

# Unveiling the wound healing potential of umbilical cord derived conditioned medium: an *in vitro* study

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## SUMMARY

Chronic wounds pose a serious threat to an individual's health and quality of life. As such, therapeutic intervention is often needed to mitigate the improper closure of wounds. Large amounts of research and funding have been directed towards finding a suitable treatment. However, due to the severity and morbidity of such wounds, many pre-existing treatments are inefficient or costly. While the use of skin grafts and other such biological constructs in chronic wound healing has already been characterized, the use of umbilical cord tissue has only recently garnered interest, despite the cytokine-rich composition of Wharton's jelly (cord component). Our current study aimed to characterize the use of an umbilical cord derived conditioned medium (UC-CM) to treat chronic wounds. We hypothesized that UC-CM contains higher amounts of wound healing cytokines, supporting cell proliferation, migration, and better wound healing properties compared to fetal bovine serum (FBS). Cytokine analysis demonstrated that UC-CM consistently contained desirable levels of HGF, EGF, VEGF, and TGF- $\beta$ , all of which are vital to the normal wound healing process, whereas there were negligible amounts of growth factors in FBS except for TGF- $\beta$ . MTT assay confirmed that UC-CM is not cytotoxic and promotes cell proliferation, while the scratch assay demonstrated that UC-CM supports migration of MSCs into the wound site. Taken altogether, our study showed that UC-CM was equally as effective as FBS-containing medium at promoting cell proliferation and wound healing.

## INTRODUCTION

Wounds may be defined as a breakdown in the integrity of skin, mucous membrane or organ tissue (1). Wound healing, the natural physiological response to wound detection, involves a complex orchestration of the action of signaling molecules called cytokines, mediators, various cell types and the vascular system (2). It comprises four main overlapping stages: hemostasis, inflammation, cell proliferation, and tissue remodeling.

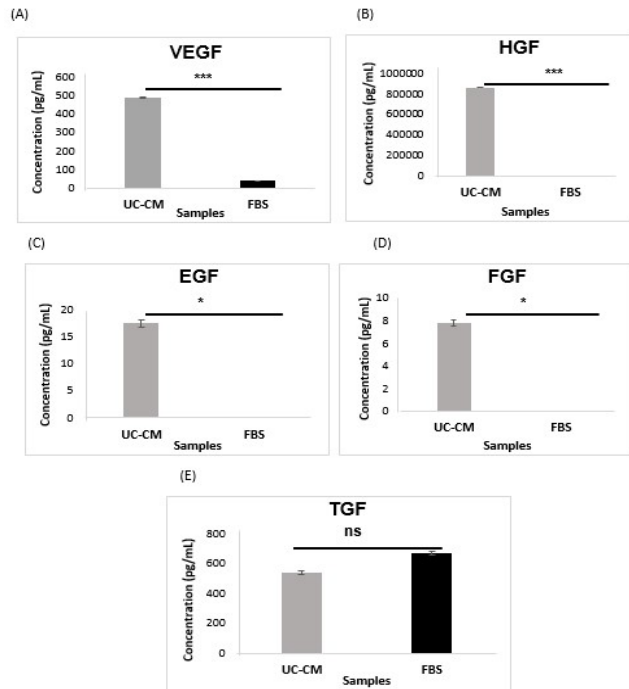
Platelet activation upon detection of a ruptured endothelium results in the formation of a fibrin clot and secretion of an array of growth factors including endothelial growth factor (EGF), platelet derived growth factor (PDGF), and transforming growth factor-beta (TGF- $\beta$ ) from alpha

granules. Inflammation is marked by PDGF and TGF- $\beta$  triggering directed cell movement (chemotaxis) of immune cells including neutrophils and macrophages, which adhere to the fibrin scaffold and cleanse the wound site by engulfing large particles of debris and bacteria in a process called phagocytosis. Macrophages also play a key role in wound healing by secreting growth factors such as TGF- $\beta$ , FGF-1, and EGF, promoting cell proliferation and granulation tissue formation. These factors also promote secretion of extracellular matrix (ECM) components including collagen and glycosaminoglycans, which form the core of the wound, from fibroblasts (3).

At this stage, TGF- $\beta$  triggers phenotypic changes in fibroblasts, converting them to myofibroblasts involved in wound contraction. Re-epithelialization, the migration and proliferation of epithelial cells to cover and protect the wound site, is initiated by the migration of cells from the wounds periphery by the action of EGF and FGF-1 (4). This is followed by VEGF and HGF triggering neovascularization, which occurs both through angiogenesis, the formation of new blood vessels from preexisting ones, and vasculogenesis, the formation of new blood vessels from endothelial progenitor cells (EPCs) (5). Neovascularization is required for the removal of granulation tissue, which is then replaced by a framework of ECM components: collagen, elastin fibers, proteoglycans, and glycoproteins (1-4). Chronic wounds, characteristic of systemic abnormalities like diabetes and venous insufficiency, are those which do not proceed through normal wound healing in a timely manner. This is caused by the up-regulation of proteases and down-regulation of cytokines, and results in the need for therapeutic intervention to facilitate proper wound closure (6, 7).

Existing treatments for chronic wounds include skin substitutes like porcine xenografts, which due to their biologically derived composition can be placed directly into the wound. However, skin grafts are often costly and come with the risk of rejection (8). Antibiotics are also widely used to mitigate bacterial infections in chronic wounds (9). However, there is limited supporting evidence for their direct impact on broader aspects of wound healing beyond their antibacterial functions, including the associated risk of resistance (10).

In order to overcome challenges presented by the existing wound treatments, researchers have investigated the potential of umbilical cord tissue to promote wound healing because of its unique cytokine-rich and extracellular matrix rich composition, cost effectiveness, and ease of procurement(11). While the effectiveness of dehydrated umbilical cord in expediting the healing of chronic wounds has been established, the efficiency of freeze-dried (lyophilized) umbilical cord, including its proteins such as growth factors, is



**Figure 1: Measurement of VEGF, EGF, HGF, FGF-1 and TGF-β by ELISA.** Graphical representation of cytokine levels of (A) VEGF, (B) HGF, (C) EGF, (D) FGF-1 and (E) TGF-β in UC-CM (N=10, 10=UC-CM samples) and FBS group. Data values are represented as Mean ± SD. cytokine levels of VEGF, HGF, EGF, FGF-1 were higher in UC-CM compared to FBS group. TGF-β levels were lower compared to FBS. Unpaired Student T-test indicated significance with \* $p < 0.05$  and \*\*\* $p < 0.001$  are considered statistically significant in comparison to FBS.

subjected to different stresses that may interfere with protein functionality (10-12). Therefore, to address this, we developed a conditioned medium called UC-CM, derived from the culture medium exposed to the entire umbilical cord tissue. This tissue includes Wharton’s jelly, a gelatinous connective tissue component rich in extracellular matrix, which would contain secreted growth factors, cytokines, hormones, and signaling molecules, distinct from those found in the tissue itself.

We hypothesized that UC-CM contains elevated levels of wound healing cytokines, thereby promoting cell proliferation, migration, and superior wound healing properties compared to FBS. We characterized UC-CM by quantifying VEGF, FGF-1, EGF, HGF, and TGF-β, key cytokines involved in epithelial repair and vasculogenesis. Subsequently, we conducted an in vitro analysis comprising cell viability, proliferation, and a scratch assay to evaluate UC-CM’s efficacy in supporting wound closure. Our findings revealed that UC-CM contained higher cytokine levels compared to FBS and supported the growth and proliferation of UC-MSCs in vitro. However, the scratch assay indicated no significant difference in migration between the two groups. To summarize, our findings indicate that UC-CM contains a wealth of wound healing factors, effectively fostering cell growth and proliferation in UC-MSCs. Furthermore, these results suggest the promising capacity of UC-CM in facilitating wound healing.

Overall, the study aimed to investigate the wound healing potential of UC-CM compared to FBS, with major results indicating higher cytokine content in UC-CM and its ability to

support UC-MSC growth and proliferation. A key takeaway is that UC-CM shows promise as a wound healing therapy, though it may not significantly affect cell migration compared to FBS.

## RESULTS

### Measurement of VEGF, EGF, HGF, FGF-1 and TGF-β by ELISA

In order to characterize the composition of UC-CM, conditioned medium was obtained after incubating umbilical cord tissue for 48 hours. Subsequently, it was evaluated for the presence of various wound healing cytokines such as VEGF, EGF, HGF, FGF-1, and TGF-β using ELISA, with FBS utilized as a control (N=10).

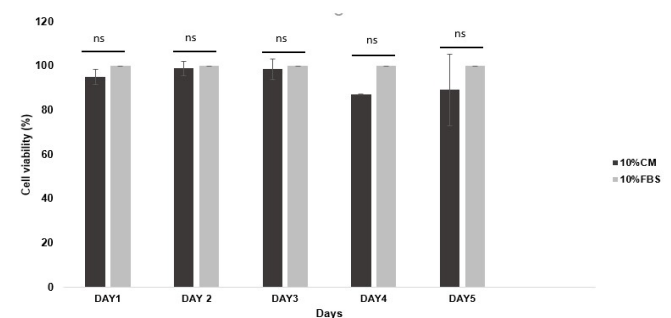
We observed significantly higher concentration of cytokines (VEGF:  $488.58 \pm 2.003$  pg/ml, EGF:  $17.55 \pm 0.657$  pg/ml, HGF:  $862,587 \pm 585.131$  pg/ml and FGF-1:  $7.805 \pm 1: 0.275$ ) in comparison to FBS (Figure 1). Additionally, we found the levels of TGF-β to be higher in FBS ( $669.90 \pm 9.93$  pg/ml) than in UC-CM ( $541.02 \pm 12.68$ ) pg/ml. These data demonstrate the cytokine profile of UC-CM, which is crucial for understanding its potential role in accelerating wound healing process.

### Percentage Viability assay

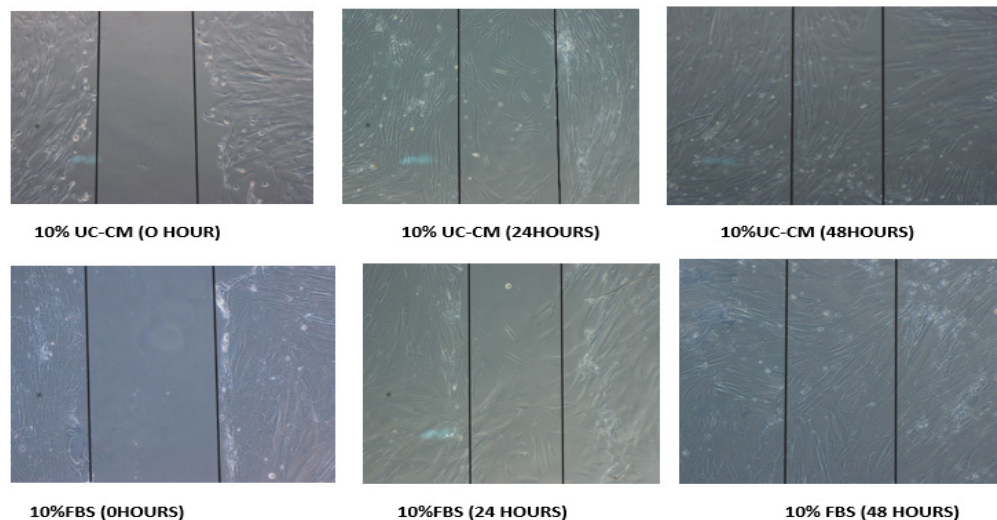
We assessed the ability of 10% UC-CM as a growth supplement for umbilical cord-derived mesenchymal stem cells using an MTT assay. MTT is a colorimetric assay used to assess the cell viability and proliferation by measuring mitochondrial dehydrogenase activity. We cultured UCMSCs ( $0.3 \times 10^6$ ) in a 96 well plate, changed the medium to 10% UC-CM the following day, and then assessed viability for five consecutive days. Our results demonstrated that 10% UC-CM supported the growth of UC-MSCs equivalent to the UC-MSCs grown in 10% FBS, as evidenced by more than 85% viability for the five consecutive days of observation (Figure 2). The results suggest that 10% UC-CM effectively promotes the growth and viability of UC-MSCs, similar to 10% FBS over the observed time period.

### Scratch assay

To evaluate the impact of UC-CM on accelerating wound closure following external injury, we conducted an in vitro



**Figure 2: Percentage viability assay using MTT.** Graph showing the percentage viability of UC-MSCs cultured with either 10% UC-CM or 10% FBS in DMEM for five days. There was no significant change in UC-CM group compared to FBS. Data values are represented as Mean ± SD (N=2, two individual setups). Student t –test was used to calculate the statistical significance between 10% UC-CM and 10% FBS. ns =  $P > 0.05$  is non-significant.



**Figure 3: In vitro scratch assay.** Images showing UC-MSCs grown in 10% UC-CM and 10% FBS (N=2) were captured at 0 hours, 24 hours and 48 hours using a phase contrast microscope. Lines drawn at the time of scratch and the rate of migration was qualitatively measured from the edge of the scratch toward the center of the scratch. All images were taken using 10X magnification.

wound healing assay using a scratch assay in UC-MSCs. Our findings demonstrated that 10% UC-CM facilitated the migration of UC-MSCs to the wound area over time. At 48 hours, we observed that UC-MSCs grown in UC-CM migrated at the same rate compared to that of the control group when observed under the phase contrast microscope, implying that 10% UC-CM promotes the migration of UC-MSCs, contributing to wound closure in vitro (**Figure 3**). However, due to lack of more replicates further experimental work would need to be done to confirm these results.

## DISCUSSION

Our study explored the potential of conditioned medium derived from umbilical cord as a therapeutic option for wound healing. We found consistent concentrations of various growth factors across different cord samples, except for FGF-1. Previous studies by Gupta et al. and Sobolewski et al. have shown that Wharton's jelly, found within the umbilical cord, is rich in wound healing cytokines (13, 14). Our cytokine analysis results reinforce this knowledge, indicating that these growth factors solubilize to form a conditioned medium when minced umbilical cord is suspended in plain DMEM media. Notably, our conditioned medium consistently contains EGF, HGF, and TGF- $\beta$ , with concentrations ranging from 17.55 pg/mL, 862587 pg/mL, and 541.02 pg/mL, respectively. HGF levels in UC-CM were significantly higher than in FBS, potentially linked to its role in fetal growth and organ development (15). The observed elevation of HGF may be attributed to its mitogenic action on hepatocytes crucial for fetal liver development. (16,17). Variability in growth factor levels could stem from procedural differences, but our methodology consistently detects key wound-healing factors in cord extract, demonstrating efficacy in in vitro wound healing models.

Previous experiments with human umbilical cord extract have showcased its efficacy in wound healing by enhancing fibroblast proliferation and migration in in vitro settings (18-20). Also, studies show that Wharton's jelly suspension, promotes cell mobilization to ruptured tissue and exhibits mitogenic and motogenic action on fibroblasts, as seen in an

in vitro experiment (18-21).

In our study, the conditioned medium (UC-CM) exhibited non-cytotoxicity in a MTT assay, supporting the growth and proliferation of umbilical cord-derived mesenchymal stem cells (UC-MSCs). However, in the study of UC-CM functionality for wound healing properties through in vitro scratch assays, the results demonstrated comparable wound closure capabilities to those observed with FBS, leading to the rejection of our hypothesis.

The possible reasons for the observed similarity in wound closure capabilities between UC-CM and FBS in the in vitro scratch assay could be attributed to the complex interplay of growth factors and cytokines or potential synergistic effects of both. For effective wound healing orchestration, an optimal concentration of growth factors may be necessary, and further increases might not significantly enhance their effects due to saturation of cellular receptors. Additionally, the role of other wound healing growth factors such as PDGF (Platelet derived Growth Factors), IGF (Insulin Like Growth factor), ICAM-1 (Intercellular adhesion molecule-1), MCP-1 (Monocyte chemotactic protein-1), and G-CSF3 (Granulocyte-Colony-stimulating factor 3) in 10% UC-CM and 10% FBS has yet to be explored, which could contribute to a more comprehensive understanding of the wound healing process.

Our findings revealed consistent presence of key factors such as HGF, EGF, VEGF, and TGF- $\beta$  in UC-CM, all crucial for effective wound healing. In contrast, FBS exhibited minimal levels of growth factors, primarily TGF- $\beta$ . The MTT assay confirmed UC-CM's non-toxic nature and its ability to enhance cell proliferation, while the scratch assay demonstrated its capacity to facilitate MSC migration to wound sites. Overall, these in vitro experiments established that UC-CM was comparably effective to FBS-containing medium in promoting both cell proliferation and wound healing processes.

Our study has limitations, particularly in drawing conclusive results from the in vitro scratch assay with less number of replicates. As the wound healing process involves numerous cell types, cell signalling and complicated physiological interactions, our future prospects would be to further validate

the wound healing properties of 10% UC-CM in other endothelial or fibroblast cell lines through in vitro assays like invasion assays, tubule formation assays, inflammatory response assays and other relevant in vitro approaches in our future studies. In summary, our results demonstrated that UC-CM is enriched with wound healing factors and can effectively support cell growth, proliferation in UC-MSCs and also suggesting its wound healing potential.

## MATERIALS AND METHODS

### Preparation of Umbilical Cord derived conditioned medium (UC-CM)

Ten Donors gave their signed consent forms for collection of Umbilical Cord samples following Caesarean section births from V-Care Maternity Hospital, Bangalore, India. These samples were pre-screened for infectious diseases and donated for research purposes. The whole umbilical cords (cellular and acellular parts) were washed with phosphate buffered saline and were equally cut into small pieces. Then, it was transferred into a 50 ml tube containing DMEM (Dulbecco's Modified Eagles Medium) (Gibco- catalog no. 11320-33). The umbilical cords were incubated for 48 hrs at 37°C in a 5% CO<sub>2</sub> incubator. After 48 hrs, the contents were centrifuged at 1500 rpm for 10 minutes and the supernatants were filtered through a 0.2 µm membrane filter. The supernatants (UC-CM) were stored at -20°C for further experimental use. The control group for the study was DMEM supplemented with fetal bovine serum, which is commonly used to promote cell growth in culture.

### Quantification of cytokines by ELISA

Elisa was performed in the UC-CM of ten umbilical cord samples to check for the presence of growth factors including VEGF (Vascular endothelial growth factor), EGF (Epithelial Growth Factor), HGF (Hepatocyte Growth Factor), TGF-β (Transforming growth factor) and FGF-1 (Fibroblast Growth Factor) (Elab Sciences). The samples were diluted 1000-fold for HGF. Fold dilutions were not done for EGF, FGF-1, VEGF and TGF-β. FBS was used as the control group.

Briefly, the procedure is summed up as follows: 100 µL each of standards, samples, and a blank were added to the precoated wells before they were incubated at 37 °C for 90 minutes. After the liquid was taken out, 100 µL of biotinylated detection antibody was added to each well, and each well was then left to incubate for an hour at 37°C. 100 µL of horseradish peroxidase conjugate solution was added to the wells after the plate had been rinsed three times with wash buffer. The plate was then incubated at 37°C for 30 minutes. 90 µL of substrate was then added after repeating the washing process five times, and it was then left at 37 °C for 15 to 30 minutes. The optical densities (O.D.) of the samples at 450 nm were then quantified using an ELISA plate reader (Lisquant) after 50 µL of stop solution had been added to each well. By using MS Excel to display the standard and blank values and then fitting the data with a linear fit, the concentration of each cytokine in pg/mL was calculated. The standards served as the positive control, the sample diluent as the negative control, and the experimental samples were adjusted by deducting the O.D. values from the blank wells. The concentration of each growth factor in pg/ml of total protein were estimated from the respective standard curves.

### Statistical Analysis

Statistical analysis was performed using MS-Excel 2016. All data obtained were from two independent experiments. The data values were represented as mean ± SD. Student unpaired two-tail, t-test was used to determine the statistical significance between the two groups (UC-CM and FBS).

### Cell culture

UC-MSCs (Umbilical cord derived mesenchymal stem cells) were obtained from Lonza. Passage (P6) of these cells were used for all the experiments. Cells were grown in DMEM (Gibco- catalog no. 11320-33) supplemented with either 10% FBS (Fetal Bovine Serum) (Gibco- catalog no. 10270-106) or 10% UC-CM and 1% antibiotic-antimycotic solution (HiMedia Laboratories- catalog no. A002). All cultures were maintained at 37°C in 5% CO<sub>2</sub> incubator.

### Percentage viability assay

A percentage viability assay was performed to analyze the cytotoxicity of UC-CM on UC-MSCs. 0.3×10<sup>6</sup> UC-MSCs at passage 6 were seeded in a 96 well plate supplemented with 10% FBS and were kept for 24 hrs at 37°C in 5% CO<sub>2</sub> incubator. The following day, the medium was changed to 10% UC-CM and cell viability was assessed from 1, 2, 3, 4, and 5 days respectively at the above mentioned culture conditions. UC-MSCs grown in 10% FBS served as the control group for this experimental setup. For the next five consecutive days, 20 µl of MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) was added to each well and the plate was incubated at 37°C for four hours. After four hours, DMSO was used as the solvent to dissolve the formazan crystals. Absorbance was measured at 545nm, with DMSO as the negative control. Percentage viability was calculated in MS-Excel 2016 using the formula:

$$\text{Percentage Viability} = \frac{\text{Blanked out value of respective UC - CM}}{\text{Blanked out value of 10\% FBS}} * 100$$

Where, blanked out value= O.D. of the sample (UC-CM) – O.D. of blank (DMSO-dimethyl sulfoxide)

The inbuilt excel function (two tailed student t-test) was used to calculate the statistical significance between the two groups. P < 0.05 is considered as statistically significant. Two independent sets of experiments were carried out for the assay.

### In vitro Wound healing assay

A scratch assay was carried out to determine whether the UC-CM will facilitate wound closure in UCMSCs. US-MSCs (P6) grown in 10% UC-CM and 10% FBS were seeded at a seeding density of 0.01×10<sup>6</sup> per well in a 24 well plate. When the cells reached a confluency of 70-80%, a scratch was made using a standard 200 µL microtip above the cell surface. Cells were washed with normal saline and serum free medium was added to each well. Cultures were incubated at 37°C in 5% CO<sub>2</sub> incubator for 48 hrs. The images were taken at the initial time of scratch using a phase contrast light microscope and the same field was taken at 24 hrs and 48 hrs. The scratch was analyzed qualitatively. The data obtained were from two independent sets.

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