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Review

Wound fluid sampling methods and analysis of cytokine mRNA expression in ulcers from patients with diabetes mellitus

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ABSTRACT

The development of diabetic foot ulcers is a common and severe complication of diabetes that can significantly affect quality of life. The physiological healing cascade does not progress tissue repair in diabetic foot ulcerations in a timely manner. Serum markers from foot ulcers have been used to characterize the healing process of the diabetic foot using various collection techniques. This study aimed to compare the use of cervical brushes and the Levine technique to collect wound fluid from foot ulcers of people with diabetes in order to determine the presence of cytokines. The collected material was used for gene expression analysis of macrophage/monocyte-associated cytokines IL1-β, IL-6, TNF-α, regulatory cytokine IL-10 and growth factor TGFβ, via quantitative polymerase chain reaction (qPCR). Both collection methods produced sufficient amounts of RNA, but significantly more RNA was collected using a cervical brush (brush 224.82 ng/μL vs. Levine 80.90 ng/μL $p = 0.0001$). Significantly higher levels of expression of the following cytokine genes were detected in samples collected using a cervical brush: IL1-β ($p = 0.0001$), IL-6 ($p = 0.0106$), IL-10 ($p = 0.0277$) and TGFβ ($p = 0.0002$). Understanding why some wounds are difficult to heal is important for developing more effective treatments, and biomarkers may be useful for predicting the healing trajectory. These results demonstrate that it is possible to collect material from the wound bed for RT-qPCR analysis, and the cervical brush proved to be a simple and rapid method for monitoring cytokine gene expression.

Contents

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1. Introduction

Foot ulcers in people with diabetes mellitus (DM) are an important public health problem, and are the main cause of non-traumatic amputations. The prevention and treatment of these wounds remains a challenge for healthcare professionals [1].

In wounds of patients with DM, the healing phases are stagnant [2], which leads to a delay in skin closure. Studies have identified a lower amount of cytokines released by macrophages, and a decrease in vascular endothelial growth factor (VEGF) in such patients enduring diabetic foot ulcer [3,4]. In addition, due to the high level of reactive oxygen species (ROS) in a hyperglycemic environment, the production of interleukin 8 (IL-8) by keratinocytes is increased, leading to augmented infiltration of neutrophils recruited by IL-8, which is one of the causes of the prolonged inflammatory phase [4,5].

A study that compared the expression of genes between diabetes and normal fibroblasts found that the diabetic cells had lower growth factor gene expression than the healthy cells, which decreased the proliferative response [6]. Thus, analysis of the cytokine gene expression in the wound bed is essential for a better understanding of the biological state of its microenvironment for the purpose of characterizing the progression of healing, the chronic conditions, and the evolution of the injured area in order to develop new therapies.

In this context, a laboratory technique that has gained notoriety in recent years is the quantitative polymerase chain reaction (qPCR), which is characterized by the conversion of messenger RNA (mRNA) into complementary DNA (cDNA) for subsequent amplification and quantification of target genes [7]. However, an appropriate method of sample collection is essential for reliable results.

A study that used the qPCR technique to investigate the expression of cytokine mRNA collected in Pap smears using a cervical brush obtained positive results [8]. Most of the samples expressed Ifn-γ, IL-10 and IL-12; the expression levels of IL-14 were extremely low. It is known that when performing a Pap smear, a cervical brush collects more cells (93.1%) than a wooden spatula (61.8%) [9].

One of the methods used to study cytokine gene expression in wounds involves collecting the wound fluid (WF) [10,11]. WF exhibits a wide range of biomarkers that can be investigated. The composition of the WF reflects the extracellular space, which allows the investigation of the current wound conditions, responses to topical treatments, and the effects of interventions [10]. Biomarkers can give an indication of a person's biological state and may be useful for understanding, or predicting, the healing trajectory of a wound [10].

WF can be obtained through different techniques, such as aspiration, drainage, use of absorbent materials, occlusive dressings, and direct collection by external devices. However, not all of them allow the adequate collection of material for the analysis of biomarkers in the wound bed [11].

Among the techniques used for WF collection is swabbing [11]. Despite being an easy technique, the amount of fluid extracted is low, often compromising the results. The wound aetiology also interferes with the amount of exudate; for example, lesions in the lower limbs of people with diabetes may in some cases present little exudate, which makes it difficult to read the biomarkers [12].

Therefore, this study aimed to compare the collection of wound fluid from foot ulcers of patients with diabetes using cervical brushes and by swabbing using the Levine technique to determine the presence of cytokines.

2. Methods

2.1. Patient and wound characteristics

This study was conducted from April 2022 to September 2023. It included 29 participants with DM treated at the wound outpatient clinic at two different centres: a university hospital at University of Campinas in Sao Paulo State and another university hospital at Federal Fluminense in Rio de Janeiro.

All wounds were located below the knee and no clinical signs of infection (perilesional erythema above 2 cm, purulent exudate, edema, redness) were present.

The inclusion criteria were patients aged ≥18 years old, with at least one diabetic foot ulcer with a duration of ≥2 months. The exclusion criteria were allergies to any of the materials used to collect the WF. This study was approved by the Research Ethics Committee of the State University of Campinas (43782721.4.1001.5404).

2.2. Technique for WF sampling from diabetes-related foot ulcers

WF was collected using a cervical brush and/or by swabbing using the Levine technique[12]; both were rotated over a 1-cm^2 area of the wound with sufficient pressure to express fluid from within the wound tissue (Fig. 1a and b). The first sample of WF was collected using a cervical brush (cervical brush with nylon bristles – Sterile Cervical Brush 10.1351, Kolplast, Itupeva, Brazil). After this the wound was washed with saline solution and the WF was collected by swabbing using the Levine technique (rayon-tipped sterile polystyrene swab, without transport medium – rayswab P0430, INLAB, São Paulo, Brazil), cleaning the lesion area before collection as recommended in the Levine technique [13], and as shown in Fig. 1b.

The tip of the brush and swab were cut and placed in plastic tubes containing 500 μL of Trizol buffer. Then the materials were transported to the laboratory and kept under refrigeration at −20 °C until RNA extraction. The collected material was used for gene expression analysis of macrophage/monocyte-associated cytokines IL1-β, IL-6, TNF-α, regulatory cytokine IL-10 and growth factor TGF-β, via quantitative polymerase chain reaction (RT-qPCR). In order to analyse the integrity of the isolated RNA, RT-qPCR of the housekeeping genes 18S and Ppia was performed.

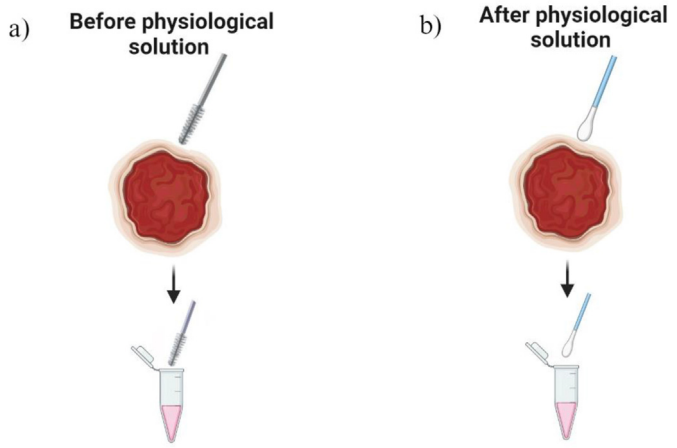

Fig. 1. a) Collection of wound fluid using cervical brush before washing with physiological solution, followed by immersion in Trizol for qPCR analysis. b) Collection of wound fluid using swab after washing with 0.9% physiological solution, followed by immersion in Trizol for qPCR analysis (figure created using Biorender.com).

2.3. Total RNA extraction

The samples were taken out of the freezer and kept at room temperature (RT) until completely defrosted. They were then lysed using a vortex for approximately 15 s or until mixed. The tip of the brush and the swab were gently pressed against the tube wall and then discarded. The liquid contents were transferred to a new 1.5 ml tube and kept at RT for 5 min for incubation until the complete dissociation of the nucleoprotein complex.

After homogenization, chloroform was added to the samples and then incubated for 2–3 min. Then, the tubes were centrifuged at 10,500×*g* at 4 °C for 15 min. After centrifugation, the mixture was separated into a lower pink organic phase, a whitish interphase, and an upper aqueous phase [14].

The aqueous phase containing RNA was transferred to another 1.5 ml tube. After that, 250 μl of isopropyl alcohol was added to precipitate the RNA present in the aqueous phase followed by centrifugation at 10,500×*g* at 4 °C for 10 min.

The supernatant was then discarded and 500 μl of 70% ethanol was added to the precipitate and vortexed for a few seconds. This step was followed by the addition of 100% ethanol to the samples. The tubes were then centrifuged at 8400×*g* at 4 °C for 10 min. The supernatants were discarded and the RNA pellets were air-dried. The precipitate was resuspended with 15–30 μl of milli-Q water, according to the size of the RNA pellet.

The sample absorbances were determined at 230 nm, 260 nm and 280 nm wavelengths using a spectrophotometer and the software Gene 5®. The ratio of absorbance at 260 nm/230 nm and 260/280 nm was used to assess the purity of DNA and RNA. A ratio between 1.8 and 2.0 indicates DNA and RNA that is free of impurities.

2.4. Gene expression analysis

To convert RNA to cDNA in a single 20 μl reaction, the enzyme High Capacity MultiScribeTM Reverse Transcriptase (Applied Biosystems®) was used. The RT-qPCR reactions were performed in duplicate.

Gene expression analysis was performed using the StepOne™ Real-Time PCR System (Applied Biosystems). Initially, the cycling conditions were 95 °C for 2 min for denaturation and then 45 cycles at 95 °C for 5 s and 60 °C for 30 s. Each PCR well contained 20 ng of cDNA, 0.25 μl of specific primer, 3.0 μl of Master Mix (LuminoCt® qPCR ReadyMix™- L6669-2000RXN – Sigma-Aldrich) and 0.25 μl H_20 Milliq. Gene specifications, assays, primers, and exon boundaries are listed in Figure 2. They were purchased from Integrated DNA Technologies or Thermo Fisher.

Gene Name/ IDT DNA Technologies	Assay	Ref Seq	Exon Boundary
IL1-β	Hs.PT.58.1518186	NM_000576(1)	1-3
IL-6	Hs.PT.58.40226675	NM_000600(1)	4-5
TNF- α	Hs.PT.58.45380900	NM_000594(1)	1b-4a
Gene Name/ Thermo Fisher Scientific			
IL-10	Hs00961622_m1	NM_000572.2	4-5
TGF-ß	Hs00998133_m1	NM_000660.5	6-7
Ppia	Hs99999904_m1	NM_021130.4	4
18S	Hs99999901_s1	X03205.1	1

Endogenous controls: Ppia, 18S; Interleukin 1 β (IL-1 β); Transforming growth factor β (TGF-β); Interleukin 6 (IL-6); Interleukin 10 (IL-10); Tumour Necrosis Factor α (TNF- α).

Fig. 2. The name of the genes, the assay number, the nucleotide sequence (Ref Seq) and the exon (Exon Boundary) used to construct the primers. The assays were purchased from Thermo Fisher Scientific and IDT DNA Technologies. Endogenous controls: Ppia, 18S; Interleukin 1 β(IL-1 β); Transforming growth factor β (TGF-β); Interleukin 6 (IL-6); Interleukin 10 (IL-10); Tumour Necrosis Factor α (TNF- α).

It is important to mention that in this study the cycle threshold value was used to represent the data obtained in the qPCR. The cycle threshold is the cycle number at which the generated fluorescence signal is significantly above the background fluorescence. Their relationship is inversely proportional, thus the smaller the number of cycles required, the greater the amount of target gene present in the sample, which supports our observation [15].

2.5. Statistical analyses

The paired Student's t-test or the paired Wilcoxon test were used to compare the quantitative measurements obtained using the two techniques, depending on the data distribution. Data distribution was evaluated using the Shapiro-Wilk test. The correlations between wound area and cytokine mRNA expression were evaluated using the Spearman correlation coefficient [16]. For all analyses, the statistical software SAS version 9.4 and SPSS version 25 (SPSS, Inc., IL, USA) were used, and a significance level of 5% was considered.

3. Results

3.1. Characteristics of the participants

A total of 29 patients were included in the study. The mean age was 55.48 years, mostly of the patients were men (68.97%), married (79.31%), and received up to 2 minimum wages (89.66%). Regarding education status, most have completed primary school (51.72%) (Table 1). Regarding clinical clinical data, the mean diabetes duration was 20.48 years, and the duration of the wound was 16.48 months on average. The wound surface area was 4.09 cm^2 on average. Regarding the cause of the wounds, six wounds were due to previous amputation and 23 wounds due to trauma. The average HbA1c among participants was 9.13% (Table 1). According to Wagner's classification [17], 28 individuals had grade I lesions and one individual had grade II lesions.

In the endocervical brush technique (Table 2), there was a positive, significant and moderate correlation between the wound area and RNA 260nm/280 nm ratio ($p = 0.0131$; r 0.4553) and endogenous 18s ($p = 0.0088$; r 0.4778) and a strong correlation with IL-10 ($p = 0.0449$; r 0.5429). Therefore, the larger the wound area, the higher the value of 260nm/280 nm RNA, endogenous 18s and IL-10. In Levine technique, there was only one positive,

Table 1
Patients' sociodemographics and clinical characteristics (n = 29).

Sociodemographics Characteristics		
Age, Mean (SD)	29	55.48 (11.44)
Gender, %		
Male	20	68.97
Income, nº of minimum monthly wages[a], %		
Unemployed	2	6.90
Up to 2	26	89.66
More than 2	1	3.45
Current marital status, %		
Single	4	13.79
Married	23	79.31
Without partner	2	6.90
Educational Status, %		
Primary school	15	51.72
Secondary school	11	37.93
College	13	10.34
Clinical Characteristics		**Mean (SD)**
Diabetes Duration (years)	29	20.48 (15.04)
Wound Duration (months)	29	16.48 (22.50)
Wound Surface Area (cm^2)	29	4.09 (3.39)
HbA1c (mg%)	29	9.13 (1.58)

[a] US$ 288.00; HbA1c, glycated haemoglobin.

significant and strong correlation between the wound area and RNA ng/μl ($p < 0.0015$; r 0.5621) which the amount of RNA (ng/μl) collected through this technique is correlated with largest wound areas.

3.2. RNA quantification by spectrophotometer

The results for RNA quantification are presented in Fig. 3. The samples collected from the wound fluid by the cervical brush or swab were subjected to the extraction process, followed by quantification of the total RNA by spectrophotometer.

All genes were evaluated in all patients (n = 29), however only 18S expression was detected in 100% of the samples in both groups. 18S was used as a house-keeping gene as it is widely adopted for normalising RT-qPCR data in biodiversity studies [18]. Expression of the other genes was not detected in all samples collected using both techniques. However, in samples in which the expression of a specific gene was detected, the mean cytokine mRNA expression was always lower in samples collected using a cervical brush.

The spectrometry values showed that the samples had a 260 nm/280 nm ratio within the expected range, with a mean of 1.95 (1.65–2.54). Both collection methods resulted in samples with sufficient RNA in ng/μL, but with significantly more RNA in samples collected using a cervical brush (brush 224.82 ng/μL vs. Levine 80.90 ng/μL, p = 0.0001). All genes were amplified from samples collected using a cervical brush, except TNFα (p = 0.092) (Fig. 3).

Table 2
Spearman coefficient correlations of 260nm/280 nm ratio, RNA concentration (ng/μL) and cytokines expression with wound area in both techniques collection methods.

	Wound Area		
	Endocervical brush	n	Levine
RNA - 260nm/280 nm			
r	0.4553	29	−0.3672
p-value	**0.0131**		0.0501
RNA - ng/μL			
r	0.3636	29	0.5621
p-value	0.0526		**0.0015**
Ppia			
r	−0.0086	26	−0.1183
p-value	0.9669		0.5649
18s			
r	0.4778	29	−0.0660
p-value	**0.0088**		0.7337
IL-1ß			
r	−0,0334	28	−0.1314
p-value	0.8660		0.5052
Tgf-ß			
r	−0.0809	24	−0.3644
p-value	0.7072		0.0801
IL-6			
r	0.3000	15	0.2929
p-value	0.2773		0.2895
IL-10			
r	0.5429	14	0.1517
p-value	**0.0449**		0.6048
Tnf-α			
r	0.2518	12	0.3088
p-value	0.4299		0.3288

r-Spearman coefficient correlations.

4. Discussion

Wound fluid is a good source for investigating the proteomics and biomarkers of the wound bed microenvironment with the aim of understanding the cellular and metabolic events involved in the healing process, as well as the response to topical treatments [19,20]. However, ensuring that a collection method can obtain an adequate quantity of fluid for analysis is one of the main challenges. One of the most used collection methods involves swabbing the wound bed [21,22], known as the Levine technique [23]. The search for other, improved collection methods is ongoing, with a view to making the analysis of the microenvironment of the lesions more reliable. Low levels of exudate make it difficult to collect reproducible samples of WF, particularly from diabetic foot lesions [12].

A study that compared the modified Levine technique, using commercially available nylon-flocked swabs, observed that the quality of WF collected is not inferior to that collected by covering the wound with an occlusive transparent dressing [12] in the evaluation of cytokines in wounds of people with diabetes. Schmohl M et al. [12] compared two wound fluid collection techniques, swabbing and aspiration in diabetic foot ulcers. They identified that both sampling methods yielded a similar qualitative protein recovery, with a tendency toward analyte enrichment through swabbing. Swabs were used in the sampling process without any effect on the recovery of analyte, except for interleukin IL-8, thymus and activation-regulated chemokine, IL-17A, interferon-induced protein 10, and IL-4. Our findings are in agreement with the results of previous studies [12,24,25]. It's not an easy task to determine the components of wound fluids, particularly in chronic wound fluid. Distinct studies have compared acute wound fluids with chronic ones to determine differences in the way these wounds heal [26–29].

According to a study [30] that investigated the relationship between CXCL 6 levels, a pro-angiogenic chemokine, and diabetic foot ulcers healing found out that the higher CXCL 6 levels the faster the wound closure. The authors also elucidated a negative association between body mass index (BMI), HbA1C, disease duration, advanced age, wound area and exudate CXCL-6 levels, which means that the healing becomes more difficult under these clinical conditions. In our study, we found a correlation between IL-6 and wound size. However, the sample collection was made in a single time point and a follow-up study should be conducted to have a better understanding of these findings.

Therefore the assessment and treatment of chronic wounds could be improved in clinical practice by using biomarkers to predict healing. Fluid from chronic wounds, as demonstrated by a

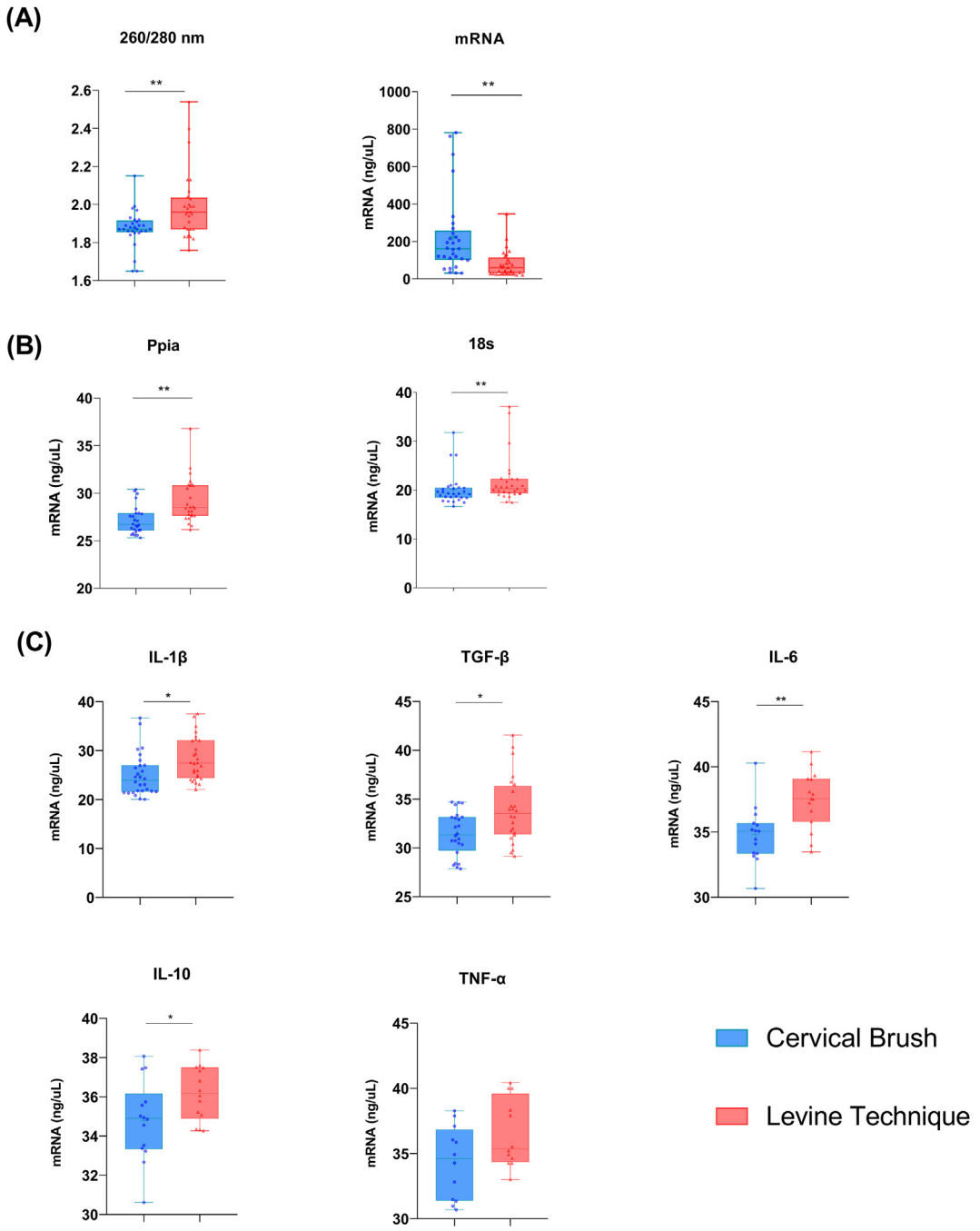

Fig. 3. Comparison of cytokine mRNA expression between wound fluid collection methods. Exudate was sampled from diabetic foot ulcers of 29 individual patients, using a cervical brush and swabbing using the Levine technique. **(A)** Box plot of RNA purity showed by the absorbance ratio 260nm/280 nm and total RNA extracted (ng/μL), **(B)** RNA concentration (ng/μL) of the house-keeping genes Ppia (n = 29) and 18S (n = 26), **(C)** and cytokines IL-1β β (n = 28), TGF-β (n = 24), IL-6 (n = 14), IL-10 (n = 15) and TNF-α (n = 12) quantified using RT-qPCR and total mRNA and protein extract were isolated as described. * p-value obtained using the paired Student's *t*-test; ** p-value obtained using the paired Wilcoxon test; $p < 0.05$. Ribosomal ribonucleic acids (RNA); Endogenous controls: Ppia, 18S; Interleukin 1 β(IL-1 β); Transforming growth factor β (TGF-β); Interleukin 6 (IL-6); Interleukin 10 (IL-10); Tumor Necrosis Factor α (TNF- α).

study [31], decreases the active Ras levels, which is a protein that regulates cell growth in eukaryotic cells [32]. Wound fluid analysis is a manner to detect and quantify cells and biochemical markers, being a promising way to monitor healing and to investigate the tissue inflammatory response without using invasive techniques.

Comparison of the levels of cytokines present in WF collected using the two different collection techniques of the present study showed higher amounts of RNA in the samples collected with a cervical brush than in those collected using the Levine technique. This was illustrated by the average threshold cycle (Ct) of the target genes, which was lower in samples collected with a cervical brush than in the Levine technique.

The cervical brush method was able to amplify the target gene in WF representatively, indicating that this method can be used to monitor the wound bed environment during the healing process. Another positive point is that the cervical brush method contributed to the identification of target genes even in environments with little exudate [33].

The amount of WF varies depending on the aetiology of the wound. For example, ulcers on the feet of the people with DM produce low amounts of exudate when compared to venous ulcers and burns. The amount of WF can also be related to the healing phase. In the inflammatory phase, it is usually higher due to cellular activity. It may also be related to the wound size [34]. As a result, the cervical brush is a good option for collecting samples from wounds with low WF, such as foot ulcers related to diabetes.

The cervical brush may collect fragments of cells and whole cells along with the WF without causing discomfort to the patient, as may happen when performing biopsies. In our opinion, this is also a very positive aspect, as this may allow the evaluation of other markers of re-epithelialization such as growth factors after a new treatment, probably without the need for an invasive procedure. Collecting this mixture from the wound bed therefore opens up the opportunity to investigate multiple target genes using a small sample.

Future investigations should include a larger number of participants and other types of cytokines in order to establish personalized treatments and to evaluated the effects of advanced wound dressings which could actively promote wound healing and shorten time-to-healing in patients with non-infected and predominantly neuropathic diabetic foot ulcers.

4.1. Limitations of the study

The small sample size of cytokines examined is one of the limitations of the present study, as is the fact that other biomarkers were not evaluated. Another limitation was the need to carry out studies to prove the presence of whole cells in the collected samples. This could open up new possibilities for evaluating the wound such as histological analyses, which could in some situations require a biopsy of the lesion.

5. Conclusion

The use of an appropriate sample collection method is a fundamental step in the research process and, therefore, it may affect the results obtained. Understanding why some wounds are difficult to heal is important for developing more effective treatments, and biomarkers can give an indication of a person's biological state and may be useful for predicting the healing trajectory. The data from this study demonstrate that it is possible to collect material from the wound bed for RT-qPCR analysis, which is a sensitive technique, requiring only a small sample to investigate multiple target genes. In the current study the cervical brush proved to be a simple, rapid, effective, and low-cost method for monitoring cytokine gene expression in the wound bed.

Declaration of competing interest

No conflicts of interest declared.

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