USAGE OF EXTRACELLULAR MICROVESICLES AS A NOVEL AND PROMISING THERAPEUTIC TOOL IN WOUND HEALING

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

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2017

Wright State University
I HEREBY RECOMMEND THAT THESIS PREPARED UNDER MY SUPERVISION
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as a Novel and Promising Therapeutic Tool in Wound Healing. BE ACCEPTED
IN PARTIAL FULLFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
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Abstract

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**Introduction** Extracellular Microvesicles (EMVs) can carry genetic messages and biologically active proteins throughout tissues and the body. Because of their transport capabilities, EMVs play an important role both diseased and healthy conditions. For example, EMVs play an important regenerative role in many damaged tissues. In the current studies, we examine the role of EMVs in epithelial wound healing. The potential use of EMVs as drug delivery vehicles has gained considerable scientific interest because they can be delivered in circulation, can be targeted to specific areas/cells, and can pass natural barriers. In the current work, we investigate the potential of EMVs or EMVs loaded with growth factors as a tool to enhance cell migration in order to accelerate epithelial wound healing.

**Material and Methods** Spontaneously immortalized skin keratinocyte and macrophage cells were stressed for 48 h by serum free media to enhance the release of the EMVs from keratinocytes (KMVs) and macrophages (MMV). The EMVs from both cell lines were isolated and collected using a centrifugation process. Specifically, the collected serum free media were centrifuged at 4 °C (500 × g for 10 minutes followed by 2,000 × g for 20 minute). The supernatant was then centrifuged at 24,000 × g for 2 hours to isolate EMVs. EMVs were “loaded” with growth factors by incubating them for 1.5 h at room temperature
with PDGF, TGF-β, VEGF, and FGF (25ng/ml per each). These “loaded” EMVs were then ultra-centrifuged at 176,000 x g for 3 h to re-pellet the loaded microvesicles derived from keratinocytes KMVs (LKMVs) or macrophages MMVs (LMMVs).

In order to evaluate the role of microvesicles on cutaneous wound healing, we chose the in vitro wound scratch assay to evaluate the cell migration rate and the wound healing percent after adding of KMVs, LKMVs, MMVs, and LMMVs separately to Epidermal keratinocytes culture. Epidermal keratinocytes were plated into 6 well plates, and wound scratch was made using 10 µl pipette tip. The model was visualized by 10 x magnification power of EVOS XL Core Cell Imaging System and analyzed using Mat lab software to measure wound area.

MTT assay was used to evaluate the proliferative effect of KMVs, LKMVs, MMVs, and LMMVs on Epidermal keratinocytes. The loading was confirmed by using BioPlex Pro cytokine assays.

Results after 72 h, the wound area in the EMVs (KMVs & MMVs) and LEMVs (LKMVs & LMMVs) treated groups showed a significant decrease in wound area and a remarkable ability to repair the wound area as compared with the control group ($P < 0.0001$). The percent of wound healing was almost three times more in KMVs and MMVs treated groups ($57.85 \% \pm 3.13$, $69.84 \% \pm 4.87$, respectively), and four times in LKMVs and LMMVs treated groups ($80.10 \% \pm 3.50$, $90.87 \% \pm 2.00$, respectively) when compared to the control groups ($21.74 \% \pm 2.389$) ($P < 0.0001$). Furthermore, the migration rate in the presence of KMVs and MMVs ($0.008810 \pm 0.0006856$, $0.01085 \pm 0.0007964$ mm²/h, respectively)
and LKMVs and LMMVs (0.01470 ± 0.0009428, 0.01767 ±0.001163 mm²/h, respectively) were enhanced when compared to the control group (0.003820 ± 0.0003760 mm²/h).

**Conclusion** EMVs and Loaded EMVs have a potential regenerative effect in wound healing, which promotes and enhances cell migration and proliferation, resulting in accelerated wound closure. Based on these finding, we suggest that EMVs are a novel and promising therapeutic tool for epithelial wound healing.
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Sincerely yours,

Sami Gamaleddin F Alsabri
Introduction

Skin is the largest organ in the body. It works as a protective layer against toxins and microorganisms, and provides chemical protection against invasion by toxins and microorganisms. Skin also plays an important role in thermoregulation and prevention of dehydration. (Choi, Uyama, Lee, & Sung, 2015)

Wounds

A wound can be defined as a breakdown in the protective function of the skin. Wounds can also be defined as disruption in the epithelial lining of the skin or mucosa due to either physical or thermal injury (Dhivya, Padma, & Santhini, 2015). Generally, wounds are classified as acute or chronic. Clinically, acute wounds are defined as wounds with high tendency to heal in a short period of time (< 3 months), while chronic wounds have little or no tendency to healing (≥ 3 moths).

Chronic wounds

Chronic wounds have increased in prevalence over the last few decades. Chronic wounds affect 1-2 % of the population in the United States, which results in an increase in the health care burden. These injuries cost the US health care system around 50 billion dollars yearly, with an average cost of $3.349 to 9.358 per wound. Furthermore, current treatments for chronic wounds do not guarantee wound closure, and recurrence is common.
Therefore, the development of effective chronic wound treatments is an important issue that could increase the effectiveness of health care resources worldwide (Gainza et al., 2015). Scientists have not only focused on developing new modalities of treatment, but also improving the efficacy of existing treatments. Here we focus upon the use of growth factors (GFs) for wound healing (Gainza et al., 2015).

**Wound healing**

Epithelial wound healing occurs in stages. Understanding the stages of wound healing at the molecular level is essential to developing treatment. This may helps in not only reduce morbidity and mortality related to abnormal or prolonged wound healing but also in finding and introducing new approaches and therapeutics tools. Cutaneous wound healing is a multistep and highly sophisticated systematic process. It includes many cell types, soluble mediators, and extracellular matrices (Robson et al., 2001) along with a highly dynamic coordination between complex cascades of cellular events. These events begin when wounding occurs in a process meant to restore and/or replace the damaged and/or missing tissues (Rieger et al., 2014). Furthermore, wound healing is characterized by a series of overlapping time dependent phases. These include the hemostatic phase (time of injury up to several hours after injury), inflammatory phase (1 to 3 days), proliferative phase (4 to 21 days), and remodeling phase (21 days up to 18 months) (Landén et al., 2016). Disruption in any of these phases results in healing impairment and the potential for chronic wounds (Landén et al., 2016, Shi et al., 2013). Unfortunately, current therapeutic tools for chronic wounds still do not achieve the complete healing, and do not prevent wound recurrence. Thus, the development of a novel, effective treatment is needed. (Dhivya et al., 2015; Rosique, Rosique, & Farina Junior, 2015).
Growth factors in wound healing

The wound healing process is affected by growth factors. Therefore, growth factors have been extensively studied over the past decades. Growth factors contribute and regulate the cell migration and proliferation in order to accomplish healing as a response to tissue injury (Molloy, Wang, & Murrell, 2003; Rosique et al., 2015). Several growth factors have been proven to have a role in inflammation (explained below) where they have been shown to improve wound healing (Efron and Moldawer 2004). Furthermore, down regulation of growth factor proteins has been reported in chronic wounds and has been hypothesized to be one possible cause of wound chronicity (Barrientos et al., 2008).

Platelet derived growth factor (PDGF)

PDGF is produced by secreted by platelets, macrophages, vascular endothelium, fibroblasts, and keratinocytes. PDGF induces cell proliferation, angiogenesis and chemotaxis. In fact, PDGF has a significant role in each phase of healing process (Efron and Moldawer 2004).

During the inflammatory phase, PDGF serves as a strong stimulant for mitogenicity and chemotaxis of neutrophils, macrophages, fibroblasts, and smooth muscle cell migration to the wound site (Barrientos et al., 2008). PDGF also enhances the release of several growth factors. For example, TGF-β can be produced by macrophages as a result of direct induction of PDGF. Later, TGF-β along with PDGF enhance macrophage mediated tissue cleanup, and formation of granulation tissue in the proliferation phase.

Regarding to angiogenesis, in vitro PDGF, synergistically with hypoxia, shows ability to induce formation of new blood vessels by enhancing of VEGF expression, and it is particularly essential in in blood vessel maturation (Hu & Huang, 2015). In vitro PDGF
has been shown to induce reepithelialization through upregulation of IGF-1 and thrombospondin-1 synthesis, which increase keratinocyte motility, delays proteolytic degradation, and indorses a proliferative response in keratinocytes in the wound healing (Barrientos et al., 2008). Similarly, PDGF can increase fibroblast proliferation and regulate collagen production, which are required during the proliferative phase of wound healing (Hu & Huang, 2015).

In the remodeling phase, PDGF can increase the degradation of collagen by regulating matrix metalloproteinases (Jinnin et al., 2004). In all stages of wound healing, PDGF plays a vital role by stimulating the release of the pro-healing cytokines at site of injury.

**Transforming growth factor beta (TGF-β)**

TGF-β is a family of pluripotent cytokines. TGF-β has three isoforms: TGF-β 1, 2, and 3 with a dominant role of TGF-β 1 in cutaneous wound healing. It can be released by keratinocytes, platelets, monocytes, macrophages, and fibroblasts (Rolfe et al., 2007 and Barrientos et al., 2008)

In homeostasis normal conditions, TGF-β has been found to regulate the keratinocyte cell cycle and inhibit proliferation. Therefore, TGF-β plays a role in maintaining skin homeostasis. TGF-β is a crucial key factor in the wound healing process (Siegel & Massagué 2003, Ramirez et al., 2014). It plays a significant regulatory role at all the tissue regeneration stages, including, the inflammation, re-epithelialization, angiogenesis, and granulation tissue formation (Efron and Moldawer 2004, Guasch et al., 2007, Barrientos et al., 2008).
In wounds, TGF-β has been found to have pleiotropic effects, and regulates the functions of keratinocytes, fibroblasts, endothelial cells, monocytes, and other cell types that are required in the regenerative process (Ramirez et al., 2014).

In nonhealing wounds, both *in vitro* and *in vivo* data show that TGF-β can suppress the growth and maintenance of epidermal homeostasis (Guasch et al., 2007). Multiple studies have also demonstrated that TGF-β expression is suppressed in the epidermis of chronic wounds (Ramirez et al., 2014). Several mouse models have shown that exogenous application of TGF-β enhanced wound healing by activating TGF-β signaling (Barrientos et al., 2008). Clinically, in chronic ulcers, suppression of TGF-β signaling may contribute to the loss of tissue homeostasis because of the hyperproliferation and the inability of keratinocytes to migrate, epithelialize, and close the wound.

During the inflammatory stage, TGF-β plays a pro-inflammatory role as a chemotactic agent for monocytes infiltration to the wound site. It can also differentiate macrophages that clean up the wound site, and play an anti-inflammatory role. During the proliferative stage. In proliferative stage, TGF-β is involved in angiogenesis and initiates granulation tissue formation. This occurs by up-regulating the expression of fibronectin, collagen types I and III, vascular endothelial growth factor (VEGF), and protease inhibitors. TGF-β also down regulates MMP expression, which further promotes the accumulation of collagen fibers (Barrientos et al., 2008). Furthermore, TGF-β induces the wound contraction by inducing smooth muscle alpha actin expression in fibroblasts and myofibroblast differentiation (Van De Water et al., 2007).
Vascular endothelial growth factor (VEGF)

VEGF, also called vascular permeability factor, is a homodimeric glycoprotein, and one of the most important vascular regulators of vasculogenesis and angiogenesis during the injury healing process (Hoeben et al., 2004). VEGF is secreted from platelets, keratinocytes, macrophages, neutrophils, endothelial cells, smooth muscle cells, and fibroblasts (Barrientos et al., 2008). VEGF is involved in wound repair by triggering angiogenesis, chemotaxis, and inducing vascular permeability (Bao et al., 2009) during the wound healing process (Cooper et al., 1999).

VEGF has distinctive effects on many components of the wound healing cascade, comprising angiogenesis, epithelialization, and collagen deposition (Tomic-Canic et al., 2007). Since angiogenesis has a vital role in the wound reconstructing process, VEGF (alone or in combination therapy) may be utilized in the future for patients with non-healing chronic wounds. According to in vitro studies, VEGF initiates the early events in angiogenesis, chiefly migration and proliferation of endothelial cell (Lamalice et al., 2007).

Fibroblast Growth Factor (FGF)

Since its discovery as a family of growth factors, FGFs are known to play a role in a variety of biological processes including differentiation, migration, proliferation, angiogenesis, and wound healing (Nakamichi et al., 2016). FGFs are released from many cells including macrophages, mast cells, keratinocytes, fibroblasts, smooth muscle cells in the wound environment. During the inflammatory stage, FGF has a role in recruiting several inflammatory cells, such as neutrophils and monocytes to the wound site. In addition, FGF induces the expression of several chemokines, and encourages mitogenesis of endothelial cells (Nakamichi et al., 2016). As a growth factor, FGF plays a critical role
in re-epithelialization, formation of granulation tissue and tissue remodeling. Several in vitro studies have revealed that FGF controls extracellular matrix deposition, enhances both keratinocyte migration and proliferation augments fibroblast migration, activates the release of collagenase, and additionally improves endothelial cell growth and migration. (Teven et al., 2014). In vivo studies, FGF administration can improve healing in diabetic, ischemic, and bacterial contaminated wounds. In clinical trials, FGF administration has proven to be efficacious for earlier wound closure in pressure ulcers, burns, chronic dermal ulcers, and operative wounds (Barrientos et al., 2008).

**Extracellular microvesicles (EMVs)**

EMVs are lipid membrane-bound vesicles, which are shed by almost all cell types into the extracellular environment. The release of EMVs is one of the ways cells communicate in both healthy and pathological conditions (Camussi et al., 2010). These EMVs can deliver many bioactive molecules such as cytokines and growth factors (Fais et al., 2016). Likewise, they can deliver a biological message to recipient cells in normal physiological and diseased conditions (Fais et al., 2016, Waldenström et al., 2012). Moreover, EMVs have been found to have a regenerative effect in several tissues from several origins. Recently, phase IV double-blinded clinical trial conducted by Simman et al. showed that there is a correlation between EMVs and the rate of wound healing (Simman et al., 2016). The decline of growth factors in chronic wounds, the need for a more effective treatment for wounds, and the evidence for the potential role of EMVs in tissue repair, have prompted us to investigate, the potential effect of growth factors loaded EMVs may have to enhance and accelerate wound healing.
**EVs as drug delivery systems**

Today, EMVs are extensively under scientific investigation as a potential drug delivery system because of their capabilities to cross the biological barriers and targeting specific cells. Furthermore, loading of the therapeutic agents into EMVs increased the circulation time, preserve the therapeutic activity, and improve the drug solubility (Somiya et al., 2017). In order to load EMVs, different methods have been invented and validated. These methods include: incubation at room temperature (RT) with or without saponin permeabilization, freeze-thaw cycles, and sonication. Loading of EMVs has been exploited in several investigations, anticancer such as Paclitaxel (Saari et al., 2015), antioxidant such as catalase enzyme to treat Parkinson disease (Haney et al., 2015) was successfully loaded, and loaded EMVs showed a strong effect compared with free drug in in vivo and in vitro model (Ha et al., 2016). In this work, we chose incubation at room temperature to load growth factors into EMVs because the other methods mentioned above may affect the nature and activity of the growth factor proteins.

**Hypothesis**

“The usage of EMVs as a novel and promising therapeutic tool in the wound healing area will induce and enhance tissue repair process.”

**Specific Aims**

1. Evaluate the effect of EMVs on the wound healing process (cell migration and proliferation).
2. Load the EMVs with growth factors (TGF-β, PDGF, FGF, and VEGF).
3. Assess the outcome of the loaded EMVs on wound healing process (cell migration and proliferation) with compare to unloaded EMVs.
Materials and methods

Reagents

Optimized Dulbecco’s Modified Eagle Medium (DMEM) was purchased from AddexBio. Corning™ Regular Fetal Bovine Serum (FBS). HyClone™ Penicillin-Streptomycin, HyClone™ Dulbecco’s Phosphate Buffered Saline (DPBS), Gibco™ Trypsin-EDTA (0.25%), and Thermo Scientific™ Biolite 6 Well were purchased from Fisher Scientific.

Cell Culture

The spontaneously immortalized skin keratinocyte cell line (HaCaT) from AddexBio was cultured in optimized DMEM (contains 2 mM L-glutamine, 2 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate) supplemented with 10 % FBS, 1 % penicillin-streptomycin and incubated at 37 °C with 5 % CO₂ in a humidified atmosphere.

EMVs release and isolation

HaCaT and Macrophage cells were cultured respectively in serum free DMEM and Ham's F-12 media for 48 h to enhance the release of keratinocytes derived microvesicles (KMVs.) and macrophages derived microvesicles (MMVs) (Silva et al., 2015). After 48 h, the medium was collected and centrifuged at 500 x g for 10 mins followed by 2000 x g for 20 minutes to remove cell debris using Eppendorf® centrifuge 5810 R. Then the supernatant was Centrifuged at 24,000 x g for 2 hours using a SORVALL® Discovery M120 SE ultracentrifuge to isolate the KMVs and MMVs. Isolated KMVs and MMVs were reconstituted in DMEM/0.5 % FBS and stored at -80 °C.
EMVs Loading

The EMVs were incubated for 1.5 hours at room temperature with PDGF, VEGF, FGF, and TGF–beta (25 ng/ml of each). They were then ultra-centrifuged for 3 hours at 176 K G to re-pellet the EMVs., followed by 2-time wash with PBS to remove any extra vesicles growth factors remained (Saari et al., 2015).

Nanoparticle tracking analysis (NTA)

NanoSight (NS300) with a 405-nm laser instrument was used to detect the presence of EMVs particles (Figure 1). 10 microliters of EMVs sample was diluted 70 times in 690 µl Filtered PBS and three 30 second videos were recorded using camera level 13 at 25 frames per second. The data were analyzed using NTA software 3.0 software (Malvern Instruments) which was optimized to first identify and then track each particle on a frame-by-frame basis. The detection threshold optimized for each sample and screen gain at 5 to track as many particles as possible with minimal background (JinjuWang et al., 2016, Saari et al., 2015).

![Average Concentration / Size of Extracellular microvesicles](image)

**Figure 1:** Average Concentration / Size of Extracellular microvesicles.
**Wound-scratch assay model**

In this model, Epidermal keratinocytes were seeded into 6 well plates (150,000 cell/well). After wells Confluency, cells were starved in DMEM media supplemented with low FBS concentration (0.5 %) overnight. On the next day, wound scratches were made using a 10 µl pipette tip, and single dose of EMVs in DMEM/0.5 % FBS was added (2ml/well). The wells were visualized by 10 x magnification power of EVOS XL Core Cell Imaging System with CMOS camera sensor at zero time and after 72 hours. The images were analyzed using Mat lab software to measure the wound area in pixels. To convert area in pixels to microns, we used the following equation (Stpierre, & Shetty 2008):

\[(\mu m^2/pixel) = \frac{\text{Physical length of a pixel on camera sensor}}{\text{total magnification power}}\]

\[(10) = (1.2 \mu m^2 = 1 \text{ pixel}).\]

**Cell Proliferation Assay (MTT Assay)**

Colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit (5 mg/ml in RPMI-1640 without phenol red) from Sigma was used to assess the metabolic (reduction) activity of NAD(P)H-dependent cellular oxidoreductase enzymes on converting yellow MTT dye to purple formazan, which reflect the number of viable cells. MTT stock solution (5 mg/cc) is appended to each culture being assayed to equal one-tenth the original culture volume and incubated for 4 hours. At the final stage of the incubation period the medium was removed and the converted dye was solubilized with acidic isopropanol (0.1 N HCl in absolute isopropanol). Absorbance of converted dye was measured at a wavelength of 570 nm using micro plate reader (Gumustas, et al., 2016).
Growth Factors Analysis

In order to analyze the growth factors levels inside the microvesicles, we used the Bio-Plex system. 100μl 1XPBS was added to each sample, and protein concentration was determined using the Bradford assay. A (Bio-Rad) BioPlex 96 well plate was set up with 50 μl of the sample and duplicated for cytokine analysis using Bio-Plex (Bio-Rad) Human Cytokine 27-Plex Group 1 assay kits. To each well, Buffer and magnetic beads were added and washed on the magnetic plate washer. The plate was run on the Bio-Plex 200 system at a low PMT setting followed by high PMT according to Bio-Rad instructions.

Figure 2. shows the level of FGF-b inside the loaded and un-loaded EMVs from keratinocytes and macrophages. “*” means that the level of FGF-b is more than standard range.

Figure 3. shows the level of PDGF-bb inside the loaded and un-loaded EMVs from keratinocytes and macrophages. “*” means that the level of PDGF-bb is more than standard range. “#” means that the level of PDGF-bb is less than the standard range.
Figure 4. shows the level of VEGF inside the loaded and un-loaded EMVs from keratinocytes and macrophages. “#” means that the level of VEGF is less than standard range.

Statistical analysis

The data analysis was conducted by using the Prism 6 software. The Experiment was performed and repeated three times with independent HaCaT cultures. Wound area, wound healing, and migration rate data are represented as mean ± SEM, analyzed using t-test with unequal variances and one-way ANOVA test. The results were considered significant at $P \leq 0.05$.

Results

Several studies have discussed the potential role of microvesicles in tissue repair due to their role in intercellular communication (Saari et al., 2015). Therefore, in this study, the wound - scratch assay was performed to examine whether treatment with EMVs accelerate the wound closure of a wounded monolayer of differentiated and living skin keratinocytes (HaCaT). The wounded areas in both groups were created by scratching the plates with monolayer cells. The initial wounded area in both groups was similar with no significant difference ($P > 0.05$) (Figure 5).
Figure 5: Shows the Initial Wound Area in the Control, KMVs treated, LKMVs treated, MMVs treated, and LMMVs Groups at zero time with no significant difference in wound area between the Groups ($P > 0.05$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>S.E.M ±</th>
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<tr>
<td>Control</td>
<td>1.320</td>
<td>0.4421</td>
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<tr>
<td>MVs</td>
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<td>0.4219</td>
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<tr>
<td>LKMVs</td>
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<tr>
<td>MMVs</td>
<td>1.154</td>
<td>0.2767</td>
<td>0.06187</td>
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</tr>
<tr>
<td>LMMVs</td>
<td>1.372</td>
<td>0.3143</td>
<td>0.08400</td>
<td>14</td>
</tr>
</tbody>
</table>

ANOVA: single factor: F= 1.908, df (4, 117), ($P > 0.05$).

In the control group, the cells showed little ability to repair the wound area, whereas in EMVs and LEMVs treated groups, the cells were shown to be able to repair the wound area (Figure 6 A-J).
<table>
<thead>
<tr>
<th>Group</th>
<th>Time</th>
<th>0 h</th>
<th>72 h</th>
<th>Effect</th>
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<tbody>
<tr>
<td>Control</td>
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<tr>
<td>Keratinocytes</td>
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<td>Unloaded EMVs</td>
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<td>Keratinocytes</td>
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<tr>
<td>Loaded EMVs</td>
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A | B | C | D | E | F | G | H | I | J | K | L | M

15
<table>
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<tr>
<th>Time</th>
<th>0 h</th>
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<th>Effect</th>
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<td>Macrophage Unloaded EMVs</td>
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**Figure 6.** Wound scratch assay shows EMVs and LEMVs from macrophages and keratinocytes accelerate wound closure. Representative inverted microscope images depicting EMVs and LEMVs potential impact on cell migration in differentiated, live HaCaT cells. In the control group (A, B), cells cultivated in 0.5 % FBS/DMEM from the time of wounding: 0 h (A) and 72 h (B) after wounding. In EMVs and LEMVs treated groups (C - J), cells cultivated in 0.5 % FBS/DMEM with single dose of EMVs or LEMVs from the time of wounding: dose of EMVs and LEMVs from keratinocytes 0 h (C, E), and 72 h after wounding (D, F). dose of EMVs and LEMVs from macrophages 0 h (G, I), and 72 h after wounding (H, J). (K- O) show the new cells file the wound area of (B, D, F, H, J) after 72 h (n = 3).

Wound area was measured after 72 hours. There was a significant decrease in wound area in LEMVs (LKMVs & LMMVs) and EMVs (KMVs & MMVs) treated groups in comparison with the wounded area in the control group ($P < 0.0001$), whereas, LEMVs was substantially declined as compared with EMVs ($P < 0.01$) (**Figure 7**).
Figure 7: Shows Wounded Area Post 72 Hours in the Control, KMVs treated, LKMVs treated, MMVs treated, and LMMVs treated groups. There is a significant decrease in wound area in the Treatment Groups as compared with the Control Group ($P < 0.001$), and substantial drop in wound area in LKMVs and LMMVs as compared with control group ($P < 0.0001$), and KMVs and MMVs ($P < 0.01$).

Table 2: Average of wound area post 72 h

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>S.E.M ±</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.045</td>
<td>0.4129</td>
<td>0.06882</td>
<td>36</td>
</tr>
<tr>
<td>KMVs</td>
<td>0.5054</td>
<td>0.3232</td>
<td>0.05900</td>
<td>30</td>
</tr>
<tr>
<td>LKMVs</td>
<td>0.3256</td>
<td>0.2958</td>
<td>0.06307</td>
<td>22</td>
</tr>
<tr>
<td>MMVs</td>
<td>0.3938</td>
<td>0.3013</td>
<td>0.06736</td>
<td>20</td>
</tr>
<tr>
<td>LMMVs</td>
<td>0.1369</td>
<td>0.09597</td>
<td>0.02565</td>
<td>14</td>
</tr>
</tbody>
</table>

ANOVA: single factor: $F= 29.55$, DF (4, 117), ($P <0.0001$).

In addition, the average percentage of wound healing was around three times higher in the presence of EMVs. (57.85 % ±3.13, 69.84 % ± 4.87) when compared with the control group (21.74 % ± 6.12) ($P < 0.0001$). Wound healing was four time higher in presence of LEMVs (80.10 % ± 3.50, 90.87 % ± 2.00) as compared with the control, and around one
time more \((P < 0.01)\) as compared with EMVs group (57.85 \% \pm 3.13, 69.84 \% \pm 4.87) (Figure 8).

![Percentage of Wound healing](image)

**Figure 8:** Shows the Percent of Wound Healing in Control, KMVs treated, LKMVs treated, MMVs treated, and LMMVs treated Groups with a significant increase in the wound healing percentage in KMVs and MMVs Groups as compared with the Control Group \((P < 0.0001)\), and substantial increase wound healing percentage in LKMVs and LMMVs groups as compared with control, KMVs, and MMVs \((P < 0.0001)\).

Table 3: Average of wound healing percentage

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>S.E.M ±</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.74</td>
<td>14.33</td>
<td>2.389</td>
<td>36</td>
</tr>
<tr>
<td>KMVs</td>
<td>57.85</td>
<td>16.86</td>
<td>3.130</td>
<td>30</td>
</tr>
<tr>
<td>LKMVs</td>
<td>80.10</td>
<td>16.45</td>
<td>3.506</td>
<td>22</td>
</tr>
<tr>
<td>MMVs</td>
<td>69.84</td>
<td>21.25</td>
<td>4.876</td>
<td>20</td>
</tr>
<tr>
<td>LMMVs</td>
<td>90.87</td>
<td>7.070</td>
<td>2.041</td>
<td>14</td>
</tr>
</tbody>
</table>

ANOVA: single factor: \(F= 70.22\), DF (4, 117), \((P <0.0001)\).

In addition, we noticed a clear change in cell morphology in EMVs and LEMVs treated groups when compared with Control group. The cells residing at the edge of the wounds became more stretched and elongated (Figure 6 K-O). Cell migration is defined as cell movement into the wound area. For this reason, we calculated the cell migration
rate for all groups, and it was significantly higher in EMVs and LEMVs treated group ($P < 0.0001$) when compared with the control group, and it was higher in LMVs groups as compared with EMVs groups ($P < 0.0001$) (Figure 9).

![Migration Rate](image)

**Figure 9:** Shows the Cells Migration Rate in the Control, KMVs, LKMVs, MMVs, and LMMVs Groups.

The cells migration rate in KMVs and MMVs Group were significantly higher as compared with the Control Group ($P < 0.0001$), LKMVs and LMMVs groups were substantially remarkable as compared with KMVs, MMVs, and Control ($P < 0.0001$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>S.E.M ±</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.003820</td>
<td>0.002256</td>
<td>0.0003760</td>
<td>36</td>
</tr>
<tr>
<td>KMVs</td>
<td>0.008810</td>
<td>0.003692</td>
<td>0.0006856</td>
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<tr>
<td>LKMVs</td>
<td>0.014700</td>
<td>0.004422</td>
<td>0.0009428</td>
<td>22</td>
</tr>
<tr>
<td>MMVs</td>
<td>0.010850</td>
<td>0.003472</td>
<td>0.0007964</td>
<td>20</td>
</tr>
<tr>
<td>LMMVs</td>
<td>0.017670</td>
<td>0.004030</td>
<td>0.001163</td>
<td>14</td>
</tr>
</tbody>
</table>

ANOVA: single factor: $F= 53.86$, DF (4, 117), ($P < 0.0001$).
Furthermore, we calculated the area covered by the migrated cells in the both groups based on their migration rate, this covered area was approximately threefold higher in EMVs treated group, and four time in LEMVs treated groups \( (P < 0.0001) \) as compared to the control group at 24, 48, and 72 hours \( (P < 0.0001) \). The migration rate in LEMVs was one time more than EMVs groups \( (P < 0.01) \) (Figure 10).

**Figure 10:** Shows the wound area covered by migrated cells was calculated at 24, 48, and 72 hours in the Control, KMVs, LKMVs, MMVs, and LMMVs, this covered area was approximately threefold higher in EMVs treated groups as compared with the control group at three time points \( (P < 0.0001) \). LEMVs groups was almost four times more as compared with the control group \( (P < 0.0001) \), and one time more when compared with the EMVs groups \( (P < 0.01) \).

<table>
<thead>
<tr>
<th>Table 5: Average of wound covered area</th>
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</thead>
<tbody>
<tr>
<td>Dependent variable: wound covered area</td>
</tr>
<tr>
<td>Group</td>
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<tr>
<td></td>
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<td>Control</td>
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<tr>
<td>KMVs</td>
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<tr>
<td>48</td>
</tr>
<tr>
<td>72</td>
</tr>
</tbody>
</table>

ANOVA: single factor: $F= 72.75$, DF (14, 351), (P <0.0001).

**Figure 11.** shows the proliferation effect of EMVs

**Discussion and Conclusion**

In addition to the role in cell communication, EMVs have shown a regenerative role in damaged tissues. For instance, K. R. Vrijsen *et al.* has reported the ability of EMVs to improve endothelial cell migration and increase capillary formation in cardiac tissue (Vrijsen et al., 2010). Camussi *et al.* has established that EMVs enhance the regeneration process in liver tissue in rat models after 70 % hepatectomy (Camussi et al., 2009). In kidneys, S. Bruno et al. has found that EMVs encourage tissue regeneration after acute kidney injury in *in vitro* and *in vivo* models (Bruno et al., 2009).
Clinically, keratinocytes play an essential role in wound epithelization process, and without them wounds cannot be healed. Moreover, in chronic non-healing wounds, it has been found that compromised keratinocytes at wound edges are responsible for impaired wound epithelization and closure (Stojadinovic et al. 2005).

The role of EMVs in conveying messages and transferring genes horizontally between cells has been revealed by Ratajczak et al. and Waldenström et al., in addition to their ability to induce the re-programming of recipient cells (Waldenström et al., 2012, Ratajczak et al., 2006).

In this study, we demonstrated that EMVs and growth factors loaded EMVs derived from keratinocytes and macrophages promoted cell migration, proliferation accelerated wound closure in epidermal keratinocytes in vitro cell model. All these findings about the role of EMVs in tissue repair, including our observation, support the hypothesis of potential regenerative and therapeutic effects of EMVs and loaded EMVs in wound healing. Further investigations are needed to study the potential therapeutic use of EMVs in wound healing.
References


Cooper, Mark; Dimitria Vranes; Sherif Youssef; Steven A. Stacker; Alison J. Cox; Bishoy Rizkalla; David J. Casley; Leon A. Bach; Darren J. Kelly; Richard E. Gilbert (1999). Increased Renal Expression of Vascular Endothelial Growth Factor (VEGF) and Its Receptor VEGFR-2 in Experimental Diabetes. *Diabetes* 48 (11): 2229–39.


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