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Wound fluid under occlusive dressings from diabetic patients show an increased angiogenic response and fibroblast migration

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ABSTRACT

Introduction: Metabolic diseases like diabetes mellitus often show prolonged healing and chronic wounds. Occlusive wound dressings are known to support wound closure by creating a moist environment which supports collagen synthesis, epithelialization and angiogenesis. We aimed to assess the effect of occlusion on diabetic wound fluid on the cellular level regarding fibroblast activity and angiogenetic response.

Material and methods: 22 split skin donor sites from 22 patients (11 patients with diabetes mellitus) were treated with occlusive dressings intraoperatively. On day 3, fluid and blood serum samples were harvested while changing the dressings. The influence of wound fluid on fibroblasts was assessed by measuring metabolic activity (Alamar Blue assay, Casey Counter), cell stress/death (LDH assay) and migration (in vitro wound healing assay) of fibroblasts. Angiogenesis of endothelial cells (HUVEC) was analyzed with the tube formation assay. Furthermore, a Magnetic Luminex Assay for multi-cytokines detection was performed focusing on inflammatory and pro-angiogenetic cytokines.

Results: The influence of wound fluid under occlusive dressings from diabetic patients showed a significantly increased angiogenic response and fibroblast migration compared to the non-diabetic patient group. Additionally, cell stress was increased in the diabetic group. Cytokine analysis showed an increase in VEGF-A in the diabetic group.

Conclusion: Occlusive dressings may stimulate regenerative effects in diabetic wounds. Our in-vitro study shows the influence of wound fluid under occlusive dressings from diabetic patients on angiogenesis, migration and proliferation of fibroblasts, which are essential modulators of wound healing and scar modulation.

1. Introduction

Wound healing aims to rapidly close a wound and restore the skin barrier while rebuilding the damaged structures in the process [1]. The different wound healing stages inflammation, exudation, proliferation and remodeling aim at tissue regeneration and result in scar tissue, which differs in tensile strength and collagen structure [2]. Metabolic diseases like diabetes mellitus (DM) often prolong different healing stages significantly resulting in chronic and complicated wounds [3]. Wound dressings try to support an ideal wound environment and facilitate healing, which is especially important in these wounds [4]. Any diabetic patient has a cumulative lifetime incidence of 25% to suffer from diabetic foot syndrome which increases morbidity, lowers quality of life and often result in chronic wounds [5]. The use of occlusive dressings (OD) could demonstrate increased collagen type I synthesis and accelerated wound closure by epithelialization by creating a moist wound environment [6,7]. The wound fluid under the occlusive dressing is rich in cytokines like Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (bFGF), shows an overall increased cytokine expression and stimulates fibroblast migration and cytokine expression [8–10]. Occlusion also seems to inhibit fibroblast overgrowth in the proliferative stage, often seen in chronic wounds, and to decrease the inflammatory response in the healing process [11]. Furthermore, OD stimulate the angiogenic response, which could be especially beneficial

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in chronic wounds like diabetes, where sufficient blood supply to the wounds is critical [5,9].

Split skin grafts are often used in surgery to cover soft tissue defects of well vascularized wound beds or as a minimal-invasive surgical option to treat chronic wounds [12]. OD can be used to cover skin grafts donor sites and improve healing [13]. The composition of the wound fluid of wounds seems to reflect to a certain extent the condition of the wound itself, especially the composition of the extracellular space [14,15]. This allows a simple means to extract important information to influence wound care effectiveness and wound healing. As the multiple positive effects of occlusive dressings on wound healing are still not fully understood we tried to assess wound fluid under OD of split skin donor sites from patients with DM compared to patients without it. Specifically, we aimed at analyzing the effect of OD on cellular responses in chronic wound environments regarding the regenerative ability, angiogenesis and fibroblast behavior.

2. Material and Methods

The study was approved by the local ethics committee (No.379/17 S). 22 split skin donor sites (11 from diabetic patients) were treated intraoperatively with occlusive dressings (Smith & Nephew IV3000), directly after split skin removal from the ventro-medial to ventro-lateral upper leg of the patients. At the 3rd day, we harvested fluid samples after obtaining informed consent, while changing the dressings (Minimum 170 μl, Median 1000 μl, Maximum 29,000 μl). This was done with a small syringe puncturing the dressing without injuring the patient's skin. Both patient groups (diabetic versus non-diabetic patients) had comparable demographics with an average age of 57.2 ± 19.4 in the control group (median 57 years) and 62.3 ± 11.2 years (median 66 years) in the diabetic group. The male to female ratio was 6:5 in the control group and 8:3 in the diabetic group. Blood serum from each patient was drawn as additional control (Minimum 1600 μl, Median 2000 μl, Maximum 3300 μl). The severity of the diabetes in the diabetic group was mild to moderate: all 11 patients suffered from non-insulin dependent diabetes type 2 without the need of regular insulin application. All patients of both groups could be ranked as ASA score 1–2.

2.1. Sample processing and cell culture

Wound fluid and serum samples were centrifuged directly after harvest for 5 min at 20,000 revolutions per minute (Hettich, Switzerland, 300 g, Rotanta 460R®). After pipetting the supernatant samples were stored in 1–3 ml portions in reaction vessels (Eppendorf GmbH, Germany) at −80 °C.

HS-27 Fibroblasts (HS-27 FB), a commercially available human-derived fibroblast cell line (ATC-LGC Standards, Germany), and Human Umbilical Vein Endothelial Cells (HUVEC) (Promocell, Germany) were cultured in DMEM (Dulbecco's Modified Eagle Medium) with High Glucose, (Biochrom, Germany) supplemented with 10% FCS (Fetal calf serum). After cell washing, they were cultivated in DMEM with 5% FCS. Finally, samples were diluted 1:20 with the respective medium required for the assay to reach a final sample concentration of 5% for each test. All experiments were performed in two test series (t1 and t2, t1 using five controls and five diabetic samples, whereas t2 tested six controls and six diabetic samples (both wound fluid and serum)).

2.2. Cell metabolism assay (Alamar Blue)

HS-27 FB were seeded in a density of 4000 cells/well in t1 and 2000 cells/well in t2 in 96 well plates using DMEM with 5% FCS. We changed the cell density from t1 to t2 due to observed cell death even before the start of the fluorescence emission, probably due to overgrowth. Medium was replaced with sample (wound fluids and serum) in a concentration of 1:20 after the cells were allowed to attach overnight. Media was washed off the cells with PBS and 200 μl of diluted samples were inserted in the wells. Alamar Blue assay (Sigma-Aldrich, Germany) was performed after three days of incubation at 37 °C in 5% CO_2 atmosphere according to the instructions of the manufacturer. After three days, 100 μl of each well was transferred into a new 96 well plate and stored at 4 °C to be used in the LDH assay (see below). Finally, the media was removed, cells were washed with PBS and 10 μl Alamar Blue reagent was added to the cells and incubated for 30 min. After one and after 2 h, fluorescence measurement was performed by the Mithras Reader® (Berthold Technologies, Germany) at Ex 560 nm/Em 590 nm. Data was analyzed with MicroWin 2000 (Labsis Laborsysteme GmbH, Germany). Four Samples were discarded due to contamination leaving 10 controls and 7 diabetic samples (wound fluid and serum) in this experiment.

2.3. Cell count measurement (Casy Counter)

HS-27 FB were seeded in a density of 10,000 cells/well in t1 and 5000 cells/well in t2 in 24 well plates using DMEM with 5% FCS and incubated for 24 h. We reduced the cell density in t2 to improve viability measurement as t1 showed high cell toxicity.

After washing the cells with PBS, samples were added in 1:20 concentration with DMEM and 5% FCS and incubated for seven days at 37 °C. Next, cells were washed with PBS twice and trypsinized. PBS with 10% FCS was added to stop further processing. 200 μl of each remaining 500 μl/well were diluted with 10 ml CASYton® (Omni Life Sciences, Germany) and cell number measurement was performed with the Casy Counter (Casy TT®, Omni Life Sciences, Germany).

2.4. In vitro wound healing assay (Scratch-assay)

After trypsinization (Trypsin EDTA Soltion®, Biochrom, Germany) and cell counting using the Casy Counter (Casy TT, Omni Life Science, Germany), we pipetted 70 μl cell suspension (HS-27 FB cells cultured in DMEM high Glucose with 5% FCS) in each of the 24 wells of Ibidi culture inserts (Ibidi, Germany). In t1, 5000 cells per slot and in t2 10,000 cells per slot were used. Then, cells were incubated at 37 °C and 5% CO_2 for 24 h to obtain a confluent cell layer. Next, culture-inserts were removed, and cell-layers washed with 500 μl PBS. Samples were then added to the plate wells with DMEM high Glucose with 5% FCS. In t1, cells were fixed after 24 h with 3.7% formaldehyde (Otto Fischer GmbH & Co, Germany) and Giemsa stained (loading solution, Roth, Germany). Microscopy pictures (Avio Observer®, Zeiss, Germany) were then taken and analysis was performed using the Wimasis software (Wimasis Image Analysis®, Onimagin Technologies SCA, Spain). As in t1 a near total cell confluence was detected, we couldn't analyze this series. In t2, photos were therefore already taken after 10 h without fixation.

2.5. Cell cytotoxicity (LDH assay)

We used the Cytotoxicity Detection Kit (Roche, Germany) according to the instructions of the manufacturer. The 96 well plate with the samples, which was already prepared during the Alamar Blue assay (see above), was then incubated for 30 min at room temperature. Then the emission of Formazan was measured at 490 nm using the Mithras® reader (LB 940 Berthold Technologies, Germany).

2.6. Tube formation assay

A day prior to the experiments, Matrigel (Corning Incorporated, USA) was thawed and 1 μl angiogenesis slides (Ibidi, Germany) were cooled. At the next day, 10 μl Matrigel were pipetted in each slot of the angiogenesis slides using a cooling block as underlayer. Plates were then incubated at 37 °C. Meanwhile, HUVEC cells were trypsinized and counted using the Casy Counter (Casy TT®, Omni Life Sciences, Germany). In t1, a cell suspension of 400,000 cells per ml cultivated in RPMI media (Biochrom, Germany) were used whereas in t2, AIMV (Gibco®, Thermo-Fischer Scientific, Germany) was used as a culture medium.

Samples were diluted 1:10 with the respective media. After 1 h, 25 μl cell suspension and 25 μl of the diluted samples were pipetted on the Matrigel to reach a final sample concentration of 1:20. Next, the angiogenesis slides were incubated at 37 °C for 6 h. Then, 25 μl per slot were pipetted and replaced with 25 μl Calcein AM (ThermoFischer Scientific, Germany) in PBS (1:4000). This induced a fluorescence reaction of the cells after another 2 min of incubation. Microscopy photos were taken with a fluorescence microscope (Avio Observer, Carl Zeiss Jena GmbH, Germany). Analysis was also performed with the Wimasis software. As in t1 the RPMI media didn't show a sufficient cell formation, the software could not sufficiently differentiate tubes from cells. That's why we excluded t1 and only evaluated t2 with 6 diabetic samples and 6 control samples (wound fluid and serum).

2.7. Magnetic Luminex Assay for multi-cytokines detection

Serum and wound secretion samples were collected from both diabetic and non-diabetic patients. All the samples were placed in a 1:2 dilution for the ELISA. Fifteen serum cytokines' concentrations in picograms per millilitre were measured following the manufacturer's protocol using a magnetic bead-based ELISA, Human Premixed Multi-Analyte Kit (R&D Systems, USA): Angiopoietin-2 EGF, Endoglin, Endothelin-1, Fibroblast Growth Factor (FGF), acidic-FGF, basic-FGF/FGF-2, Granulocyte Colony-Stimulating Factor (G-CSF), Heparin Binding Epidermal Growth Factor (HB-EGF), Hepatocyte Growth Factor (HGF), Interleukin 8 (IL8/CXCL8), Leptin, Placental Growth Factor (PIGF), Vascular Endothelial Growth Factor A, C, D (VEGF-A, VEGF-C, VEGF-D). The Luminex® 100/200™ was used for the read-out of the final results of multi-plex ELISA.

3. Statistical analysis

Data are shown as mean ± SEM. Statistical analysis between the groups was performed with Microsoft® Excel version 16.44 using t-tests. P-values of less than 0.05 were considered statistically significant.

4. Results

4.1. Cell metabolism assay (Alamar Blue)

We did not detect a significant difference in the proliferation of HS-27 FB in the Alamar Blue Assay between wound fluids from controls and diabetic patients, but rather a trend towards a decreased cell metabolism in diabetic patients compared to the control group ($p = 0.09$) (figure Alamar Blue 1).

4.2. Cell count measurement (Casy Counter)

We did not observer a significant change in cell count ($p = 0.24$) and cell viability ($p = 0.76$) between diabetic and control wound fluid groups (figure Casy Counter 2).

4.3. In vitro wound healing assay (Scratch-assay)

The in vitro Wound Healing Assay showed a very significantly increased HS-27 FB migration in the diabetic wound fluid group at 10 h ($p = 0.0006$) compared to the control fluid group. It also showed a significantly increased HS-27 FB migration in the control fluid group compared to the respective serum samples ($p = 0.012$) (figure Scratch Cells 3). Also looking at the areas not yet covered by cells in the Scratch Assay we observed a significant decrease in the diabetic wound fluid group compared to the control fluid group ($p = 0.0006$) and a significant decrease in the control wound fluids versus the control sera ($p = 0.011$) (figure Scratch Scratch 4). Comparing HS-27 FB migration of all wound fluid samples together with all sera, we detected a highly significant increase in the wound fluids ($p = 0.0002$).

4.4. Cell cytotoxicity (LDH assay)

We observed increased cell cytotoxicity in the diabetic fluid group compared to the control group but without significance ($p = 0.83$)

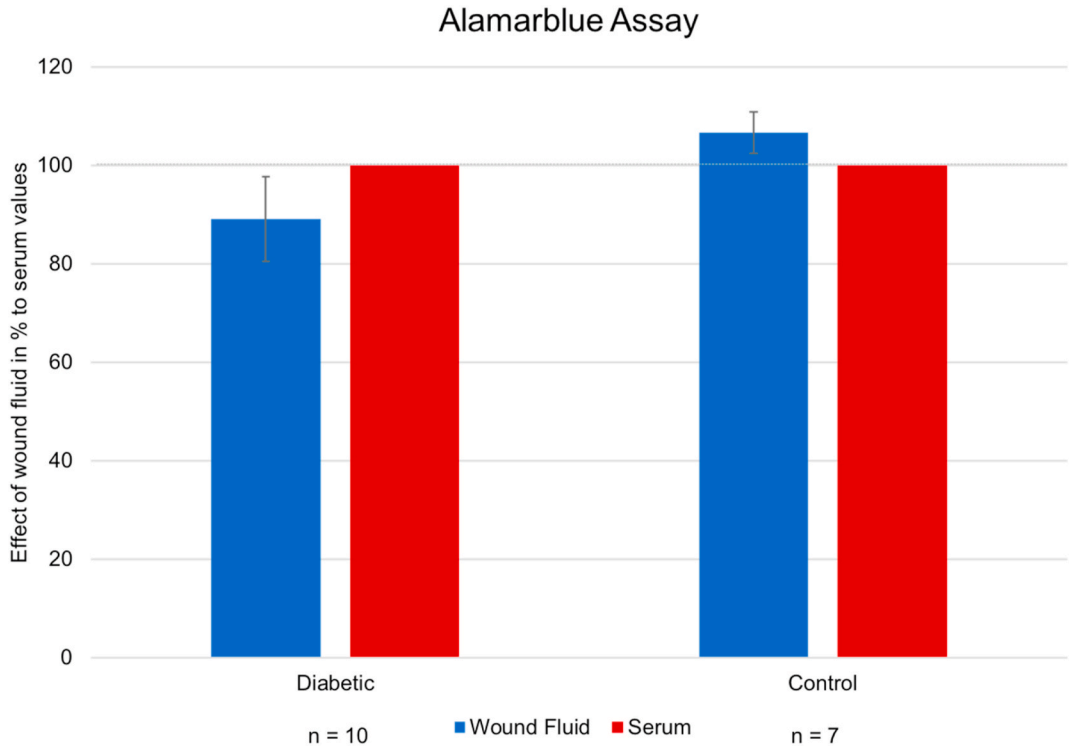

Figure Alamarblue 1. Proliferation of HS27-FB 3 days after sample incubation. Columns show wound fluid groups (control and diabetic) compared to serum values set at 100%. All groups in mean ± SEM.

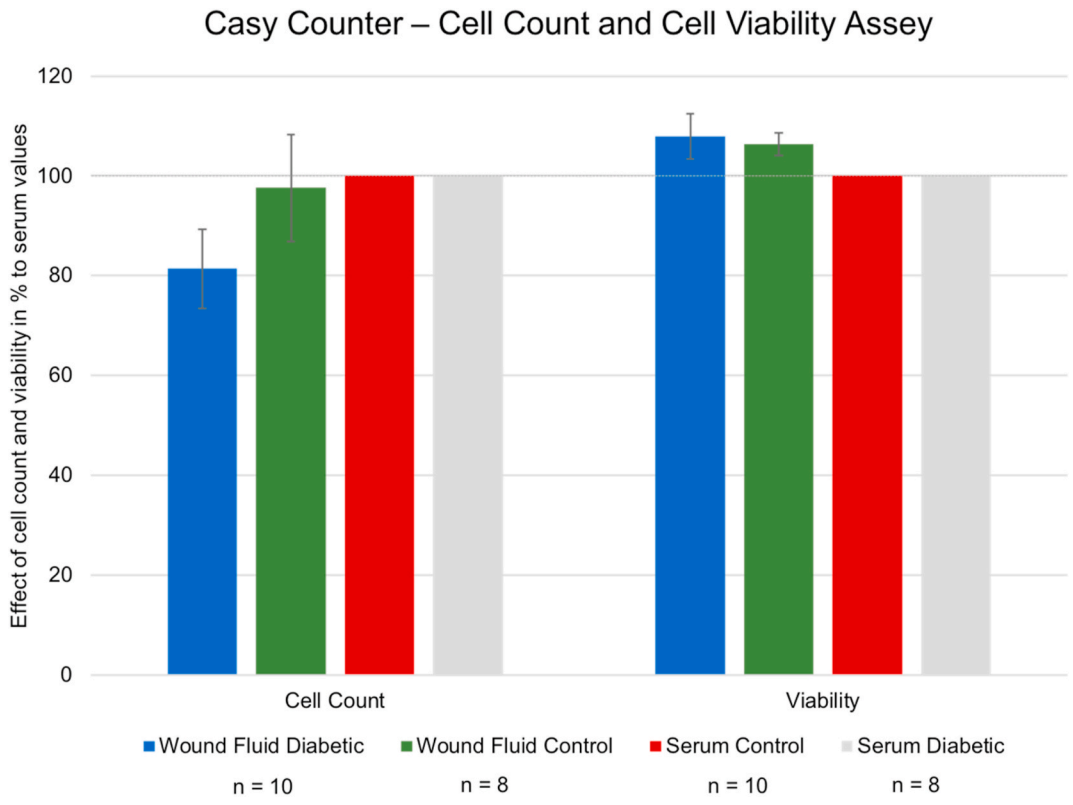

Figure CasyCounter 2. Cell Count and Cell Viability of wound fluid groups (control and diabetic) compared in % compared to serum values (set at 100%). All groups in mean ± SEM.

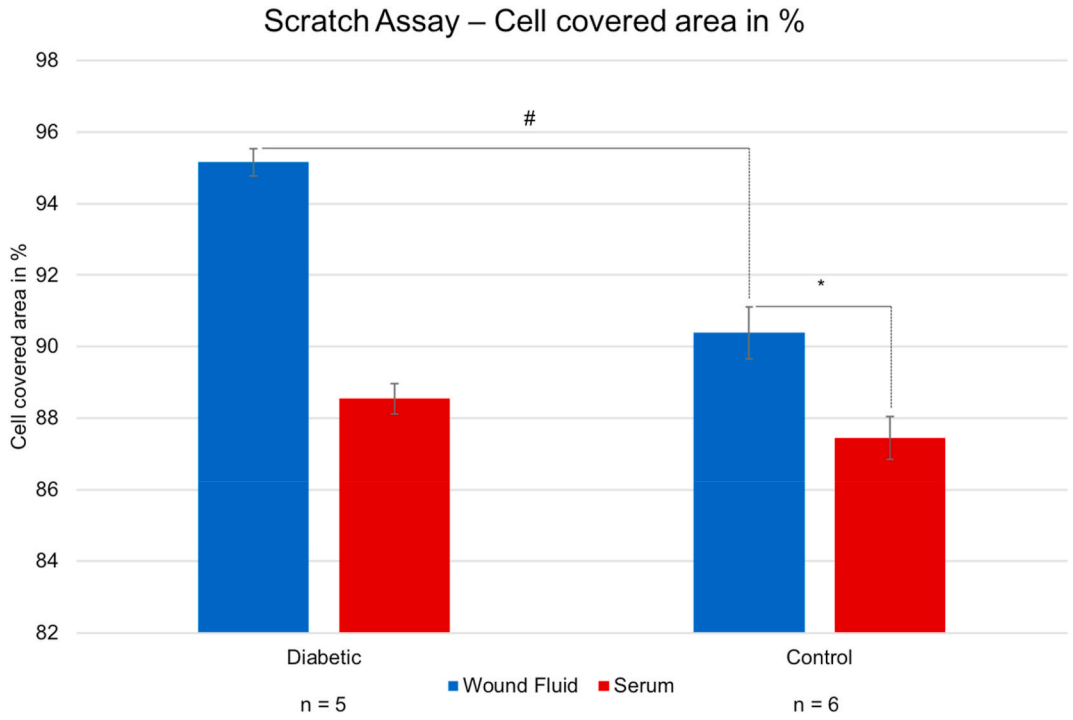

Figure Scratch Cells 3. Migration of HS27-FB in the wound healing assay. Columns show area in% already covered by cells at 10 h after cell incubation. All groups in mean ± SEM. #$p < 0.01$ mean diabetic wound fluids compared to control wound fluids. *$p < 0.05$ mean control wound fluids compared to mean control sera.

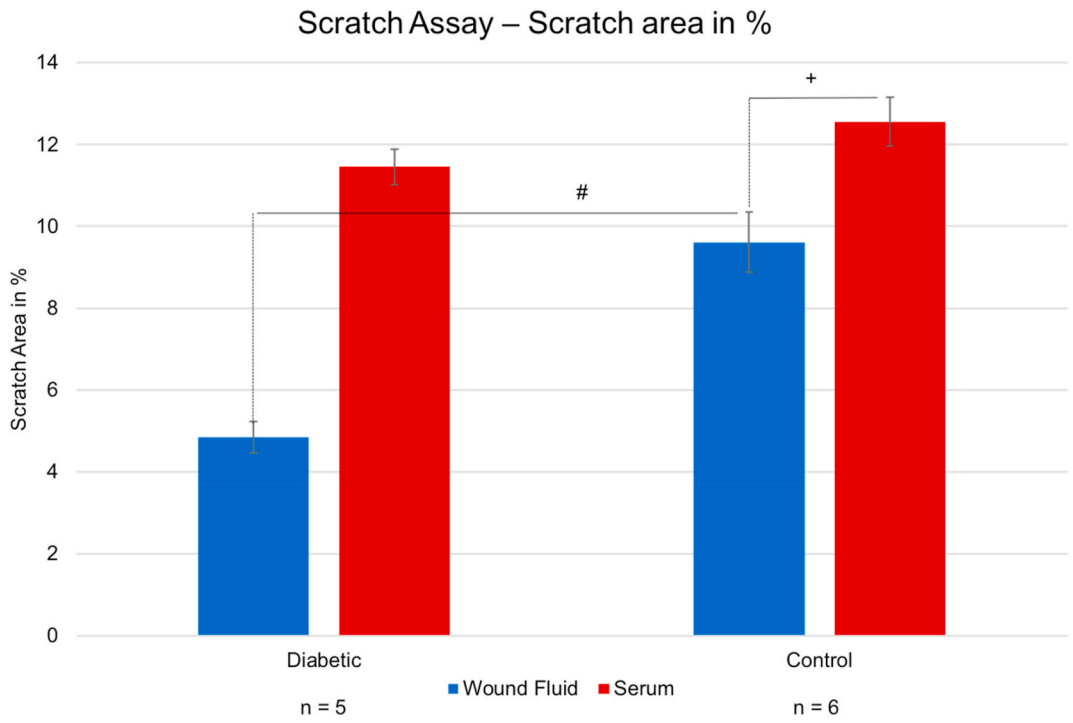

Figure Scratch Scratch 4. Migration of HS27-FB in the wound healing assay. Columns show area in% not yet covered by cells at 10 h after cell incubation. All groups in mean ± SEM. #p < 0.01 mean diabetic wound fluids compared to control wound fluids. + p < 0.05 mean control wound fluids compared to mean control sera.

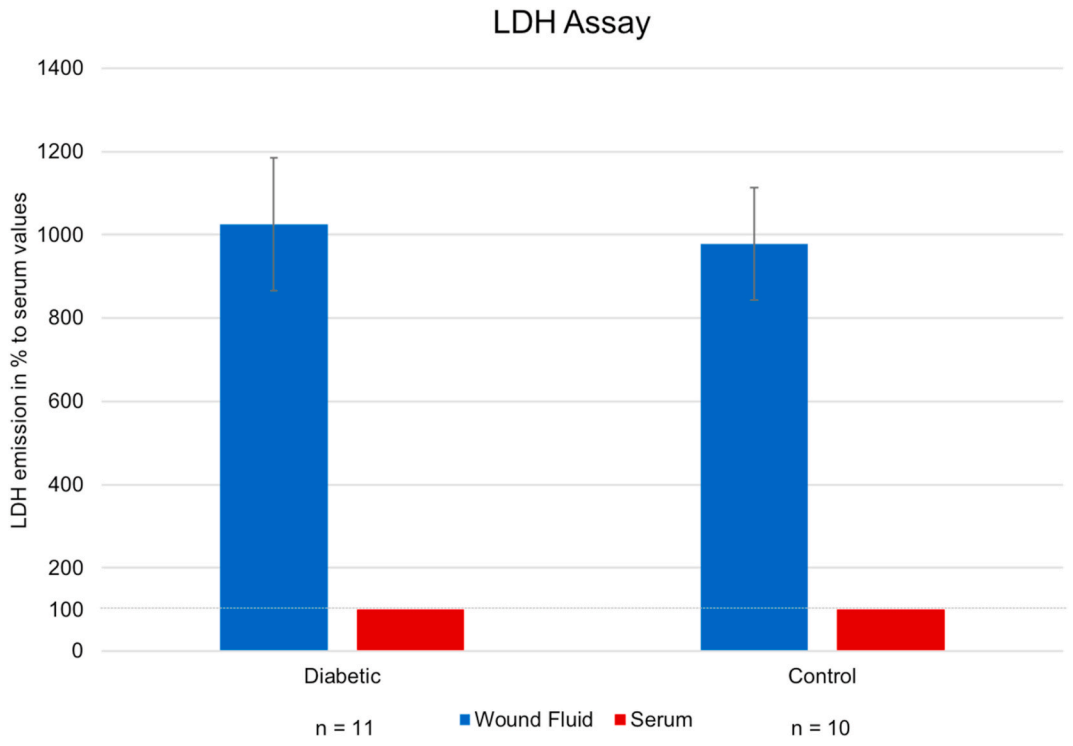

Figure LDH 5. Analysis of LDH emission in wound fluids of controls (left) versus diabetic samples (right) related to serum values set at 100%. All groups in mean ± SEM.

(figure LDH 5).

4.5. *Tube formation assay – angiogenesis*

We observed an increased growth of HUVEC cells in the diabetic wound fluid group compared to the control fluid group, but which was not significant (p = 0.094) (figure Tube formation cells covered 6). Total tubes of HUVEC showed an increased trend in diabetic wound fluids compared to control fluids (p = 0.34) as well as in total tube lengths and total branching points of HUVEC cells without reaching significance (p

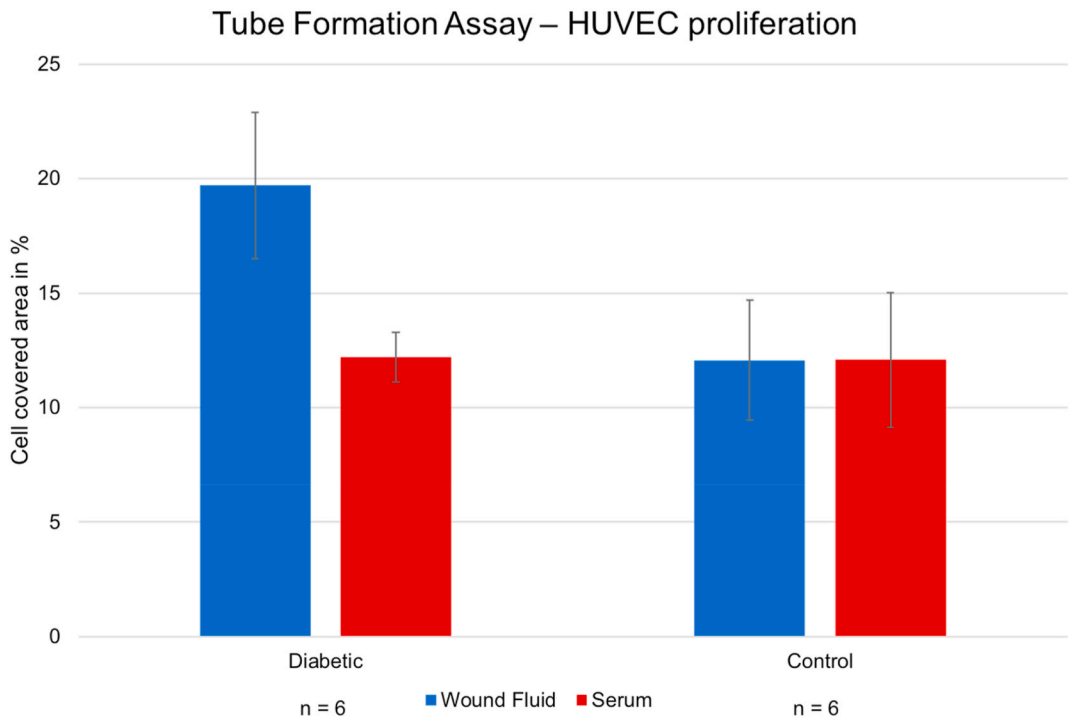

Figure Tube formation cells covered 6. HUVEC proliferation in percent of the covered area comparing diabetic wound fluids and sera with control fluids and sera. All groups in mean ± SEM.

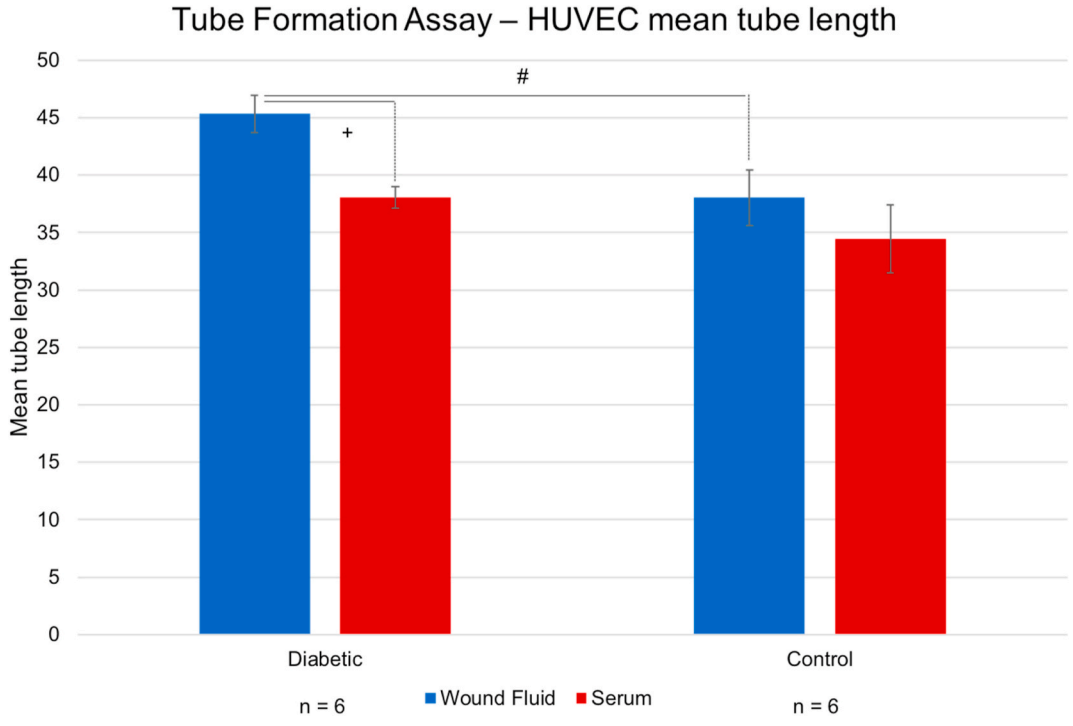

Figure Tube formation mean tube length 7. HUVEC mean tube length comparing diabetic wound fluids and sera with control fluids and sera. All groups in mean ± SEM. #p < 0.05 mean diabetic wound fluids compared to control wound fluids. + p < 0.01 mean diabetic wound fluids compared to mean control sera.

= 0.17 and p = 0.16, respectively). Mean tube length of HUVEC showed a significant increase in diabetic wound fluids compared to control fluids (p = 0.035) (figure Tube formation mean tube length 7). Total loops formed by HUVEC showed also an increase in diabetic wound fluids compared to control fluids, which were only slightly not significant (p = 0.062).

4.6. *Magnetic Luminex Assay for multi-cytokines detection*

We detected a decrease in Angiopoetin-2 in the diabetic wound fluid group compared to the control, which was not significant (p = 0.2). No significant differences nor trends were observed in cytokine levels of EGF, Endoglin, PIGF, HB-EGF, VEGF-D and Leptin. Endothelin-1, FGF

acidic and VEGF-D showed too many values below the detection range. basic-FGF/FGF-2 showed higher levels in non-diabetic wound fluids compared to the diabetic group, but this was not significant ($p = 0.23$). G-CSF, HGF and IL8/CXCL8 showed in all wound fluid samples values above the detection ranges of the assay, which didn't allow us any further analysis. VEGF-A levels were higher in non-diabetic wound fluids, which also was not statistically significant ($p = 0.66$).

5. Discussion

Diabetic foot ulcers, chronic wounds and generally wound treatment of this patient group is an ever increasing economic burden [16,17]. Diabetes in general affects every stage of the wound healing cascade and metabolic dysregulation, poor angiogenic response as well as a total increase in leucocytes are main factors that affect wound healing [18,19].

Fibroblasts and endothelial cells are two cell types, which are dominant in early stages of wound healing, creating a scaffold for wound closure, regulating cell migration and cytokine expression as well as promoting the growth of new blood vessels [20]. The use of occlusive dressings demonstrated an increase in fibroblast migration, angiogenesis, reduction of inflammatory cells and faster wound closures in non-diabetic patients [11,21,22]. As occlusive dressings are a cheap and easy method to treat wounds, which also allows external monitoring, it may be an ideal solution to treat selected diabetic wounds to lower mortality and reduce economic costs.

5.1. Stimulation of fibroblast migration

Fibroblast behaviour is most important in early wound healing. It regulates the creation of granulation tissue and later on scar formation and differs largely to air-exposed wounds [23,24]. Diabetic wounds show in general an increase in inflammatory cells and a reduced expression of cytokines [25]. We could show that fibroblast migration was significantly increased in all wound fluid samples compared to blood sera controls, while showing no increase in fibroblast proliferation, which normally occurs in diabetic chronic wounds. Diabetic wound fluids even showed a trend towards a decreased metabolic state of HS-27 FB. Regarding migration, the diabetic fluid samples showed a significant increase of fibroblast migration compared to the control, which could show a positive effect also in this patient group, which is contradictory to diabetic wound healing [26]. Velander et al. investigated the effects of diabetes on wound healing using a pig model and occlusively treated wounds [27]. Local hyperglycemia seemed to increase reepithelization compared to the control group and therefore fibroblast migration could be induced further by a moist wound environment in diabetic patients. Similar results were shown by Park et al. who investigated the reepithelialization of diabetic wounds in mice using occlusive dressings and polyethylenglycol (PEG) to stimulate cell transport [28].

5.2. Effect on wound angiogenesis

Cytokine expression and regulation is another important factor in wound healing. Occlusive dressings affect pro-regenerative cytokine levels in wounds, which was shown by Vogt et al. [22]. Our multi-cytokine detection assay showed an increase in VEGF-A in occlusive diabetic wound fluids compared to non-diabetic wound fluid and an overall increase in G-CSF, HGF and IL8/CXCL8. Especially patients with diabetic foot ulcers showed decreased levels of VEGF-A, which was argued to possibly lead to a reduction of VEGF receptor-2, a supposed cause of poor wound healing in diabetic patients [29,30]. Amoli et al. showed that diabetic foot ulcers were associated with a lower frequency of A and AA alleles in VEGF genes [26,31]. The use of occlusive dressings seems to affect VEGF-A and improve wound healing and VEGF-A may even be used a biomarker of wound healing in diabetes [26,32].

VEGF in general is assumed to be the most important factor regarding angiogenesis and is stimulated by hypoxia, which upregulates VEGF-A transcription of targeted genes by hypoxia-inducible factor-1 (HIF-1) [33]. VEGF-A is most prominent in wounds and regulates vessel growth [34]. In diabetes wound healing angiogenesis is impaired and results in decreased capillary density and low vascularity promoting chronic wounds [35]. We could show that occlusive dressings also seem to affect diabetic wound fluid, which showed a significantly increased mean tube length and number of total loops compared to the control. Furthermore HUVEC cells showed a non-significant increased growth, all which normally would not be seen in diabetic wound conditions [35]. Poor angiogenetic response in diabetic wounds results mostly from reduced cytokine secreting macrophages and the growth factor VEGF [19]. Topical application of VEGF could show a positive influence on wound healing of chronic wounds [36]. Therefore, an increased level of growth factors due to occlusion could have a positive effect on angiogenesis as well.

We observed an increased HUVEC response in the diabetic group compared to the control group. In diabetes an increased but impaired angiogenesis can be observed in diabetic microangiopathy for example [37]. Dysfunctional vessels promote a hypoxic state, which in turn stimulates further angiogenesis [37,38]. Occlusive dressings also showed to induce angiogenesis by a hypoxic stimulus [6,7]. It seems possible that occlusion affects diabetic wounds positively by promoting vessel growth and increase wound healing, which would have an immense impact in this patient group. These positive effects may be related to occlusive therapy but may also result from differences in wound healing in diabetic patients, which can't be differentiated by our study. To obtain this information another study would need to include a control group without the usage of occlusive dressing, which could show clinical results, but possibly would limit any wound fluid harvest.

The main limitations of this study are its design as in-vitro study, which only allows limited conclusions, especially regarding the quality of the vessels formed. Furthermore, the wound fluid is only harvested once from the patients, while biologically it is constantly produced in vivo and reflect the specific stage of wound healing, where a multitude of factors interact. On the other hand, this study allowed us to focus on specific cell types, which are dominant in early wound healing.

6. Conclusion

Occlusive dressings may stimulate regenerative effects in diabetic wounds. Our study shows the influence of wound fluid under occlusive dressings from diabetic patients on angiogenesis, migration and proliferation of fibroblasts, which are essential modulators of wound healing and scar modulation. Further in-vivo studies should investigate the application in this selected patient group and also analyze a possible economic impact.

Declaration of competing interest

All authors confirm that there are no competing interests. None of the authors has any financial or personal interest in the products mentioned in this article. None of the authors has any competing interests.

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