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Wound Healing – A Proteomic Analysis of the Effect of Erythropoietin on Granulation Tissue Isolated from ePTFE Implants

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Abstract

Background: Erythropoietin is a multifunctional cytokine, with recent studies indicating a positive effect on wound healing in models of incisional and burn wounds.

Objective: The objective of this study was to investigate the changes in protein expression after daily injections of recombinant human erythropoietin in granulation tissue isolated from subcutaneous implants of expanded polytetrafluoroethylene (ePTFE tube).

Methods: ePTFE tubes were implanted subcutaneously in twelve C57bl6 mice. Six of the mice were treated with daily subcutaneous injection of recombinant human erythropoietin of 1000 IU/kg. The remaining six mice, the control group, were treated with injections of 0.9% saline. The implants were removed on day 9 after implantation. The granulation tissue was isolated and analyzed for protein expression using a proteomic approach including two-dimensional polyacrylamide gel electrophoresis and mass spectrometry. Some of the results were confirmed with Western Blotting.

Results: We identified 33 differentially expressed proteins in the rhEPO-treated group. Three proteins involved in the glycolytic pathway — Glyceraldehyde-3-phosphate dehydrogenase, Alpha-enolase, and Triosephosphate isomerase — were altered in the granulation tissue from the rhEPO-treated group.

Conclusion: Daily injection of recombinant human erythropoietin of 1000 IU/kg alters the protein expression of GAPDH, ENOA and TPIS in granulation tissue from wounds on postoperative day 9. The successful combination of proteomic analysis of wound tissue and the ePTFE wound model could advance our knowledge of the complex healing process.

Key words: *Erythropoietin, granulation tissue, ePTFE tube, wound healing, protein expression, proteomics*

Introduction

The complex and highly orchestrated healing process is the basis of all surgery. Pathological wound healing represents as a broad variety of clinical situations, ranging from the mere delayed healing and insufficient deposition of a connective tissue matrix and resulting in dehiscence to the excessive healing in hypertrophic scars and keloids, fibrosis, strictures, and adhesions.

In the delicately tuned healing cascade there are distinct roles for a large number of different types of cells, growth factors, cytokines, and receptors. Disruption of pathways and events during the healing cascade may result in inferior healing. Therefore, it is crucial to gain knowledge of the biology of the tissue repair process and the factors in-

fluencing it: how to optimize the healing process while diminishing the postoperative complications.

Erythropoietin (EPO) was identified almost a century ago as a humoral substance with a profound influence on red blood cell production and has for many years been used for treating patients with anemia [1]. Today, EPO is recognized as a type 1 cytokine utilizing different receptor isoforms. In addition to the hematopoietic properties, erythropoietin is involved in a multitude of mechanisms in areas of tissue protection, restoration and angiogenesis.

Several investigators have reported a promoting effect of EPO in in vitro and in vivo models of wound healing. Studies have demonstrated a positive effect on migration of fibroblasts [2], formation of granulation tissue [3] and collagen synthesis shown by an increase in breaking strength in incisional wounds [4,5] and colonic anastomoses [6-8], as well as an increase in collagen content in ischemic incisional wounds [9]. Furthermore, studies have shown a faster re-epithelialization in excisional wounds [10,11] and burns [12]. In addition, several studies indicate an effect on neovascularization in wounds [4,9,10,12].

These studies have investigated wound properties from collagen content and breaking strength to neovascularization and epithelialization. The different studies, although studying different objectives, do indicate that EPO could influence wound healing and may be of use in reconstructive surgery as well as in treatment of acute and chronic wounds.

It is not yet fully understood how erythropoietin influences the complex cascade of wound healing. If erythropoietin alters the protein expression during formation of granulation tissue, knowledge of these alterations may elucidate elements behind the positive effect on wound healing.

Previous strategies for unraveling the mechanisms of wound repair mechanisms have generally been based on evaluations of a single or a few cytokines per study. Proteomic analyses can provide a panoramic view of posttranslational expression and changes in steady-state protein levels in granulation tissue.

To ensure only studying de novo synthesized granulation tissue, an implant wound model - the expanded polytetrafluoroethylene (ePTFE) tube model - was combined with proteomics (Figure 1). The insertion of ePTFE tubes [13-17] is a well-established implant model of wound healing in humans and other mammals.

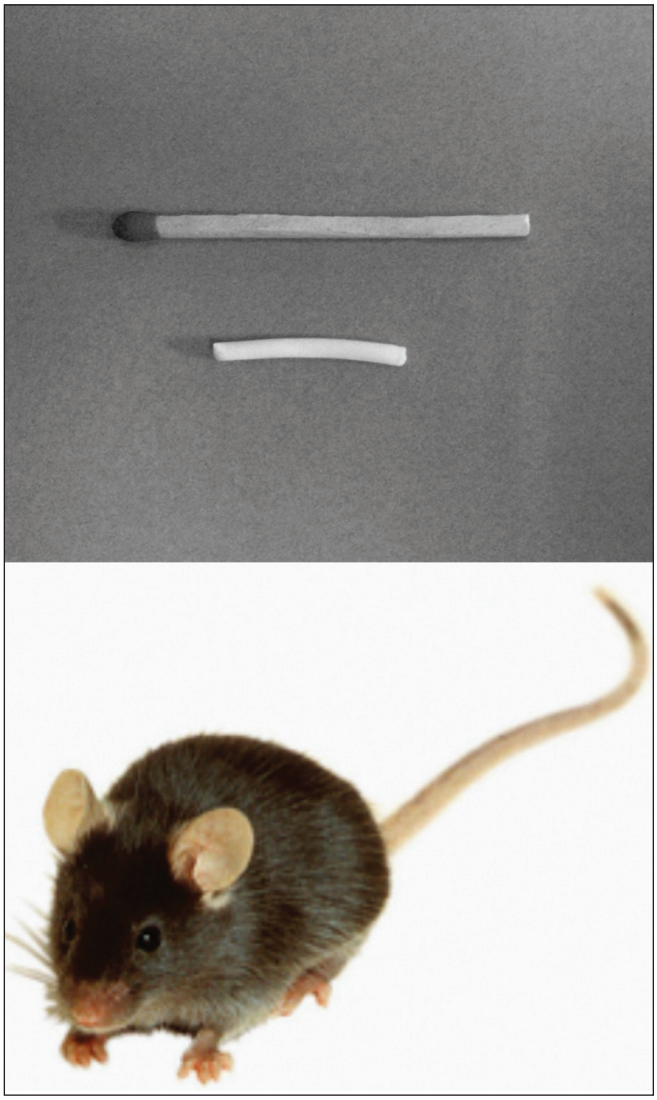

Figure 1. This figure shows the ePTFE tubes which were implanted in the subcutaneous tissue on each side of the mouse spine.

The aim was to study the influence on protein expression in an acute wound after treatment with subcutaneously injected recombinant human erythropoietin (rhEPO).

Material and Methods

Twelve C57bl6 mice (males and 8 weeks old) were studied. During the study the animals were housed one per cage and maintained under controlled environmental conditions with a 12-hour light/dark cycle. The temperature was kept at approximately 23°C, and the mice had free access to water and standard laboratory rodent food.

The Danish Animal Experiments Inspectorate approved the study, with all animal procedures being

done in accordance with institutional guidelines for care and use of laboratory animals.

For surgical procedures we used a mixed anesthesia of Ketamine and Xylazine of 10/1mg/ml and 0.1ml/10g bodyweight, respectively, based on 1 ml of Ketaminol vet, Intervet (50 mg/ml) and 0.25 ml of Rompun vet, Bayer (20 mg/ml) diluted in 3.75 ml of isotonic saline (9 mg/ml).

To relieve postoperative pain the mice were subcutaneously given buprenorphine of 0.05 mg/kg three times a day on the first 2 days after implantation of the tubes.

The expanded polytetrafluoroethylene (ePTFE) tubes (International Polymer Engineering, Arizona, USA) have an outer diameter of 2.5 mm, an inner diameter of approximately 1.2 mm, and a pore size of 90–120 μm. The tubes were threaded on a 4.0 nylon suture (Ethilon) and sterilized by autoclaving at 120°C for 30 min.

On both sides of the spine an incision of 5 mm was made; a cannula was introduced through the incision, passed through the subcutaneous tissue for 3 cm, and then brought out through the skin. The ePTFE tubes were implanted in the subcutaneously created tunnels. The wound edges were closed with discontinuous nylon 7.0 sutures (Figure 1).

Recombinant Human Erythropoietin, rhEPO, and saline

There is no therapeutically established level for the use of EPO in wound healing, and there are few data on dose response. In studies of the cytoprotective effect of EPO the applied amount of EPO ranges from 500 to 20,000 IU/kg as bolus injection, whereas in studies of EPO and wound healing there is a trend toward a dose of 500 IU/kg on a daily basis [5,8,18]. However, some, i.e., Kaemmer et al. in their study on the influence of EPO on colonic anastomotic healing, use a higher dose of 5000 IU/kg, which is applied 24 hours prior to surgery and again after surgery. In studies focusing on collagen synthesis, i.e., studies of collagen content and wound breaking strength, the effect has been shown on days 5–7 postoperatively [5,8,18] but not prior to day 5.

Based on these studies, we chose in this study a dosage of 1000 IU/kg/day and a termination day which was 9 days postoperative.

In the rhEPO-stimulated group (n=6) Eprex® (epoetin alpha) by Janssen-Cilag was diluted to a concentration of 200 IU/ml, giving an injection volume of 0.05 ml per 10 grams of bodyweight. RhEPO of 1000IU/kg bodyweight was administered by subcutaneous injections on a daily basis from the day the wounds were created.

In the control group (n=6), 0.9% saline was administered in the same fashion with an injection volume of 0.05 ml/10 g of bodyweight.

The hemoglobin (Hb) was determined prior to the first injection of either rhEPO or saline and again after nine days by sampling blood from the tail (Table 1).

The mice were weighed prior to the first injection of either rhEPO or saline and again after 9 days.

Tissue sampling and preparation

Most of the fibrous capsule was sheared off when the ePTFE tube was removed, with any excess tissue being removed under a stereomicroscope. The ePTFE tubes were stored at -20°C until analysis.

Proteomic analysis

The ePTFE tubes were homogenized in lysis buffer, and the supernatants were removed. Two-dimensional gel electrophoresis, image analysis and identification of differentially expressed protein spots by mass spectrometry were essentially performed, as previously described [19].

The dry and transparent gels were scanned in the transmissive mode on a GS-710 Imaging Densitometer from Bio-Rad (Hercules, CA, USA) using the Quan-

Table 1: Hemoglobin levels.

rhEPO group Hemoglobin, mmol/l		Control group Hemoglobin, mmol/l	
Day 0	Day 9	Day 0	Day 9
8.6	11.6	9.4	8.6
8.8	12.1	8.7	8.9
8.4	10.6	8.4	9
8.4	10.7	8.1	7.7
8.5	11	9.5	8.7
8.6	11.7	8.2	7.9
Mean 8.8	Mean 11.3	Mean 8.7	Mean 8.5

Hemoglobin (mmol/l) for each animal in the rhEPO-stimulated group and the control group on day 0 and day 9. On day 0, there is no difference in Hb between the two groups. On day 9, there is a significant rise in Hb (mean difference of 2.7 mmol) in the rhEPO-stimulated group to a mean of 11.3 mmol/l, compared to a slight fall to a mean of 8.5 mmol/l in the control group.

tity One software package. Gel images were exported as 16-bit grayscale TIFF files that were imported into the PDQuest 8.0 2D Analysis Software (Bio-Rad). After background subtraction the protein spots were automatically defined and quantified. Spot intensities were expressed as optical densities (OD) and were normalized against the total density in the gel image. One of the gels used in the analysis comparison was selected as a reference gel to which the other gels used in the analysis were aligned and matched using landmarks, as described in the manual. In the reference gel, each spot (feature) was assigned a number. The quality of the match made by the computer was critically evaluated in each case, and necessary editions and corrections were done manually.

Searches were performed in the SwissProt database release 56.0 or 2012_07. Searches were performed with a peptide mass tolerance of 20 or 50 ppm, a fragment mass tolerance of 0.05 Da, max missed cleavages of 1 or 2, and Carbamidomethyl (C) as fixed modification.

Western Blotting analysis

The protein concentrations were measured with a Non-Interfering Protein Assay (Geno Technology, Inc., St. Louis, MO, USA). Ten µg of each sample was loaded and run on 4–20% gradient polyacrylamide gels. After transfer by electroelution to nitrocellulose Hybond C-extra membranes, blots were blocked with 5% milk, 5% fetal calf serum, and 0.05% Tween-20 in 80mM Na2HPO4, 20mM NaH2PO4, and 100mM NaCl, pH 7.5 (PBS-T), and incubated overnight at 4°C with anti-triosephosphate isomerase (Everest Biotech, Oxfordshire, UK) diluted at 1:1,000 as well as Anti-GAPDH and Anti-non-neuronal enolase (Abcam, Cambridge, UK) diluted at 1:1,000. The labeling was visualized with horseradish peroxidase-conjugated secondary antibodies (P217, P260 or P449, DAKO, Glostrup Denmark, diluted at 1:1,000 to 1:5,000) by using the enhanced Chemiluminescence system (Amersham International).

Statistical analysis

The identity of the groups was blinded to the investigator through sample preparation, performance of 2D gel electrophoresis, selection of protein spots, and identification of proteins.

The protein spots were automatically defined and quantified using PDQuest 8.0 2D Analysis Software (Bio-Rad).

The statistical analysis of the western blot data was performed in Excel. The data were analyzed by a student's unpaired t-test. The level for significance was set at $p \leq 0.05$.

Results

The gels displayed more than 500 protein spots that were common to all tissue samples (Figure 2). The spots were distributed throughout the gels, ranging from 10–100 kDa.

To identify changes in protein expression between the two groups, only the well-defined spots were included in the analysis.

33 spots were found to differ significantly between granulation tissue isolated from the rhEPO-stimulated group and the control group. These 33 proteins were excised from the gels and analyzed with mass spectrometry.

Of these 33 spots, 6 proteins were successfully identified as Alpha-enolase (ENOA/ENO1), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Hemoglobin subunit alpha (HBA), Triosephosphate isomerase (TPIS/TIM), Serotransferrin (TRFE), and Transthyretin (Prealbumin, TTHY) (Table 2). Thirteen spots contained albumin, two spots contained actin, and 12 spots contained an insufficient quantity or quality of protein for identification.

The 13 spots containing albumin were downregulated in the EPO-treated group.

We selected three proteins all involved in the glycolytic pathway, which were well-defined and significantly different between the two groups, for further analysis: TPIS/TIM, ENOA/ENO1 and GAPDH (Figure 2). The three proteins were all downregulated in the EPO group. Verification of the selected proteins was performed with western blotting.

TPIS and ENOA were both confirmed as being significantly downregulated between the two groups with western blot, with a p-value < 0.001 for TPIS and p-value of 0.05 for ENOA (Figure 3).

The commercial antibody for western blotting and GAPDH was too unspecific to confirm our results.

Data for Hb are presented in table 1. There was no difference in Hb levels between the two groups on day 0. There was a significant rise in Hb, with a mean differ-

Table 2: Identification numbers, names and a summary of protein function .

Protein spot number	Identity	SWISS-PROT (primary accession number)	Gene	Functional association
9002	Hemoglobin subunit alpha	HBA_MOUSE	HBA	Involved in oxygen transport from the lung to the various peripheral tissues
6105	Triosephosphate isomerase	TPIS_MOUSE	TPIS	Aerobic glucose metabolism, gluconeogenesis, glycolysis
6304	Alpha-enolase	ENOA_MOUSE	ENOA	Multifunctional enzyme that, as well as its role in glycolysis, plays a part in various processes, such as growth control, hypoxia tolerance and allergic responses. Stimulates immunoglobulin production.
8003	Serotransferrin	TRFE_MOUSE	TRFE	Transferrins are iron binding transport proteins. They are responsible for the transport of iron from sites of absorption and hemodegradation to those of storage and utilization. Serum transferrin may also have a further role in stimulating cell proliferation.
8201	Glyceraldehyde-3-phosphate dehydrogenase	G3P_MOUSE	G3P	Glyceraldehyde-3-phosphate dehydrogenase is a key enzyme in glycolysis that catalyzes the first step of the pathway Also participates in nuclear events including transcription, RNA transport, DNA replication and apoptosis.
6002	Transthyretin (Prealbumin)	TTHY_MOUSE	TTHY	Thyroid hormone-binding protein.

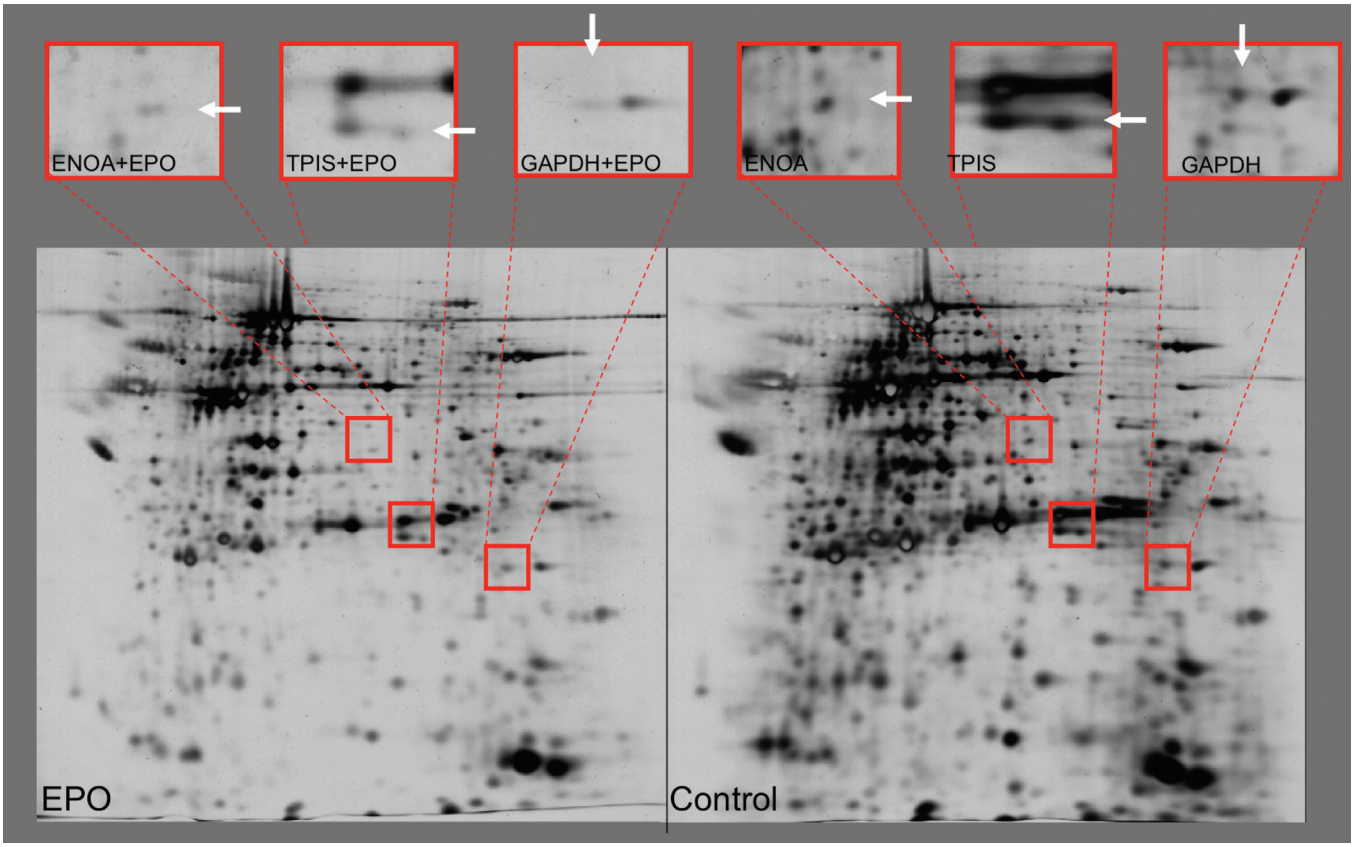

Figure 2. This figure shows a gel from the control group and the EPO group, with the arrows indicating the three proteins: ENOA, TPIS and GAPDH. The isoelectric point is on the x-axis, while the molecular mass is on the y-axis.

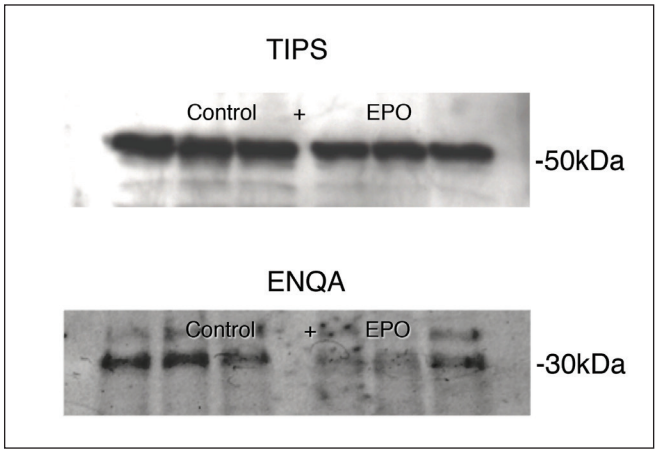

Figure 3. TPIS and ENOA were both confirmed as being significantly different between the two groups with Western blotting analysis.

ence of 2.7 mmol/l on day 9 in the rhEPO-treated group to a mean of 11.3 mmol/l compared with a mean of 8.5 in the control group. There was no change in weight of the animals during the experiment in either group.

Discussion

The ePTFE tube model is a well-established and validated implant model in both human and animal studies of wound healing [13,14,16,17]. In this study we found the ePTFE tube model to be excellent in providing de novo synthesized granulation tissue for proteomic analyses. Proteomics is a discovery-based analysis and is, therefore, suitable to analyze complex biological processes without prejudice on which specific proteins are involved in the processes.

The combination of the well-established ePTFE tube model and proteomic analysis enabled us to examine protein activity in granulation tissue during the time course of wound healing and to investigate alterations in protein expression under experimental settings, i.e., stimulation with rhEPO.

In this study, 33 proteins, isolated from granulation tissue, were found to be significantly downregulated in the rhEPO-treated group. Not all relevant proteins will be identified by the technique used in this study. This may be due to molecular weight and solubility coefficients; the detection range of this technique was between 10 kDa and 100 kDa. Also, the proteins may be less than 2-fold differentially expressed. We chose to use a cut-off level of 2; however, an up- or down-regulation of less than 2-fold might also represent important differences in the healing potential of a wound. Furthermore, relevant proteins may be present amongst those protein spots we failed to identify. Generally, a visible silver-stained spot contains sufficient amounts of protein to be identified. However, some proteins/peptides may give MS spectra of an insufficient quality for identification; moreover, if the sample has large albumin content, it can cloud smaller proteins. Thus, in practice only a part of the visible spots can be identified. Finally, there is a time course in the wound healing process, and other interesting/exciting differences may have been present earlier or will present later than day nine.

None of the identified proteins were growth factors or receptors. There is no apparent direct functional link between the observed alterations in protein expression, thus confirming that the influence of rhEPO on the early wound healing process is the result of several complex series of molecular events.

In several previous studies of EPO and wound healing, EPO has been shown to have a positive effect on protein synthesis regarding collagen [4-9]. Interestingly, we detected a downregulation of albumin in the group treated with rhEPO. This infers that the effect of EPO on protein synthesis is not a generalized stimulation.

Among the downregulated proteins, we selected three proteins (Figure 2), all involved in the glycolytic pathway, for further analysis: TPIS, ENOA and GAPDH. TPIS and ENOA were both confirmed as being significantly different between the two groups with western blot, whereas the antibody for GAPDH was too unspecific to confirm our results (Figure 3).

TPIS and GAPDH have a central role in glycolysis and gluconeogenesis. GAPDH catalyzes the conversion of Glyceraldehyde 3-phosphate (GAP), thereby enhancing the production of energy (ATP). Oxygen is not necessary to produce energy, but it enables pyruvate to be completely oxidized, thereby producing energy which is much more efficient and requires less GAPDH and TPIS/TIM.

ENOA encodes a 47 kDa protein which, besides its well-established role in glycolysis, has many other cellular functions and sub-cellular locations. GAPDH, ENOA and TPIS are well known in cancer research as hypoxia upregulated genes, and have been demonstrated to contain hypoxia-inducible transcription factor (HIF) response elements and to be upregulated by HIF and hypoxia [20].

These differentially expressed proteins can be categorized as proteins involved in the formation of energy compounds. Whether rhEPO by a direct mechanism changes metabolism and energy consumption in healing wounds or whether the downregulation of proteins involved in glycolysis is a consequence of yet other processes will need further studies. There is no doubt that the supply of oxygen for the granulation tissue is better in this study on day nine in the rhEPO-treated group. There is a significantly elevated level of Hb, and perhaps even more important is EPO improving neovascularization [4,9,10,12]. Thus, the presence of exogenous EPO and the elevated level of Hb will undoubtedly influence and lessen the hypoxia in the wound healing process, thus downregulating gene expression of hypoxia upregulated genes.

A strong up-regulation of a glycolytic enzyme might be associated with an increased glucose metabolism of wounded individuals. The up-regulation could also be explained by other as-yet-unknown functions of TPIS. As an example, ENOA, a glycolytic enzyme, is in fact a multifunctional protein that possesses additional extracellular functions besides the well-known intracellular function [21].

The application of the proteomic technology represents an important step in the process of elucidation of the multiple and dynamic alterations at work in the poorly understood influence of EPO on wound repair. We believe that this technology combined with the ePTFE tube wound model provides a new experimental approach to gauge the molecular alterations of the repair process in wounds treated with different agents aimed at accelerating the wound healing process.

In conclusion, EPO produces distinct and measurable changes in protein expression in granulation tissue. The ePTFE wound model combined with the proteomic technique could identify alterations in granulation tissue. The identified proteins were all downregulated in the EPO-treated group and involved in glycolysis. Presumably, hypoxia in the wound was diminished by the rhEPO treatment, resulting in a downregulation of proteins/enzymes involved in glycolysis.

Technologies such as proteomics are needed to advance both the science and practice of wound care, as wound providers are currently limited to external remedies that activate and advance the different phases of wound healing.

Future research applied to a larger cohort can further validate these data and further highlight and confirm characteristic patterns within wounds.

Acknowledgements

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Abbreviations

EPO	Erythropoietin
ENOA	Alpha-enolase
G3P	Glyceraldehyde-3-phosphate dehydrog nase
ePTFE tube	Expanded polytetrafluorethylene
HBA	Hemoglobin subunit alpha
rhEPO	Recombinant Human Erythropoietin
TPIS	Triosephosphate isomerase
TRFE	Serotransferrin
TTHY	Transthyretin (Prealbumin)

Conflict of interest statement

The authors have no conflicts of interest to declare.

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