single-dose application to the CAM as follows: phosphate-buffered saline (PBS) 50 μL for the control group, atorvastatin at 0.1 μmol/L for the atorvastatin group, 50 μL of hUCB-MSC-derived exosome preparation for the exosome group, and the corresponding exosome preparation combined with atorvastatin for the combination group (Baharara et al., 2012).

The principal controlled experimental conditions included embryonic age at intervention, egg source and incubation batch, intervention timing, sample volume, CAM observation area, image acquisition and analysis procedures, and incubation conditions. The incubation temperature was maintained at 37–38°C with relative humidity of 55–65%.

Instruments

The instruments used in the study included a biosafety cabinet for aseptic handling of biological materials, a CO_2 incubator or cell-culture incubator for hUCB-MSC maintenance, an incubator for embryonated chicken eggs, and an egg candler for assessment of embryonic viability and CAM vascularization.

For vascular assessment, images of the CAM were acquired using a standardized imaging procedure. A fixed region of interest (ROI) measuring 800 × 800 pixels was used across all experimental groups and observation time points. Vascular density was quantified using Fiji/ImageJ image-analysis software with the Trainable Weka Segmentation plugin. Vascular density was operationally defined as the percentage of the ROI occupied by blood vessels.

The use of a standardized ROI and identical image acquisition and analysis procedures across groups was intended to minimize analytical variability. Each CAM was longitudinally assessed relative to its own baseline vascular density, allowing within-sample changes to be quantified before between-group comparisons.

Procedure

Following incubation, embryonated eggs were screened for viability and appropriate CAM vascularization. At ED-8, the CAM was exposed and the respective intervention was administered as a single dose according to the assigned experimental group. The control group received PBS, whereas the intervention groups received atorvastatin, hUCB-MSC-derived exosomes, or their combination with atorvastatin.

Vascular images were obtained at three predefined time points: D0 (ED-8, immediately following intervention/baseline assessment), D1 (ED-9; 24 hours), and D2 (ED-10; 48 hours). The same CAM was followed over time to allow longitudinal assessment of vascular development. The use of the same experimental unit at each observation point enabled normalization of vascular density against the individual baseline and reduced the potential influence of inter-egg differences in baseline vascularization (Piotrowska et al., 2025; Zhang et al., 2025; Mardi et al., 2025).

For image analysis, a standardized 800 × 800-pixel ROI was applied to the CAM images.

Vascular structures within the ROI were segmented using Fiji/ImageJ with the Trainable Weka Segmentation plugin. Vascular density was calculated as the percentage of the ROI occupied by vascular structures. Changes in vascular density were subsequently calculated as ΔD1–D0 and ΔD2–D0 to characterize the early and cumulative angiogenic responses, respectively.

Data Analysis

Continuous variables were summarized as mean ± standard deviation (SD). Vascular density was evaluated longitudinally at D0, D1, and D2. The change from baseline was calculated for each experimental unit to account for differences in initial vascular density.

The longitudinal effect of treatment was evaluated using a mixed-design repeated-measures analysis of variance (ANOVA), with time as the within-subject factor and treatment group as the between-subject factor. The analysis assessed the main effects of time and treatment group, as well as the time × treatment group interaction.

To compare the cumulative change in vascular density from D0 to D2 (ΔD2–D0) among the four experimental groups, one-way ANOVA was performed. When a significant overall group effect was identified, Tukey's honestly significant difference (HSD) test was used for post-hoc pairwise comparisons. Effect sizes were expressed using partial eta squared (partial η^2) for the repeated-measures analysis, eta squared (η^2) for the one-way ANOVA, and Cohen's d for pairwise comparisons.

The primary analytical endpoint was the difference in cumulative vascular density change (ΔD2–D0) between experimental groups. Statistical significance was defined as a two-sided p-value < 0.05.

3. Results

A total of 28 embryonated chicken eggs were included in the final analysis and equally distributed among four experimental groups: control, atorvastatin, hUCB-MSC-derived exosome, and exosome plus atorvastatin (n = 7 per group). Vascular density was quantified longitudinally at baseline (D0), D1, and D2 as the primary outcome of angiogenic response. Rather than evaluating treatment effects solely at a single endpoint, the analysis characterized both the temporal trajectory of vascular development and the cumulative change from baseline. The results therefore describe the baseline vascular profile, the evolution of vascular density over time, and the magnitude and direction of treatment-related differences.

Baseline Vascular Profile Across Experimental Groups

The baseline vascular profile of each experimental group is presented in Table 1. At D0, mean vascular density ranged from 5.114 ± 0.793% in the control group to 6.097 ± 0.500% in the atorvastatin group. The exosome and combination groups had baseline vascular densities of 5.214 ± 0.815% and 5.644 ± 0.978%, respectively. All groups were analyzed using an identical 800 × 800-pixel region of interest.

Divergence of Vascular Density Over Time

The longitudinal measurements revealed a progressive divergence in vascular density among treatment groups. As shown in Table 2, vascular density increased from D0 to D2 in all groups, but the magnitude of increase was markedly different. The control group demonstrated only a modest increase, whereas the three treatment groups exhibited greater increases, with the exosome group showing the highest values at both D1 and D2.

At D2, vascular density reached 10.000 ± 1.306% in the exosome group, compared with 9.292 ± 1.161% in the atorvastatin group, 8.286 ± 1.416% in the combination group, and 5.786 ± 0.899% in the control group. The cumulative increase from baseline to D2 followed the same hierarchy: exosome (4.786 ± 0.828 percentage points), atorvastatin (3.196 ± 0.734), exosome plus atorvastatin (2.642 ± 0.683), and control (0.671 ± 0.647).

Table 1. Baseline vascular density across experimental groups

Variable	Control (n=7)	Atorvastatin (n=7)	Exosome (n=7)	Exosome + Atorvastatin (n=7)
Vascular density at D0 (%)	5.114 ± 0.793	6.097 ± 0.500	5.214 ± 0.815	5.644 ± 0.978
ROI	800 × 800 px	800 × 800 px	800 × 800 px	800 × 800 px

Table 2. Longitudinal vascular density and change from baseline

Group	D0	D1	D2	ΔD1–D0	ΔD2–D1	ΔD2–D0
Control	5.114 ± 0.793	5.457 ± 0.824	5.786 ± 0.899	0.343 ± 0.553	0.329 ± 0.309	0.671 ± 0.647
Atorvastatin	6.097 ± 0.500	7.329 ± 1.017	9.292 ± 1.161	1.232 ± 0.725	1.964 ± 0.620	3.196 ± 0.734
Exosome	5.214 ± 0.815	7.414 ± 0.982	10.000 ± 1.306	2.200 ± 0.424	2.586 ± 0.986	4.786 ± 0.828
Exosome + Atorvastatin	5.644 ± 0.978	6.737 ± 1.190	8.286 ± 1.416	1.093 ± 0.522	1.549 ± 0.646	2.642 ± 0.683

Table 3. Longitudinal analysis of vascular density using mixed-design repeated-measures ANOVA

Effect	F	df	P value	Partial η^2
Time	256.199	2, 48	<0.001	0.914
Treatment group	7.73	3, 24	<0.001	0.491
Time × Treatment group	23.278	6, 48	<0.001	0.744

Table 4. Overall comparison of cumulative

Treatment group	ΔD2–D0 (mean ± SD)	One-way ANOVA
Control	0.671 ± 0.647	
Atorvastatin	3.196 ± 0.734	
Exosome	4.786 ± 0.828	$F(3,24) = 38.302$; $p < 0.001$
Exosome + Atorvastatin	2.642 ± 0.683	$\eta^2 = 0.827$

Table 5. Pairwise comparison of cumulative vascular response

Comparison	Mean difference	95% CI	Adjusted P	Cohen's d
Atorvastatin vs Control	2.524	1.454 to 3.595	<0.001	3.649
Exosome vs Control	4.114	3.044 to 5.185	<0.001	5.538
Exosome + Atorvastatin vs Control	1.97	0.900 to 3.041	<0.001	2.961
Exosome vs Atorvastatin	1.59	0.519 to 2.661	0.002	2.033
Exosome vs Exosome + Atorvastatin	2.144	1.073 to 3.215	<0.001	2.826
Exosome + Atorvastatin vs Atorvastatin	−0.554	−1.625 to 0.517	0.495	−0.782

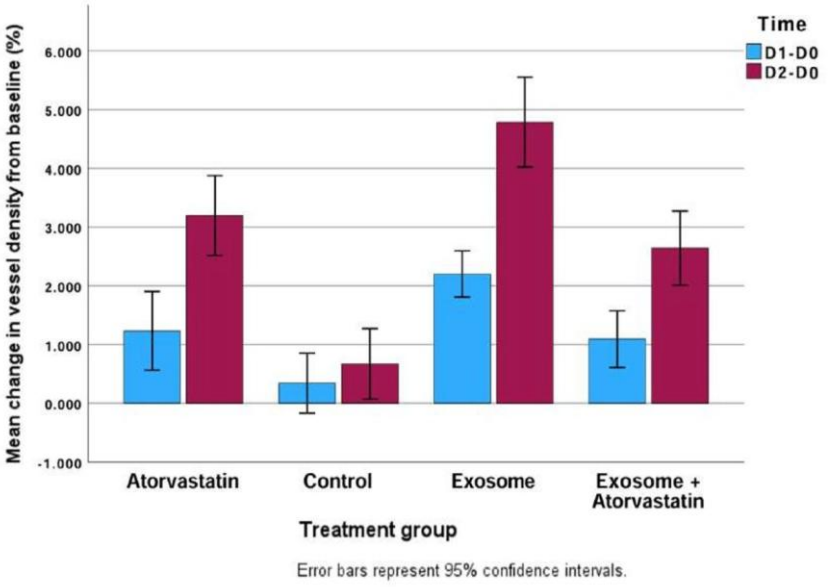

Figure 1. Changes in vascular density from baseline (ΔD1–D0 and ΔD2–D0) according to treatment group

Values are presented as mean ± SD. D0, baseline; D1, first observation; D2, second observation; ΔD1–D0, change from D0 to D1; ΔD2–D1, change from D1 to D2; ΔD2–D0, cumulative change from D0 to D2; SD, standard deviation.

The increasing separation between groups was also evident when changes from baseline were examined. The exosome group showed the largest increase during both observation intervals, followed by atorvastatin, exosome plus atorvastatin, and control. This progressive separation suggested that the angiogenic trajectories were treatment-dependent rather than representing a uniform temporal increase across all groups.

Figure 1 illustrates the progressive divergence in vascular density changes from baseline across the treatment groups. The exosome group demonstrated the greatest increase in vascular density at both D1 and D2, followed by the atorvastatin, exosome plus atorvastatin, and control groups. At D1, the mean changes from baseline were 2.200, 1.232, 1.093, and 0.343 percentage points for the exosome, atorvastatin, combination, and control groups, respectively. By D2, these increases reached 4.786, 3.196, 2.642, and 0.671 percentage points, respectively. Thus, the separation between treatment groups became progressively more pronounced over time, with exosome treatment demonstrating the largest cumulative vascular response.

The observed pattern was consistent with the longitudinal inferential analysis, which demonstrated a significant time-by-treatment interaction ($F(6,48) = 23.278$, $p < 0.001$, partial $\eta^2 = 0.744$). This finding indicates that the rate of increase in vascular density differed significantly according to treatment group, rather than reflecting a uniform increase over time across all groups.

Treatment Modified the Trajectory of Vascular Development

To formally determine whether the observed divergence represented a treatment-dependent longitudinal effect, a mixed-design repeated-measures ANOVA was performed. This analysis simultaneously evaluated the effects of time, treatment group, and their interaction. The results are summarized in Table 3.

A significant main effect of time was observed ($F(2,48) = 256.199$, $p < 0.001$, partial $\eta^2 = 0.914$), demonstrating a substantial change in vascular density across the observation period. A significant main effect of treatment group was also identified ($F(3,24) = 7.730$, $p < 0.001$, partial $\eta^2 = 0.491$). Most importantly, a significant time × treatment group interaction was observed ($F(6,48) = 23.278$, $p < 0.001$, partial $\eta^2 = 0.744$). Thus, the magnitude of vascular development over time was significantly different across treatment groups.

Magnitude of the Cumulative Angiogenic Response

Because the longitudinal analysis demonstrated different trajectories among treatment groups, the cumulative change from baseline to D2 (ΔD2–D0) was subsequently used to quantify the overall magnitude of vascular response. The cumulative response differed significantly among the four groups (one-way ANOVA, $F(3,24) = 38.302$, $p < 0.001$, $\eta^2 = 0.827$), indicating that treatment group accounted for a substantial proportion of the variability in vascular density change.

The magnitude of the response was greatest following exosome treatment, with a cumulative increase of 4.786 ± 0.828 percentage points. Atorvastatin produced an increase of 3.196 ± 0.734 percentage points, while the combination treatment produced an increase of 2.642 ± 0.683 percentage points. The control group showed the smallest change, at 0.671 ± 0.647 percentage points.

Comparative Treatment Effects

To determine which treatment groups accounted for the overall difference, Tukey HSD pairwise comparisons were performed. The exosome group demonstrated a significantly greater cumulative increase in vascular density than the control group (mean difference, 4.114; 95% CI, 3.044–5.185; $p < 0.001$; Cohen's d = 5.538). The atorvastatin group also showed a significantly greater increase than control (mean difference,

2.524; 95% CI, 1.454–3.595; p < 0.001; Cohen's d = 3.649), as did the combination group (mean difference, 1.970; 95% CI, 0.900–3.041; p < 0.001; Cohen's d = 2.961).

Direct comparison between active treatment groups further demonstrated that the cumulative increase following exosome treatment was significantly greater than that following atorvastatin treatment (mean difference, 1.590; 95% CI, 0.519–2.661; p = 0.002; Cohen's d = 2.033). Exosome treatment also produced a significantly greater increase than the combination treatment (mean difference, 2.144; 95% CI, 1.073–3.215; p < 0.001; Cohen's d = 2.826). In contrast, the difference between atorvastatin and the combination treatment was not statistically significant (mean difference, −0.554; 95% CI, −1.625 to 0.517; p = 0.495; Cohen's d = −0.782).

Taken together, the treatment response demonstrated a consistent hierarchy in cumulative vascular response: **exosome > atorvastatin > exosome plus atorvastatin > control**. The exosome group not only produced the largest absolute increase in vascular density but also demonstrated statistically larger responses than both atorvastatin monotherapy and the combination treatment. Conversely, adding atorvastatin to exosome treatment did not produce a greater vascular response than atorvastatin alone at the doses evaluated.

4. Discussion

The present study demonstrated that both hUCB-MSC-derived exosomes and atorvastatin exerted significant proangiogenic effects in the in ovo chorioallantoic membrane (CAM) model. Importantly, hUCB-MSC-derived exosomes produced the strongest angiogenic response among the treatment groups, whereas the addition of atorvastatin to exosomes did not provide an additional angiogenic benefit compared with exosome monotherapy. A significant time-by-group interaction was observed for vascular density ($F(6,48) = 23.278$, $p < 0.001$, partial $\eta^2 = 0.744$), indicating that the trajectory of vascular development differed according to treatment. The cumulative change in vascular density from D0 to D2 also differed significantly among groups ($F(3,24) = 38.302$, $p < 0.001$, $\eta^2 = 0.827$).

4.1 Proangiogenic effect of hUCB-MSC-derived exosomes

The most prominent finding of this study was the superior angiogenic response following administration of hUCB-MSC-derived exosomes. The cumulative increase in vascular density from D0 to D2 was 4.786 ± 0.828 percentage points in the exosome group, compared with 0.671 ± 0.647 percentage points in the control group. The exosome group also demonstrated significantly greater vascular density changes than the atorvastatin and combination groups.

This finding is consistent with previous studies demonstrating the proangiogenic properties of extracellular vesicles derived from human umbilical cord mesenchymal stem cells. Zhang et al. (2015) demonstrated that exosomes derived from human umbilical cord MSCs enhance angiogenesis through activation of the Wnt4/β-catenin pathway. Similarly, Divband et al. (2022) reported that small extracellular vesicles derived from human umbilical cord MSCs have potential as cell-free therapeutics for promoting angiogenesis.

The strong angiogenic response observed in the present study may be explained by the multifactorial biological activity of MSC-derived extracellular vesicles. Rather than acting through a single growth factor, exosomes can transfer a complex combination of proteins, lipids, messenger RNAs, and microRNAs to recipient cells. Nawaz et al. (2018) emphasized the role of extracellular vesicles and matrix-remodeling enzymes in tissue repair, while Divband et al. (2022) demonstrated that hUC-MSC-derived extracellular vesicles can enhance the expression of angiogenic mediators, including FGF, VEGF, and VEGFR-2, in endothelial cells.

In addition, Zhang et al. (2015) demonstrated that exosomal transfer of Wnt4 activates β-catenin signaling in endothelial cells, thereby promoting endothelial migration, proliferation, and angiogenesis. These mechanisms provide a

plausible biological explanation for the pronounced vascular response observed following exosome administration in the present study.

The proangiogenic effect of MSC-derived extracellular vesicles may also extend beyond direct stimulation of endothelial cells. Mabotuwana et al. (2022) reviewed the paracrine effects of mesenchymal stem cell-derived factors in cardiovascular disease and highlighted their potential roles in endothelial responses, tissue repair, inflammation, and vascular remodeling. Thus, the angiogenic effect of hUCB-MSC-derived exosomes may reflect coordinated modulation of the vascular microenvironment rather than activation of a single angiogenic pathway.

4.2 Proangiogenic effect of atorvastatin

Atorvastatin also significantly increased vascular density compared with the control group. The cumulative increase in vascular density was 3.196 ± 0.734 percentage points in the atorvastatin group, compared with 0.671 ± 0.647 percentage points in controls. This finding indicates that atorvastatin has proangiogenic activity in the CAM model under the concentration used in this study.

These findings are consistent with previous evidence regarding the pleiotropic vascular effects of statins. Zahedipour et al. (2022) reviewed the relationship between statins and angiogenesis and described the ability of statins to modulate angiogenic signaling in a context-dependent manner. In addition, Baharara et al. (2012) specifically demonstrated an effect of different atorvastatin doses on angiogenesis in the chick embryo CAM model.

The proangiogenic effects of atorvastatin may involve several endothelial mechanisms. Statins can improve endothelial function through modulation of nitric oxide bioavailability and PI3K/Akt/eNOS signaling, while also affecting oxidative stress, inflammation, and angiogenic mediators. Dulak et al. (2005) reported that atorvastatin affects several angiogenic mediators in human endothelial cells, supporting the concept that statins can directly modulate endothelial angiogenic responses.

However, the angiogenic effects of statins are dose- and context-dependent. Zahedipour et al. (2022) described a biphasic relationship in which statins may exert proangiogenic effects at lower concentrations but may become antiangiogenic at higher concentrations. This phenomenon may be related to modulation of the mevalonate pathway and downstream small GTPases, as well as changes in PI3K/Akt and eNOS signaling.

Therefore, the proangiogenic response observed in the present study should be interpreted within the context of the atorvastatin concentration used. Since only one concentration was evaluated, it remains uncertain whether different concentrations would produce a similar response or alter the interaction between atorvastatin and hUCB-MSC-derived exosomes.

4.3 Effect of the combination of hUCB-MSC-derived exosomes and atorvastatin

Contrary to the initial hypothesis, combining hUCB-MSC-derived exosomes with atorvastatin did not result in an angiogenic response greater than that observed with exosome monotherapy. Although the combination group demonstrated a significant increase in vascular density compared with the control group, it was not significantly different from the atorvastatin group ($p = 0.495$). Moreover, the exosome group demonstrated a significantly greater cumulative increase in vascular density than the combination group, with a mean difference of −2.144 percentage points (95% CI, −3.215 to −1.073; $p < 0.001$; Cohen's $d = -2.826$).

The absence of an additional benefit from atorvastatin may be explained by the distinct but potentially overlapping mechanisms of the two interventions. Exosomes provide multiple proangiogenic mediators, whereas atorvastatin primarily modulates endothelial responsiveness through intracellular signaling pathways. Zahedipour et al. (2022) described the pleiotropic effects of statins on angiogenesis, while Nawaz et al. (2018) highlighted the role of extracellular vesicles and matrix-remodeling mechanisms in tissue repair.

One possible explanation is that the angiogenic response induced by exosomes was already sufficiently strong that additional endothelial stimulation by atorvastatin provided limited incremental benefit. The broad biological activity of exosomal cargo may activate several angiogenic processes simultaneously, potentially reducing the magnitude of any additional effect produced through a separate pharmacological pathway.

Another possible explanation is the dose and treatment conditions used in this study. Only one dose of exosomes and one atorvastatin concentration were evaluated. Therefore, the absence of an additive effect at these doses does not exclude the possibility that a different dose ratio, treatment sequence, or exposure duration could produce a synergistic response.

Interestingly, previous work has suggested that atorvastatin may modify the paracrine properties of MSC-derived products. Yu et al. (2020) reported that exosomes derived from atorvastatin-pretreated MSCs enhanced angiogenesis through the AKT/eNOS pathway. This finding differs conceptually from the present study because atorvastatin in that study was used as a preconditioning stimulus for MSCs, rather than being administered simultaneously with already isolated exosomes. This distinction may be important and suggests that the timing of atorvastatin exposure relative to exosome production could influence the biological properties of the resulting therapeutic product.

Similarly, Oktaviono et al. (2021) previously reported that hUCB-MSC-derived secretome combined with atorvastatin enhanced endothelial progenitor cell proliferation and migration. The difference between that finding and the present results may be attributable to differences in the biological product evaluated, experimental model, treatment sequence, and angiogenic endpoint. The present study evaluated isolated exosomes administered concurrently with atorvastatin in the CAM model, whereas secretome-based approaches contain a broader spectrum of soluble and vesicular factors.

Thus, the current findings should not be interpreted as definitive evidence of an antagonistic interaction. Rather, they indicate that under the experimental conditions tested, atorvastatin did not provide an additional angiogenic benefit when added to hUCB-MSC-derived exosomes. Further dose-response and mechanistic studies are required to determine whether a true pharmacological interaction exists.

4.4 Possible biological explanation for the stronger effect of exosome monotherapy

The superiority of exosome monotherapy over both atorvastatin and the combination treatment may reflect the broader biological repertoire of MSC-derived extracellular vesicles. Exosomes can simultaneously influence endothelial proliferation, migration, survival, extracellular matrix remodeling, and intercellular communication. Mabotuwana et al. (2022) highlighted the importance of mesenchymal stem cell-derived paracrine factors in cardiovascular tissue repair, while Divband et al. (2022) demonstrated the angiogenic potential of hUC-MSC-derived extracellular vesicles.

Furthermore, the cargo of MSC-derived extracellular vesicles may be source-specific. Ding et al. (2024) demonstrated that the molecular characteristics of extracellular vesicles vary according to the MSC source, suggesting that their biological effects cannot be generalized across all MSC-derived products. This consideration is relevant to the present study because the observed proangiogenic activity may be particularly related to the biological composition of hUCB-MSC-derived exosomes.

4.5 Clinical and translational implications

The present findings provide preliminary evidence supporting hUCB-MSC-derived exosomes as a potential cell-free strategy for therapeutic angiogenesis. This approach may be relevant to ischemic cardiovascular disease, in which endogenous angiogenesis may be insufficient to restore adequate tissue perfusion.

The CAM model is particularly useful as an initial preclinical platform because it allows direct visualization and quantification of vascular

responses within a rapidly developing vascular network. Ribatti (2016) described the chick embryo CAM as a versatile experimental model for angiogenesis, while Ribatti et al. (2020) further highlighted its utility for evaluating the angiogenic activity of biomaterials. Shekatkar et al. (2024) also emphasized the usefulness of the CAM model in preclinical angiogenesis research.

Nevertheless, the strong angiogenic response observed in CAM should not be directly equated with therapeutic efficacy in human cardiovascular disease. Further studies in mammalian models of myocardial or peripheral ischemia are needed to determine whether the increase in vascular density translates into improved tissue perfusion, vascular maturation, and functional recovery.

4.6 Limitations and future directions

Several limitations should be considered when interpreting the findings. First, the study was conducted using an in ovo CAM model, which represents an early stage of preclinical evaluation. The CAM does not fully reproduce the complex environment of adult mammalian ischemic tissue, including chronic inflammation, oxidative stress, and tissue remodeling. Therefore, validation in mammalian models of myocardial or hindlimb ischemia is required before clinical translation.

Second, only a single effective dose of each intervention was evaluated. Consequently, dose-response relationships and the possibility of synergistic effects at different dose combinations could not be determined. Future studies should investigate multiple concentrations of both exosomes and atorvastatin, as well as different treatment sequences.

Third, vascular density was the primary outcome. Although vascular density provides a useful quantitative measure of angiogenesis, it does not fully characterize vascular branching complexity, vessel maturation, pericyte recruitment, or functional perfusion.

Fourth, the molecular mechanisms underlying the observed effects were not directly examined. The study did not characterize the exosomal cargo or directly measure VEGF, HGF, angiopoietins, proangiogenic microRNAs, HIF-1α, or Wnt/β-catenin signaling. Therefore, the proposed mechanisms should be regarded as plausible interpretations based on previous literature rather than mechanisms directly demonstrated by the present experiment.

Finally, the observation period was limited to 48 hours. Although this period was sufficient to detect early morphological changes in CAM vascularization, it was insufficient to assess longer-term vessel stabilization, maturation, pericyte recruitment, or functional perfusion.

4.7 Overall interpretation

In conclusion, the present study demonstrates that both atorvastatin and hUCB-MSC-derived exosomes exert proangiogenic effects in the CAM model, with hUCB-MSC-derived exosomes producing the strongest response. The addition of atorvastatin did not enhance the angiogenic effect of exosome monotherapy under the conditions tested. These findings are consistent with previous evidence supporting the proangiogenic properties of MSC-derived extracellular vesicles (Zhang et al., 2015; Divband et al., 2022) and the pleiotropic vascular effects of statins (Zahedipour et al., 2022; Baharara et al., 2012).

The findings suggest that hUCB-MSC-derived exosomes may represent a promising cell-free therapeutic candidate for further investigation in therapeutic angiogenesis. However, optimization of dose, treatment sequence, molecular characterization of exosomal cargo, assessment of vascular maturation and function, and validation in mammalian ischemia models are necessary before the findings can be translated toward clinical application.

5. Conclusion

This study demonstrated that both hUCB-MSC-derived exosomes and atorvastatin exerted significant proangiogenic effects in the in ovo chorioallantoic membrane (CAM) model. Among the treatment groups, hUCB-MSC-derived exosomes produced the greatest increase in vascular density. Atorvastatin also significantly increased vascular density compared with the

control group. However, the combination of hUCB-MSC-derived exosomes and atorvastatin did not provide an additional angiogenic benefit compared with exosome monotherapy. Therefore, under the experimental conditions and doses used in this study, hUCB-MSC-derived exosomes demonstrated the strongest proangiogenic effect, whereas the addition of atorvastatin did not enhance this response.

These findings support the potential of hUCB-MSC-derived exosomes as a cell-free therapeutic approach for promoting angiogenesis. Further studies are needed to evaluate dose-response relationships, treatment timing, molecular mechanisms, vascular maturation, and functional perfusion, as well as to validate these findings in mammalian models before potential clinical translation.

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