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Research article

Platelet lysate promotes the healing of long-standing diabetic foot ulcers: A report of two cases and *in vitro* study

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ABSTRACT

Long-standing foot ulcers present a great challenge in diabetes care. Platelet products have been suggested as a possible therapeutic option. However, nor the effect of an injectable form of platelet lysate on the healing of ulcers nor that on primary cells of the epidermis have been studied. In the current study, we present two cases of an ongoing clinical trial showing the positive effect of autologous platelet lysate injected perilesional. Both clinical cases treated with injections of hPL showed complete healing of previously un-healed within 8 weeks of treatment. Further, we describe the *in vitro* effect of human platelet lysate (hPL) on primary human epidermal keratinocytes (HEK) in terms of chemotaxis, migration and proliferation. *In* vitro, HEK showed enhanced chemotaxis towards the hPL compared to keratinocyte-defined media ($p < 0.0001$). Their migration was also stimulated especially at hPL concentration of 10%V/V ($p < 0.0001$). In contrast, hPL significantly inhibited HEK proliferation measured through MTT assay ($p < 0.0001$). In conclusion, the findings presented here provide preliminary evidence of an explanatory mechanism for the effect of hPL on primary keratinocytes and therefore of their potential use in a clinical setting. hPL promotes keratinocyte migration and therefore closure of foot ulcers.

1. Introduction

Diabetic foot ulcers (DFUs) are a chronic problem for 15% of diabetes mellitus (DM) patients. DM-associated wounds are slow or non-healing, frequently getting infected and leading to amputation. Defects in re-epithelialization, angiogenesis, as well as cytokine and growth factor deficits are the main impediments to proper healing [1, 2, 3, 4].

Wound healing is a multiplex and dynamic process requiring a series of fibroblast and keratinocyte functions to renovate the integrity of the skin barrier [5, 6, 7]. In response to chronic cutaneous wound injury, keratinocytes at the wound edges retreat from terminal differentiation and enter a status called "keratinocyte activation" [8]. This is distinguished by cell hypertrophy and changes in gene expression as keratinocytes initiate migration to close the wound between 6 and 24 h after injury [5, 9]. Cell migration and proliferation are initiated and regulated by growth factors and cytokines that are released from the wounded epithelium [10].

Numerous recombinant growth factors have been introduced into clinical practice for wound healing for more than two decades, yet few of them are reliable for clinical use [11]. Furthermore, the limited shelf-life of recombinant growth factors and their excessive cost are practical constraints for their routine clinical use [7].

Human platelet lysate (hPL) is a hemo-derivative that is rich in biosubstances and growth factors such as: platelet derived growth factor (PDGF), transforming growth factor-beta (TGFβ) and vascular endothelial growth factor (VEGF) [12, 13]. These growth factors are particularly involved in wound healing.

Prior studies have supported the use of platelet rich plasma in healing chronic lower extremity wounds and the treatment of diabetic foot ulcers and skin lesions [14, 15, 16, 17, 18]. These studies employed platelet rich

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Table 1. Inclusion and exclusion criteria for patient selection.

Inclusion Criteria	Exclusion Criteria
• People with Type II Diabetes mellitus between the ages of 18 and 70 with an ulcer with at least 4 weeks duration. • Hb1c of less than 13 %. • Index foot ulcer located on the plantar, medial, or lateral aspect of the foot (including all toe surfaces), and wound area (length*width) measurement between 0.5 cm2 and 20 cm2, inclusive. • Wounds located under a Charcot deformity has to be free of acute changes and went through appropriate structural consolidation. • Wagner Grade II or III ulcer. • The protocol requires that post-debridement, the ulcer would be free of necrotic debris, foreign bodies, or sinus tracts. • Non invasive vascular testing Ankle Brachial Index (ABI). • Physical examination (Including a Semmes-Weinstein monofilament test for neuropathy). • Blood tests to be obtained (CBC, HbA1c) • Approved, informed, signed consent to be obtained from each patient.	• Patient currently enrolled in another investigative device or drug trial or previously enrolled (within the last 30 days) in an investigative research of a device or a pharmaceutical agent. • Ulcer area decreased >50% during the seven-day screening period. • Ulcer is due to a non-diabetic etiology. • Evidence of gangrene in ulcer or on any part of the foot. • Patient is currently receiving or has received radiation or chemotherapy within the last 3 months of randomization. • Patient has received growth factor therapy within 7 days of randomization. • Screening platelets count <100* 109/L. • Patient is undergoing renal dialysis, or has a known immune insufficiency, known abnormal platelets activation disorder (i.e. Grey Platelet Syndrome, Liver Disease, Active Cancer), eating/nutritional, hematologic, collagen vascular disease, rheumatic disease, or bleeding disorder. • History of peripheral vascular repair within 30 days of randomization. • Patient is known to have a physiological, developmental, physical, emotional, or social disorder, or any other situation that may interfere with their compliance with the study requirements and/or the healing of the ulcer. • History of alcohol or drug abuse within the last year prior to randomization. • Patient has inadequate venous access for blood withdraw.

plasma in the form of topical gel, and most of these studies excluded ulcers with challenging presentations such as in patients with severe limb ischemia. No previous work has been done with injectable hPL for wound healing in humans.

Currently, we are running two on-going clinical trials on the use of autologous and allogenic platelet lysate in diabetic foot ulcers (NCT02989961 and NCT02972528, respectively). In the current study, we present two cases enrolled in the autologous above-mentioned clinical trial, as we aimed to give an explanatory mechanism of the clinical effects observed. Therefore, we examined the effect of hPL on primary keratinocytes and primary fibroblasts *in vitro* as a potential mechanistic explanation for its *in vivo* effect.

2. Materials and methods

This study was approved by the Institutional Review Board (IRB) at the Cell Therapy Center, University of Jordan. A written informed consent was obtained from each participant in accordance with the declaration of Helsinki.

2.1. Patients and injection protocol

The cases described below are of participants enrolled in an on-going clinical trial at the Cell Therapy Center/University of Jordan (NCT02989961). Inclusion and exclusion criteria of the trial are summarized in Table 1.

2.2. Case 1

A 58-year-old male patient with a 20-year history of type 2 diabetes mellitus with a non-healing diabetic foot ulcer of 10 months on the lateral aspect of patient's right foot. The patient's initial management included measures to optimize glycemic control and the use of moisture dressings and offloading pressure from the wound. His HbA1C was 7% with controlled fasting blood sugar levels. Conventional treatment failed to heal the ulcer. Autologous platelet lysate was used as detailed in the injection protocol below, in addition to standard of care.

2.3. Case 2

A 67-year-old female patient with a 25-year history of type 2 diabetes mellitus presented with 2 months non-healing diabetic foot ulcer on the planter aspect of patient's left heel. The patient's initial management included measures to optimize glycemic control and the use of moisture dressings and offloading pressure from the wound. Her HbA1C was 11.6% with controlled fasting blood sugar levels. Conventional treatment failed to heal the ulcer. Autologous platelet lysate was used as detailed in the injection protocol below, in addition to standard of care.

2.4. Platelet lysate

Platelet lysate (PL) was prepared as previously described [19, 20, 21]. Breifly, platelet rich plasma (PRP) was obtained from 20 ml of autologous whole blood collected in citrated tubes and 5ml of platelet lysate was prepared subsequently. The PRP preparation was frozen at -80 C°, thawed once and refrozen as in first time. After thawing the second time, the sample was centrifuged at 1800g the supernatant was collected and then filtered with 0.22μl filter (BD, USA).

2.5. Injection protocol

Platelet lysate was injected peri-lesional at ulcer margins by multiple punctures per each session of therapy (a total of around 5–7 ml/session). A total of 4 sessions throughout the period of treatment were used (total blood taken from patient throughout treatment is 80 ml). The treatment was given on days 0, 14, 28, 42 and the final image was on day 56 (total duration is about 2 months).

3. In vitro experiments

3.1. Platelet parameters and growth factors

For *in vitro* experiments, pooled human platelet lysate (hPL) was prepared as described above with the exception of pooling PRP from at least 8 participants for each batch. Platelet count was normalized to 1×10^6 platelet/ml. Platelet count, plateletcrit, mean platelet volume was determined. Following freeze/thaw cycles, pooled platelet lysate was compared against autologous platelet lysate obtained from the two cases reported here using Luminex multiplex assay for the following growth factors: TGF-beta, PDGF-AB, FGF, EGF and VEGF-C.

3.2. Sample collection

Neonatal foreskin samples were acquired following new born males' routine circumcision surgery. Samples were obtained from the day care surgery unit of Jordan university hospital. For the purpose of this work,

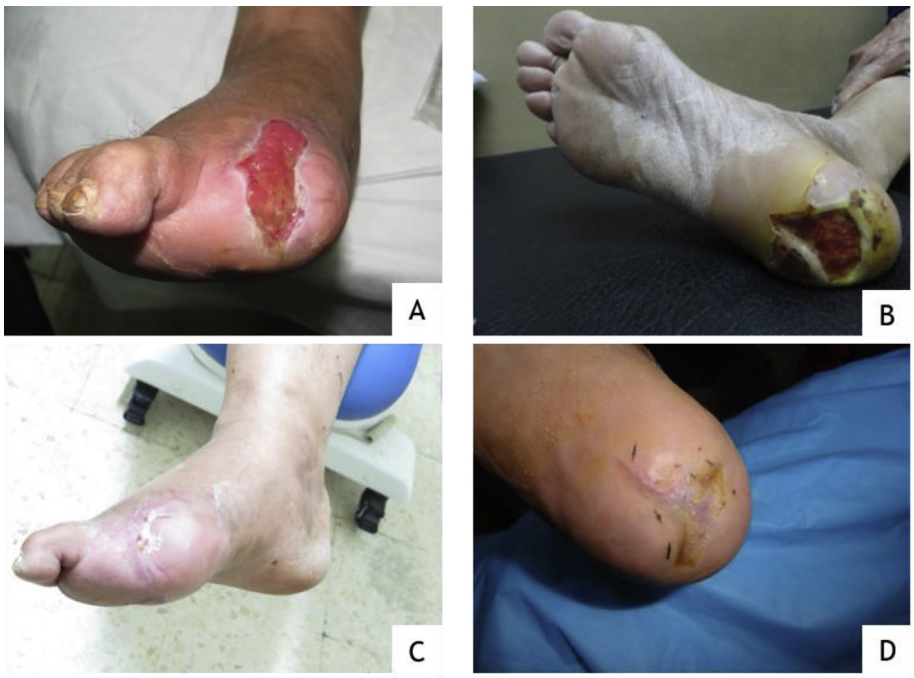

Figure 1. Representative photographs of case 1 at days 0 (A) and 56 (C), and case 2 at days 0 (B) and 56 (D).

five different samples were collected. A written informed consent for each sample was acquired from the child's parents. Collected samples were transferred into 30ml of Dulbecco's modified Eagles' medium (DMEM) (Gibco, In vitrogen Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS; Euroclone, Italy) and 100 IU penicillin/100 IU streptomycin (Gibco, In vitrogen Life Technologies, USA) and transported on ice to the processing facility at the cell therapy center within the same day.

3.3. Cell isolation and culture

Primary keratinocytes were isolated and expanded as per the protocol recommended by the media manufacturer (Invitrogen Life Technologies). Briefly, skin biopsies were incubated in dispase overnight to separate the epidermis from the dermis. A single cell suspension was obtained following incubation of the epidermis in 0.25% trypsin for 10–30 min. Keratinocytes were maintained in serum-free media (EpiLife®, Invitrogen Life Technologies, USA) as per manufacturers' recommendations. Only cells of passage three and lower were used in the following experiments.

For experiments involving the addition of hPL, keratinocyte media consisted of basal EpiLife® media supplemented with 0.1% W/V bovine serum albumin (BSA,Sigma-Aldrich,Germany), 1% antibiotic mixture (Gibco, In vitrogen Life Technologies,USA) and different amounts of hPL (5% V/V,10% V/V,15% V/V, and 20% V/V). Starvation media composed of basal EpiLife® medium supplemented with 0.1% W/V bovine serum albumin and 1% antibiotic mixture.

3.4. Chemotaxis assay

Keratinocyte chemotaxis was measured using a 6.5mm Transwell® with 8.0μm pore polycarbonate membrane insert (Corning Costar,USA). After starving cells cultured in a 25cm^2 tissue culture flask for 24 h, cells were seeded at a density of 50,000 cells and maintained in 100μl of starvation medium in the upper chamber of each well. The lower chamber was filled with 650μl of medium containing different concentrations of hPL (5% V/V, 10% V/V, 15% V/V, and 20% V/V). For the control wells, lower chambers were filled with the same amount of starvation medium. Cells were allowed to transmigrate for 6 h in a 37 °C humidified CO_2 incubator, the inserts were removed and membranes were fixed in 3.7% paraformadehyde (TEDIA) in phosphate buffered saline for 30 min. By using a cotton swab, non invading cells were removed from the upper side of the membrane. Finally, membranes were stained and mounted on microscope slides using ProLong® Gold Antifade Reagent with DAPI (molecular probes, Invitrogen Life Technologies, USA). Four fields per transwell membrane were assessed under inverted wide field fluorescent microscope (Carl Zeiss, Germany). Each treatment was performed in duplicate.

3.5. Scratch closure assay

Keratinocytes were seeded into 12 well plates. When cultures reached 80% confluence, cells were starved for 24 h in 37 °C humidified CO_2 incubator. In confluent cell monolayers, scratch wounds were developed by using a 0.1–10 μL pipette tip. Following PBS washing of suspended cells, cultures were re-fed by media containing different concentrations of hPL (5% V/V, 10% V/V, 15% V/V, and 20% V/V). Control wells were re-fed by the starvation medium. Cell migration into the denuded area was evaluated at 0, 6 and 24 h, using an inverted microscope (Nikon ECLIPSE TS100, Japan), and measured (Nikon Digital Sight DS-L3). Time zero values were subtracted from measurements at 6 and 24 h, in order to achieve a net measurement of cell migration into the wound.

Table 2. Patients DFU area measurements before and after normalization on each follow-up day.

Days	DFU area (cm^2)		Area after normalization to baseline	
	Case 1	Case 2	Case 1	Case 2
Day 0	33.15	47.81	1.00	1.00
Day 14	14.46	21.59	0.44	0.45
Day 28	9.47	8.79	0.29	0.18
Day 42	unmeasurable	0.79	unmeasurable	0.02
Day 56	0.00	0.00	0	0.00

3.6. MTT assay

Keratinocytes were seeded on 96 well plates for 24 h before performing the experiment. Cells were seeded at a density of 5,000 cells and maintained in 100µl of complete EpiLife® medium, on the next day media on cultures were removed, and cells then were washed by PBS and media containing different concentrations of hPL (5% V/V, 10% V/V, 15% V/V, and 20% V/V) were then added, and basal EpiLife® medium was added to control wells. MTT assay was performed on three different time points 24, 48 and 72 h. MTT reagent (Sigma-Aldrich,Germany) was prepared from a powder as a (5 mg/ml) concentration in PBS. MTT was added to each 96 well plate at each time point. Briefly, 20µl of MTT reagent was added to cells and left for incubation in the dark in a 37 °C humidified CO_2 incubator for 4 h. Media was removed and MTT solvent was added (Sigma-Aldrich,Germany). After 15 min incubation at room temperature in the dark, absorbance readings were obtained at 570nm using a microplate absorbance reader (sunrise basic-TECAN,Switzerland).

3.7. Data analysis

Document clinical cases were photographed and uploaded into Autocad software to measure the surface area of ulcers. Statistical analysis was performed using the Instat and Prism software package (GraphPad Software, Inc.,USA). To determine differences between five means, one-way ANOVA with Turkey's test for multiple comparisons was performed. All statistical analysis was performed at $p < 0.05$ level of significance.

Table 3. Platelet parameters for donors (mean), case 1 and case 2.

Parameter	Donors (mean ± SD)	Case 1	Case 2
PLT (103/ul)	252.9 ± 45.47	236	225
PCT (%)	0.27 ± 0.05	0.26	0.25
MPV(fL)	10.78 ± 0.79	10.7	10.8
PDW (fL)	13.2 ± 1.67	14	15

PLT, Platelet count; PCT, Plateletcrit; MPV, Mean platelet volume; PDW, Platelet distribution width.

4. Results

4.1. Case one

The patient presented to The Diabetic Foot Care Unit with a single, non-infected (4.98 * 2.12cm) elliptical shaped ulcer located on the lateral aspect of patient's right foot, with inflamed margins (Figure 1-A). Two weeks after giving the first peri-lesional autologous hPL injection, the ulcer size and the margins showed early healing, the ulcer area was significantly reduced by 56% (from 33.15 cm^2 to 14.46 cm^2). Healing continued over the next 6 weeks until the ulcer was completely healed (Figure 1-C). Details of each follow up are summarized in Table 2.

Two weeks after giving the 2nd injection, there was further decrease in the ulcer's area by 71%. Granulation tissue was noticed to fill the ulcer's floor, the edges became more slopped and the defect became unmeasurable after two weeks of giving the 3rd injection. Two weeks after the 4th injection, the ulcer was completely healed.

4.2. Case two

The patient presented to The Diabetic Foot Care Unit with a single, non-infected (3.5 * 4.35cm) triangular shaped ulcer located on the planter aspect of patient's left heel (Figure 1-B). The DFU showed an ischemic appearance characterized by a dark red base, necrotic tissue and hyperkeratotic rim. Two weeks after giving the 1st peri-lesional Autologous hPL injection, a significant decrease in the ulcer size by 55% was observed with a healthy granulation tissue covered the base of the ulcer. The margins of the ulcer looked healthier. Two weeks after the second injection, the ulcer's surface area decreased by 82% (from 47.81 to 8.79 cm^2). On the third follow up visit (two weeks after the 3rd injection), the ulcer's edges became more sloped and the ulcer's granulation tissue became more mature and the ulcer area was 0.02cm^2. On day 56 (2 weeks after the 4th injection), the ulcer completely healed and the new skin became cosmetically more acceptable (Figure 1-D). Details of each follow up are summarized in Table 2.

4.3. Platelet parameters and growth factor content

Table 3 shows the platelet parameters of the two cases reported here, as well as those of the pooled hPL used in the *in vitro* experiments. No significant difference was observed in any of the parameters.

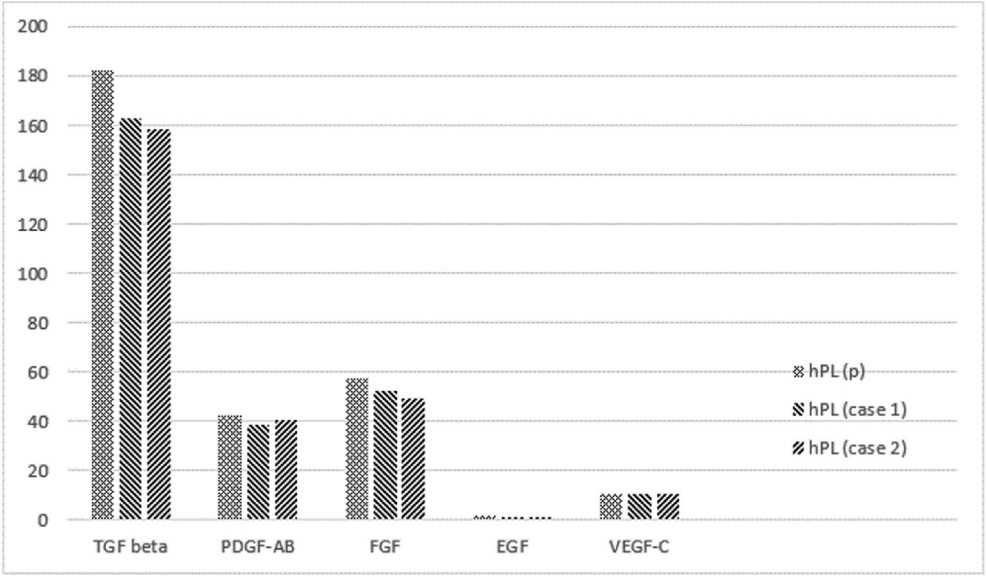

Figure 2. Growth factor content of donors' pooled hPL (hPL(p)), case 1 and case 2 measured in ng/ml. TGF, transforming growth factor; PDGF, platelet derived growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor.

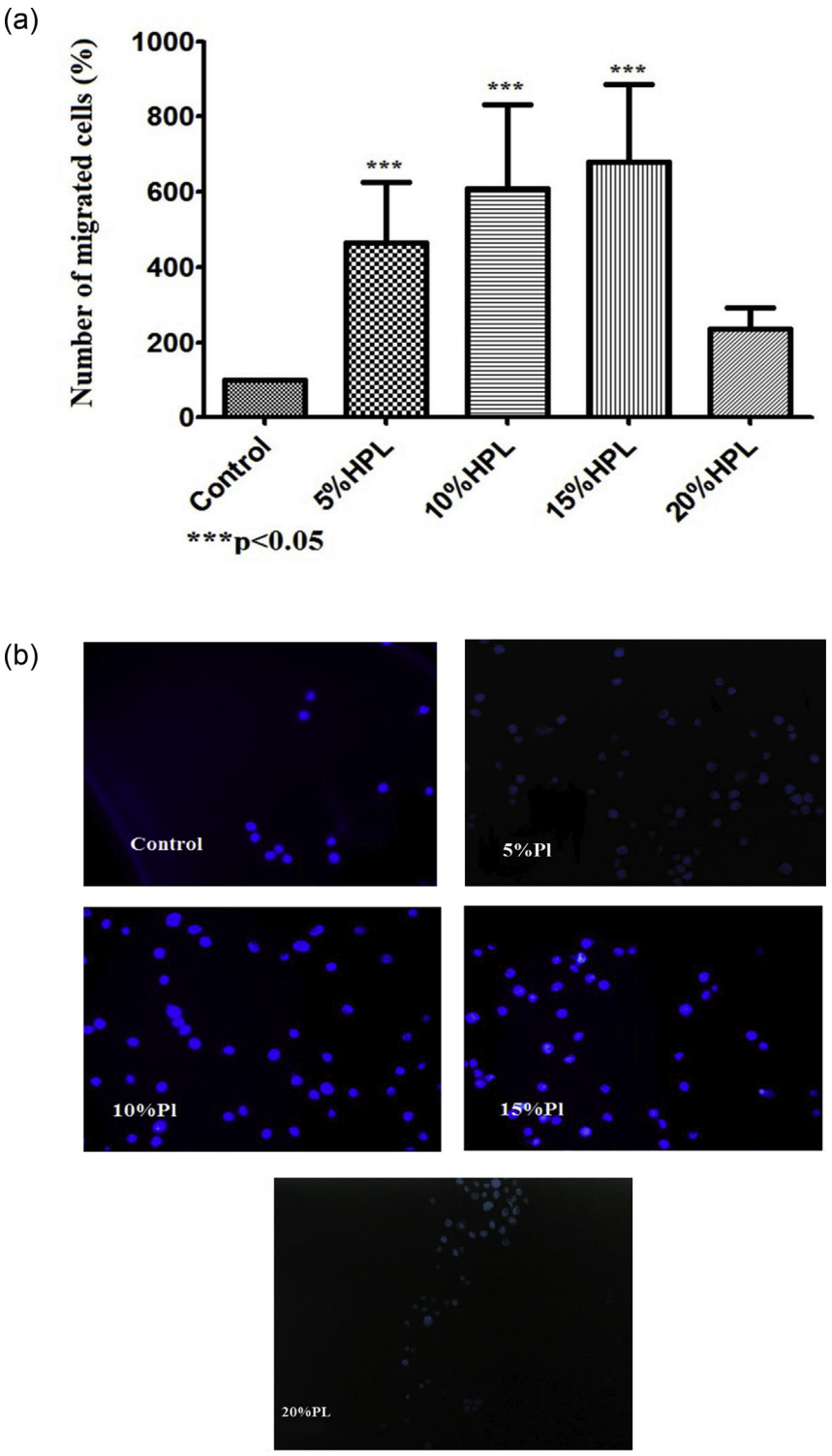

Figure 3. Effect of platelet lysate on human primary keratinocyte chemotaxis. (a) The chemotactic effect of HPL on HEK cells was assessed following 6 h of cell migration towards different HPL concentrations (5, 10, 15 and 20%). Data (n = 5) are means ± SD of duplicates. (b) Representative photo micrographs of cells that crossed the transwell membrane stained with DAPI examined under fluorescence microscope (100x magnification).

Similarly, the growth factor content did not differ significantly between the two cases and the pooled hPL used in the *in vitro* experiments, although slightly higher in the pooled hPL (Figure 2).

4.4. Chemotaxis assay

To investigate the chemotactic effect of different concentrations of hPL on primary human keratinocytes (HEK), we performed a transwell

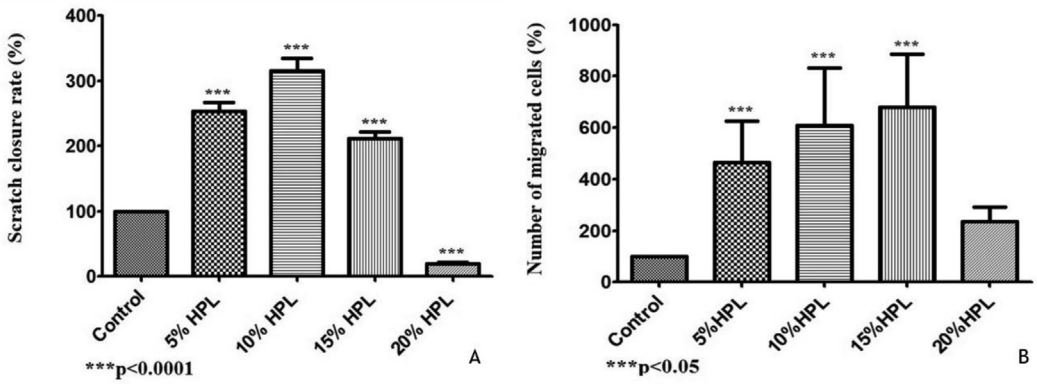

Figure 4. Effect of platelet lysate on human primary keratinocyte in wound closure assay. The chemotactic effect of HPL on HEK cells was assessed following 6 h of cell migration towards different HPL concentrations (5, 10, 15 and 20%). Data (n = 5) are means ± SD of duplicates. (*p < 0.05; ***p < 0.0001).

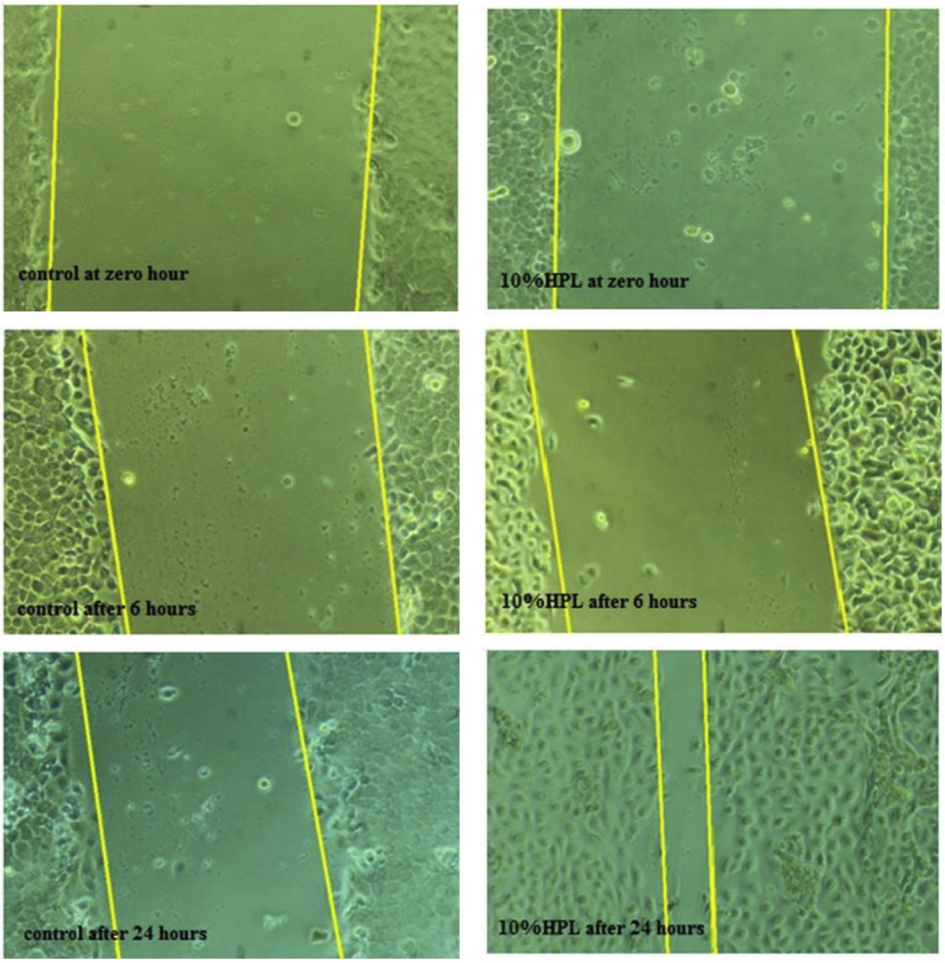

Figure 5. Representative phase contrast micrograph of 10%V/V hPL treated HEKs at 0,6,24 h (100X magnification).

assay. It was shown that different concentrations of hPL have substantial chemotactic effects on HEK ($p < 0.0001$) (Figure 3). Notably, the 5%V/V hPL containing medium has fivefold higher chemotactic effect on HEK. Even higher chemotactic effect was observed reaching sevenfold increase in cell migration for both 10% and 15%V/V, in comparison with hPL-free medium. However, the 20%V/V hPL containing medium had no significant chemotactic effect on HEK compared to other hPL concentrations.

4.5. Scratch closure assay

In vitro scratch assay was used to quantify the migration rate of the HEK in a monolayer cell model. As illustrated in Figure 4, a considerable stimulatory effect of the 5%, 10%and 15%V/V hPL containing media on HEK migration after 6 and 24 h of cells monolayer wounding compared to the control was observed; observed both as the percentage of scratch closure (Figure 4-A) and the percentage of cells migrated (Figure 4-B) (p

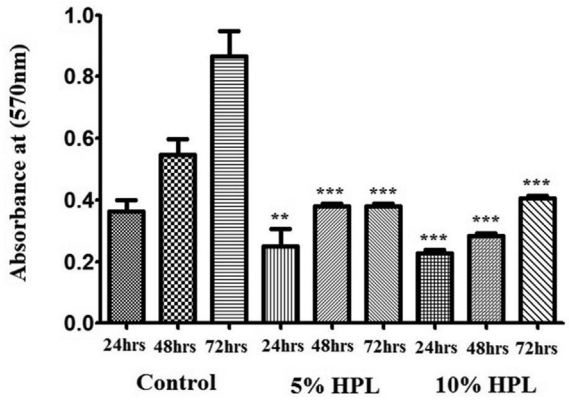

Figure 6. MTT results reflecting HEKs proliferation in the presence of hPL. Following 24, 48, 72 h of incubation with different hPL concentrations (5, 10 % V/V). Data (n = 5) are means ± SD of duplicates. (***P < 0.0001).

< 0.0001). The 10%V/V of hPL concentration showed the best effect on wound closure after 24 h (Figure 5); the wound was fully closed in many regions. The 5%V/V hPL containing medium had a good stimulatory effect, but fully closed regions of the wound were much less in number compared to the 10%V/V concentration. While wounds that were exposed to 15%V/V hPL concentration showed a statistically significant difference in the appearance of wound closure however fully closed regions of wounds were not observed. As expected from the results of the chemotaxis and migration assays, the 20%V/V hPL containing medium showed an inhibitory effect on wound closure compared to the control.

4.6. MTT assay

Cell viability and the proliferation of HEK cells exposed to increased hPL dilutions at different time points 24, 48 and 72 h were assessed by MTT assay. As illustrated in Figure 6, 5% and 10%V/V hPL containing cultures demonstrated a significant reduction in cell proliferation compared to control cultures on all time points ($p < 0.0001$).

5. Discussion

Only few reports have been published on the use of autologous platelets derivatives for the treatment of diabetic foot ulcers [8, 9]. Most of these studies excluded ulcers with "challenging presentations" such as severe limb ischemia. On account of paucity of information in the literature about the use of injectable hPL for diabetic foot ulcers, we have initiated a clinical trial to investigate this effect. In the current study, we report 2 of these case and attempt to offer an explanatory mechanism for the positive clinical impact observed so far, Needless to say, our clinical trial is still on-going and therefore, statistical significance of the results is yet to be calculated for the entire cohort.

Photographs of representative diabetic foot ulcer cases are shown in Figure 1. A DFU was observed in a 58-year-old man (case 1): the duration of the ulcer was 10 months, the ulcer measured 33.15cm^2. Time to complete healing was 28 days. A 67-year-old woman (case 2) showed a DFU that persisted for 2 months, the ulcer measured 47.81cm^2. Time to complete healing was 42 days.

After the administration of first hPL injection, the healing process was quite visible (at day 14) and at day 56 for both cases, the wound was completely healed. The skin surrounding the ulcer area became healthier, cosmetically more acceptable and more youthful in appearance. In addition, patients did not report any side effects during or after treatment.

Previous studies have shown that 10% and 20% (v/v) of pooled allogenic hPL have significant effects on cell migration and proliferation, *in vitro* [6.7]. However, these studies were conducted on cell lines rather than primary cells. Our *in vitro* results are in slight contradiction to these reports as our data revealed that lower percentages of autologous hPL (5% and 10%) showed the highest stimulatory effect on HEK migration and lower inhibitory effect on cell proliferation. This may be attributed to the preparation method of hPL we used. Higher concentrations like the 20%V/V may have cytotoxic effects on HEK, altering their pattern of migration.

In contrast to other studies that were performed by adding hPL to FBS-containing media, we investigated the role of hPL on HEK proliferation in the absence of serum and growth factors. MTT assay results denoted that HPL containing media showed significant lower cell proliferation compared to control.

The 5% and 10% V/V concentrations showed the least inhibitory effect on HEK proliferation compared to higher hPL concentrations, despite the fact that cell proliferation increased along the 72 h. This can be explained by the fact that hPL has a major effect on cell migration, so cells were induced more to migrate and less to proliferate. We showed that *in vitro*, low concentrations of hPL (5%, 10%) have a significant stimulatory effect on the migration of HEK cultures. In that regard, we considered the level of VEGF-C in hPL, as this growth factor was observed to promote migration of certain cancer [22] and mesenchymal stem cells [23]. Further work is needed to support this hypothesis. Overall, our results supported the idea that autologous hPL could be a potent therapeutic agent for the treatment of chronic diabetic foot ulcers. Further evidence to support this preliminary data is needed.

Declarations

Author contribution statement

H. Jafar: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

M. Hasan: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

D. Al-Hattab: Conceived and designed the experiments; Analyzed and interpreted the data.

M. Saleh and L. Ameereh: Performed the experiments; Analyzed and interpreted the data.

S. Khraisha: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

N. Younes: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

A. Awidi: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

The clinical trial described in this paper was registered at ClinicalTrials.gov under the registration number NCT02989961.

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