Changes in Gene Expression in Pressure Ulcers Debrided by Different Approaches – a Pilot Study

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Summary

Pressure ulcers (PUs), also known as pressure injuries, are chronic wounds that represent potential lifelong complications. Pressure ulcers of a deep category (III and IV) are often indicated for surgical treatment – debridement and surgical reconstruction. Sharp surgical debridement is widely used in the debridement of PUs; however, the Versajet® hydrosurgery system is becoming an increasingly popular tool for tangential excision in surgery due to its numerous advantages. This work focused on the expression of selected genes, especially those associated with oxidative stress, in PUs debrided by two approaches – sharp surgical debridement and debridement using Versajet® hydrosurgery system. Expression of following genes was evaluated: *NFE2L2*, *ACTA2*, *NFKB1*, *VEGFA*, *MKI67*, *HMOX1*, *HMOX2*, *HIF1A*, and *SOD2*. *ACTB* and *PSMB* were used as housekeeping genes. So far, five patients have been enrolled in the study. Preliminary results suggest no significant difference in gene expression with different pressure ulcer treatment approaches except *NFE2L2*, despite the macroscopic differences. However, the results revealed correlations between the expression of some genes, namely *HIF1A* and *SOD2*, *VEGFA* and *SOD2* and *VEGFA* and *HIF1A*. These results may indicate a connection between hypoxia, oxidative stress, pressure ulcer healing processes and angiogenesis.

Key words

Pressure ulcers • Debridement • Wound healing • Oxidative stress • Gene expression

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Introduction

A pressure ulcer (PU) is defined by the European Pressure Ulcer Advisory Panel as an area of localized damage to the skin and underlying tissue caused by pressure, shear, or friction, or a combination of these [1]. Pressure ulcers are caused by a local breakdown of soft tissue as a result of compression between a bony prominence and an external surface [2]. Soft tissue deformation beyond certain tolerance threshold also occurs, leading to direct deformation and damage of the cells due to structural failure of the cytoskeleton and plasma membrane [3,4].

The highest prevalence of PUs is in high-income North America followed by Central America, tropical Latin America and Carribean, and the lowest in the south and central Asia. Compared to high-income North America, central Europe ranks tenth with a prevalence of 7.8 per 100000 population [5]. Between 2010-2019, 264442 patient records with diagnoses L89.0-L89.9 (PUs diagnoses in ICD-International Classification of Diseases and Related Health Problems) were identified (an average of 26444 patients per year) in the Czech Republic. Numbers have increased each year, with a 40 % increase

between 2010 and 2019 [6]. Analyses of national health registries showed that the prevalence of PUs before the onset of the COVID-19 pandemic and during the 2020 pandemic was higher in patients hospitalized with SARS-CoV-2 infection [7]. It is evident that PUs represent a significant socio-economic and health problem. Management of PUs is complex and involves a change in patient care, including the use of various aids to prevent its occurrence, i.e. avoiding pressure, friction or shear (pressure-relieving strategies, repositioning or 'turning' patients), good skin-care regime and managing exacerbating factors, such as urinary or fecal incontinence, nutrition therapy, but also surgical treatment – debridement – followed by pressure ulcer healing management [8]. Multiple techniques, such as mechanical, biological or surgical, are used to debride. Determination of the most appropriate technique mandates the consideration of both host-specific (i.e. comorbidities, compliance, social support, etc.) and wound-related (i.e. infection/contamination, perfusion, viability, etc.) factors, as well as the resources available at the treatment facility. The European Wound Management Association guidelines for debridement provide specific information regarding each technique's indications, contraindications, and potential adverse effects [9,10].

New approaches and procedures are being introduced in the field of surgical debridement. One of these techniques is the hydrosurgery system, which utilizes a high-pressure parallel water jet promoting the Venturi effect. Its use was first described by Klein *et al.* in 2005 as a new tool for tangential dissection [11]. This technique has gradually spread, especially in managing chronic wounds and burns. It is gradually proving to offer a number of advantages, including lower blood loss during the procedure and faster wound healing [12]. Unfortunately, despite the use of Versajet hydrosurgery in clinical practice, our knowledge about its impact on healing is still limited. Therefore this study aimed to compare two approaches to category III and IV PUs treatment – sharp debridement (performed with a scalpel and/or electrocauter) and Versajet® hydrosurgery system. This treatment represented the first surgical step before reconstruction using flap plasty. The obtained samples were analysed for gene expression levels of genes related to oxidative stress and healing processes to gain new information about the differences between the two techniques at the molecular biological level.

Methods

Experimental design

Prospective interventional study in which a total of five patients with PUs larger than 5×5 cm of various localizations were included in the pilot phase. Basic data of patients describes Table 1. The PU bed was divided into two halves, and each half was subsequently debrided with a different approach – sharp or hydrosurgery (Versajet®) debridement. Tissue samples were collected from each half of the PU before and one week after the debridement. Samples were always collected in the same manner with respect to the size and the depth of the PU. Samples collected in this way were processed immediately after collection, i.e. placed in an RNA-later (Roche, Czech Republic).

Tissue samples were mechanically homogenized by microtube pestle. RNA was isolated from homogenized tissue using TriPure Isolation Reagent (Roche, Basel, Switzerland) according to the manufacturers' protocol and then transcribed using using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) in accordance with the manufacturer's instructions.

Analysis of tissue samples

The quantitative RT-PCR was carried out using TaqMan gene expression assays with the LightCycler®480 II System (Roche, Basel, Switzerland). The amplified cDNA was analyzed by the comparative ddCt method using *PSMB* as a reference. The primer and probe sets for *PSMB2* (Hs01009704_m1, housekeep), *ACTB* (Hs99999903_m1, housekeep), *NFE2L2* (Hs00975961_g1), *ACTA2* (Hs05005341_m1), *NFkB1*(p50/p105) (Hs00765730_m1), *EGF* (Hs01099999_m1), *VEGFA* (Hs00900055_m1), *MKI67* (Hs00606991_m1), *HMOX1* (Hs01110250_m1), *HMOX2* (Hs01558390_m1), *GPX1* (Hs07288100_g1), *HIF1A* (Hs00153153_m1), *SOD2* (Hs00167309_m1), *NOS2* (Hs01075529_m1), and *ANGPT4* (Hs00907074_m1) were selected from the TaqMan Gene Expression Assays (Life Technologies, USA). The selection of genes for expression monitoring was based on available data in the literature with respect to oxidative stress [13,14]. The qRT-PCR was executed under the following amplification conditions: total volume of 20 µl, initial incubation at 50 °C/2 min, denaturation at 95 °C/10 min, then 45 cycles at 95 °C/15 s and 60 °C/1 min.

Table 1. Basic clinical data of patients.

Gender/Age	PU category	Size of PU (length × width × depth)	PU location	Wound bed	Type of flap	Comorbidities	Swab (admission date)
F/55	IV	8×5×3 cm	Sacral	Slough, appropriate secernation, bone not exposed	FC gluteal rotation bilateral flap	Paraplegia, st. p. PU sepsis year ago	*Streptococcus alfa haemolyticus, Staphylococcus aureus, Candida albicans*
M/42	III	9×7×1 cm	Hip-left-sided	Intact fascia, bone not exposed	Tensor fasciae latae muscle flap (MTFL)	Quadriplegia, nefrotic syndrome, amyloidosis, hypothyreosis, mild ventilation disorder, polymorbidities	*Acinetobacter baumanii*
F/58	IV	5×5×2 cm	Sacral	Rolled hard edges, poor granulation, coated pseudocyst	FC gluteal rotation unilateral flap	Quadriparesis, sclerosis multiplex, polymorbidities	*Proteus mirabilis, Acinetobacter baumannii*
M/68	IV	6×6×2 cm	Ischial-right-sided	PU rather sessile, granulation poor, elbow slightly loose, bone not exposed	Dorsal thigh flap+distal portion of gluteus maximus muscle	Quadriplegia, M. Recklinghausen, polymorbidities	*Staphylococcus aureus, Pseudomonas aeruginosa, Proteum mirabilis*
M/63	IV	6×3×2 cm	Ischial-left-sided	Rolled edges, macrosopically clean, bone intact, no signs of inflammation	Dorsal thigh FC flap	Paraplegia, sideropenic anemia	*Beta-haemolytic Streptococcus group G*

Statistical analysis

The statistical analysis was performed using R 4.0.2 language with the following packages: ggplot2, tidyverse, corrplot, rstatix [15-19]. The data from qRT-PCR analysis were evaluated with the "ddCt" method, where the relative expression of each gene was referred to *PSMB* and mean values as control. Considering the distribution of the data, logarithmic values were used for statistical analysis. Multifactorial ANOVA was calculated, but better the Wilcoxon test was used to determine the significant differences between individual treatments, since the data did not meet the assumptions for parametrical testing (Levene's Test for Homogeneity of Variance, Shapiro-Wilk normality test).

Spearman correlations were computed to analyze the relations in the data. For summary, the heatmap with dendrogram was created. For the dendrogram, non-hierarchical cluster analysis was used with calculation of the Euclidean distance both for individual samples and for individual variables. Unless noted otherwise $p<0.05$ was considered significant.

Results

In expression analysis, there were 9 gene expressions in 20 samples (all in duplicates) detected. From each patient (5) in the study were collected 4 different samples (each in duplicate). The sample was

either treated with Versajet®, or collected surgically and also monitored in two individual samplings. The overall effects on expression profiles of treatment or sampling were determined with multifactorial ANOVA, but the data did not meet the requirements for parametric statistical analysis. The mutual differences between individual patients were greater than the effects of the used treatments. For example, the effect of a patient factor on *NFKB1* expression (Welch´s One-way analysis of means, not assuming equal variances, F=4.6394, num. df.=4.000, denom. df.=17.189, p=0.0101), or *VGEFA* expression (F=5.2973, num. df.=4.000, denom. df.=16.599, p=0.006126). Hence individual differences were detected with the Wilcoxon test. This analysis revealed a significant change between the expression of *NFE2L2* after the first (mean=-0.5892) and the second sampling (mean=-0.7358) that were treated with Versajet® (W=77, p=0.04507, n=10) (Fig. 1).

The correlation analysis was performed as well. Spearman correlations were computed for each treatment and sampling, and the 10 most significant ones ($p<0.05$) are presented in Figure 2. The pairs unique for each treatment are worth mentioning. The expression of *NFE2L2* correlates positively with *NFKB1* after the first sampling and surgical treatment (r=0.91, p=0.0002). The only significant negative correlation is found between *ACTA2* and *NFKB1* (r=-0.902, p=0.0004), and between *NFE2L2* and *NFKB1* (r=-0.801, p=0.005, not shown) after the second sampling and surgical treatment. This is in contrast with the results after the first sampling. Some correlations are specific for VersaJet samples, i.e. *VGEFA* and *HMOX2* (r=0.92, p=0.00016, the first sampling; r=0.86, p=0.0013, the second sampling), and *NFKB1* and *HMOX1* (r=0.88, p=0.0008, the second sampling).

All gene expression profile results were summarized in a heatmap with cluster analysis (Fig. 3). The mean expression values after all treatment combinations show several trends. The genes form three separate clusters, the ones that increased their expression after the treatment (*HIF1A*, *SOD2*), the ones that were suppressed (*NFE2L2*, *HMOX2*) and the rest that showed minor changes. Also, the samples created some clusters, where the ones after the first sampling show more similarities, regardless of the treatment.

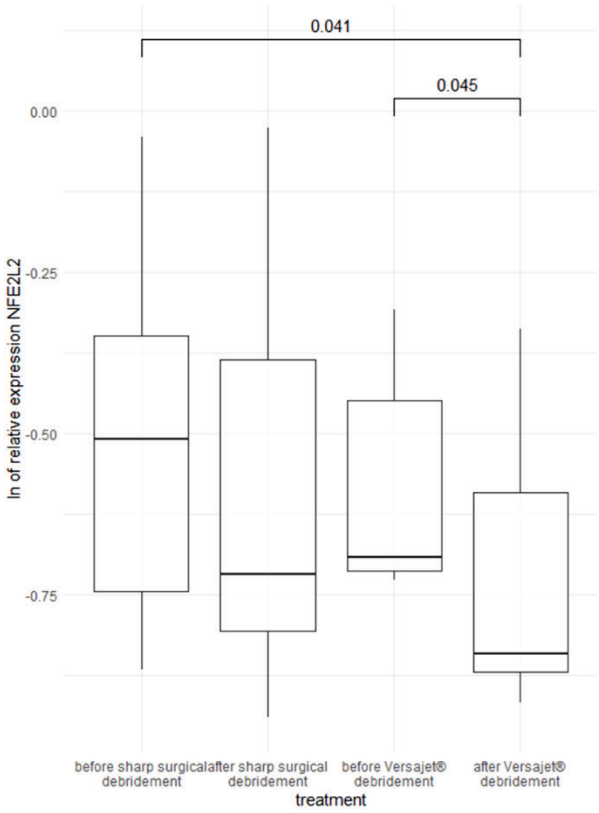

Fig 1. The log of relative expression of NFE2L2. The box and whisker plot presents the median values after all treatments and their significant differences. The hinges correspond to the 1st and 3rd quartiles and the whiskers show 1.5*IQR (interquartile range). The depicted statistical significance is result of Wilcoxon test. The samples treated with Versajet® after the first sampling have higher relative expression of NFE2L2 than the same treated samples after the second sampling (p=0.045). Also, the samples treated surgically have higher relative expression of this gene (p=0.041).

Discussion

Analysis of tissue samples taken from PUs treated with different debridement techniques – sharp surgery (scalpel or electrocauter) versus Versajet® hydrosurgery – yielded almost no differences in the expression of the studied genes. However, the expression of *NFE2L2* was significantly reduced in case of Versajet as compared to sharp surgery (the first sampling: surgery versus Versajet) and in case of the second sampling of Versajet as compared to the first sampling of Versajet. *NFE2L2* is gene coding Nuclear Factor Erythroid 2-Related Factor 2 (NF-E2-Related Factor 2, respectively Nrf-2). It is a transcription factor, which is under normal conditions relatively rapidly degraded in the cells. Still, under oxidative stress conditions, it is transported to the nucleus, where it binds to the DNA promoter region and triggers the expression of genes encoding enzyme–protein–antioxidant mechanisms. This function has been studied in case of the antioxidant action of compounds of natural origin [20,21], but particularly in the context of certain types of cell deaths, especially apoptosis [22],

Fig. 2. Ten most important correlations for each treatment and sampling combination. The length of the column expresses the size of the correlation coefficient R (Spearman's correlation), blue shows positive correlation, red is negative. All depicted correlations are significant ($p<0.05$).

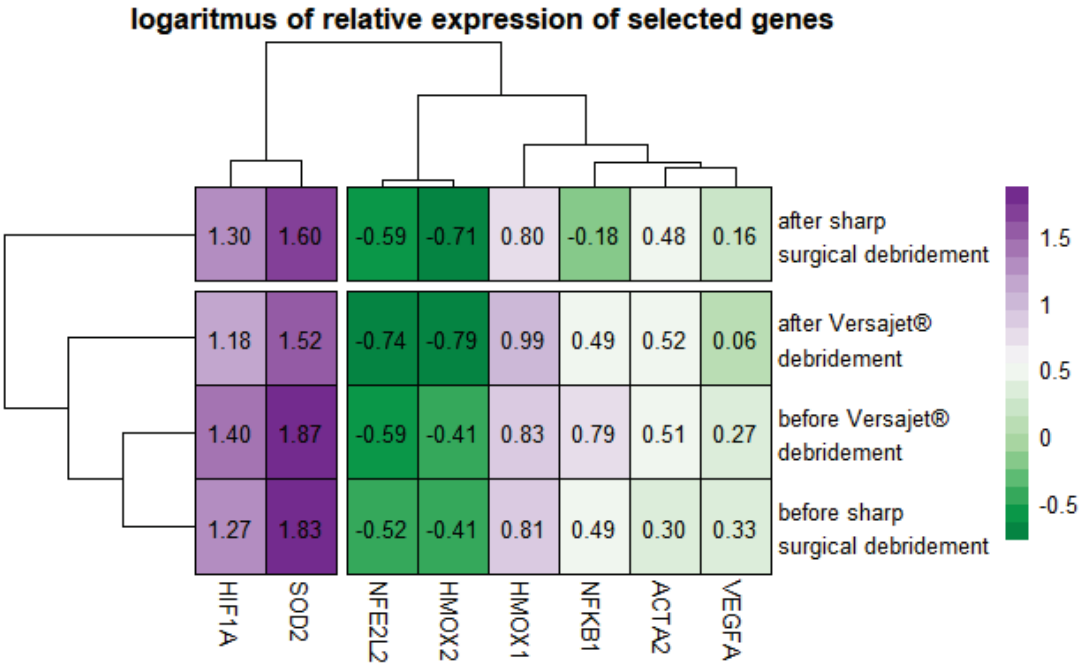

Fig. 3. The picture presents all obtained gene expression results. The mean of relative expression is depicted with color (high expression in purple, low in green). The dendrograms show the differences and similarities between individual genes and treatments. The highest relative expression has SOD2 in all samples, the lowest HMOX2, in particular after the second sampling and Versajet® treatment. Samples from the first samples collection regardless the treatment cluster together. For example, the genes HMOX2 and NFE2L2 show similar trends in expression.

autophagy [23], or pyroptosis [24]. The question arises what is the relationship between the expression of this gene and the pressure ulcer healing process. Reactive oxygen species (ROS) and nitrogen species (RNS) play an essential role in the wound healing process, being involved in different phases of the healing process. Van Huizen *et al.* reports that the first significant accumulation of ROS occurs at the wound site after the first hour, and ROS are required for wound closure [25]. Subsequently, they are implemented in the regulation of actin-mediated epithelial stretching and rearrangement of adjacent cells over the wound surface. Their importance also lies in the regulation of cell signalling during the wound healing process. The importance of ROS in the wound healing process is described and discussed in detail for example, in a review by Krizanova *et al.* [26] or Hokynkova *et al.* [27]. So far, several genes have been identified expression of which is directly controlled by ROS. The same authors [25] describe ROS-dependent jun-1 and HSP70 expression regulation. Jun is a transcription factor that, together with Fos, forms the transcription factor AP-1 (Jun and Fos are its subunits), which in turn modulates the expression of MMP (matrix metallopeptidase)-2 and MMP-9 [28]. Both are significant players in regulating extracellular matrix degradation and deposition essential for wound reepithelialization [29,30]. The importance of HSP70 in the wound healing process has also been described [31]. Nrf-2 is another important protein whose association with the previous ones has been reported, but not sufficiently studied in the wound healing process. Expression and activity of NRF-2 in wounds have been found in keratinocytes as well as in other cells in granulation tissue as a response to ROS [32]. Several studies have shown that elevated levels of Nrf-2 temporarily modulate the expression of vascular genes in wounds, which may accelerate the healing of chronic wounds. The mechanism of action of Nrf-2 is still not precisely known, but Nrf-2 is likely to be essential during the inflammation and proliferation phases of wound repair [33]. From this perspective, the pilot results could indicate a potential benefit of the Versajet technique in Pus healing.

The results of the correlation analysis are also interesting. The association between the expression of genes for neovascularization, antioxidant proteins and HIF1A is shown for each intervention. In case of the Versajet® technique, the specific correlation was found between gene expression of *VGEFA* (vascular endothelial growth factor A) and *HMOX2* (heme oxygenase 2) and *NFKB1* (nuclear factor NF-kappa-B p105 subunit) and *HMOX1* (heme oxygenase 1). Vascular endothelial growth factor A (keratinocyte-derived) binds primarily to endothelial cell receptors to promote angiogenesis which plays a key role in wound healing [34]. Correlation in *VGEFA* and *HMOX2* expression suggests the link between oxidative stress, or hypoxia, and new blood vessel formation [35].

The presented data come from a pilot study, which has so far been conducted on a small sample of patients. Although it suggests some interesting correlations in the expression of various genes, further extension of the study and additional analyses are needed to provide new insights and allow us to determine the potential benefit of Versajet technique in the debridement of PUs category III and IV.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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