Time to wound closure in trauma patients with disorders in wound healing is shortened by supplements containing antioxidant micronutrients and glutamine: A PRCT

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Original article

1. Introduction

Disorders in wound healing (DWH) are major complications in trauma patients associated with poor individual outcome. Dietary measures including micronutrient supplementation are discussed to improve the wound healing process, 1,2 but clinical data on causal relationships are lacking.

Antioxidant micronutrients, such as ascorbic acid, α-tocopherol and β-carotene, as well as cofactors of antioxidant enzymes, such as zinc and selenium, are attributed an important role in wound healing. 2–4 For example, ascorbic acid is mandatory for the formation of cross-links between collagen fibres, for fibroblast maturation, and for angiogenesis. 3 Retinol maintains the integrity of epithelial and mucosal surfaces and plays a role in fibroplasia. 25 Zinc is needed for the synthesis of the retinol binding protein which is required for retinol mobilization from hepatic stores 25 and for the formation of cross-links between collagen fibres. 2

Since injury generally leads to an increased formation of reactive oxygen species, an intra-/extracellular deficiency in these micronutrients resulting in oxidative stress may favour DWH. 7 Consequently, any measures to improve the antioxidative capacity in the injured body may be effective to avoid and/or treat DWH. In line

Abbreviations: APR, acute phase response; CRP, C-reactive protein; DWH, disorders in wound healing; MDA, malondialdehyde; ONS, oral nutritional supplement; SGA, subjective global assessment; TEAC, trolox equivalent antioxidant capacity; VEGF-A, vascular endothelial growth factor-A.

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with this hypothesis, adequate administration of antioxidant micronutrients like ascorbic acid and zinc has been proven to support regular wound healing in patients with pressure ulcer.\(^9\)

Glutamine is the major energy and nitrogen source for rapidly proliferating cells of the intestinal mucosa as well as for fibroblasts, epithelial cells, and leukocytes.\(^7\) Any intra-/extracellular deprivation of this amino acid, frequently observed in catabolic\(^10\) and post-traumatic\(^11\) situations, may further enhance disturbances in wound healing.

Based on these observations, we hypothesize that a daily oral supplementation with antioxidant micronutrients together with glutamine as energy and nitrogen donor prevents micronutrient and glutamine deprivation (primary aim) and, thus, improves wound healing (secondary aim) in trauma patients with DWH.

2. Materials and methods

2.1. Patients

In this mono-centre PRCT, adult trauma patients with DWH (defined as failure to heal, i.e. wound not closed or persisting secretion within ten days after trauma or surgery) were consecutively recruited between October 2007 and November 2008 at the Department of Orthopaedics and Trauma Surgery, University Hospital of Bonn. Exclusion criteria were: prescribed parenteral and enteral nutrition, current supplementation with vitamins/trace elements (questionnaire) and consumption of juices fortified with vitamins, multiple trauma, exclusive implant removal, pressure ulcers as primary diagnosis, HIV infection, chronic inflammatory bowel diseases, liver disease, drug abuse, pregnancy, lactation, intensive care unit stay, and sepsis. Main diagnosis and co-morbidities were obtained from the patient files. The injury severity score was determined according to Baker et al.\(^12\) All patients provided written, informed consent prior to enrolment. The study was conducted according to the Declaration of Helsinki 2004 and was authorized by the Ethics Committee of the University of Bonn (No. 123/07).

2.2. Intervention

Allocation to verum or placebo group was done by permuted-block randomization. Each block consisted of four patients (two patients per group) in a randomly selected order. Patients, physicians and nurses were blinded to treatment until data collection and analysis had been finalized. The patients assigned to the verum group were supplemented for fourteen days with two sachets of Glutamine Plus\(^7\) granulate (Fresenius Kabi, Bad Homburg, Germany) twice daily (\(2 \times 22.4 \) g) providing 500 mg ascorbic acid, 166 mg \(\alpha\)-tocopherol, 3.2 mg \(\beta\)-carotene, 100 \(\mu\)g selenium, 6.6 mg zinc, and 20 \(\mu\)g glutamine in addition to their hospital diet. The placebo group received isoenergetic sachets containing only maltodextrine (a tasteless carbohydrate; Dr. Steidle, Linden, Germany) in a double-blind manner. The patients were instructed to mix the complete content of the sachets with yoghurt, dessert or beverage and to eat or drink the enriched food immediately. Additionally, patients were asked to document the intake of the supplement in a diary.

All patients regularly received a protein-rich diet chosen from the diet catalogue provided by the hospital caterer. Juices fortified with vitamins and other micronutrients were excluded from the diet. Food intake was monitored on three days during the study using a self-completed standardized dietary record. The intake of energy, protein, ascorbic acid, \(\alpha\)-tocopherol, \(\beta\)-carotene, and zinc was calculated from the food records using commercial software (Ebis Pro 4.0, University of Hohenheim, Germany) based on the German Nutrient Data Base (Bundeslebensmittelschlüssel, BLS) version II.3.

2.3. Anthropometric data and general nutritional status

Body composition and general nutritional status were determined on d0 and d14. Patients were weighed and asked for their heights; body mass index was then calculated (kg/m\(^2\)) and classified (underweight: <18.5; normal weight: 18.5–24.9; overweight: 25.0–29.9; obesity: ≥30.0).\(^13\)

Calf and upper arm circumferences (cm) were measured twice and the triceps skin fold thickness (mm, GPM Switzerland) was measured in triplicate. General nutritional status and disease associated weight loss were determined by the Subjective Global Assessment (SGA) and classified as well-nourished (SGA A), moderately malnourished or suspected to be malnourished (SGA B), or severely malnourished (SGA C).\(^14\) The risk for malnutrition (categories: no risk, at risk, at high risk) was estimated by the Nutritional Risk Screening-2002.\(^15\)

2.4. Blood sampling

After an overnight fast, blood was drawn before and after intervention (d0 and d14, respectively) using tubes coated with EDTA or lithium heparin and free of anticoagulant. Plasma was immediately obtained by centrifugation and stored at −80 °C until analysis. Preparation of plasma samples for analysis of ascorbic acid and glutamine was done as described earlier.\(^16,19\) Heparinized plasma samples for malondialdehyde (MDA) and 8-isoprostan measurements were protected against lipid peroxidation by addition of 0.05% butylhydroxytoluol. All laboratory parameters, except for those investigated routinely, were analyzed in duplicate.

2.5. Nutrient status

The concentration of ascorbic acid in EDTA plasma was measured according to Steffan\(^17\) (CV 3.2%). \(\alpha\)-tocopherol, \(\alpha\)-carotene, and \(\beta\)-carotene were also determined by HPLC. The protocol of Erhardt et al.\(^18\) was modified by using apocarotenal as internal standard, Nucleosil\(^19\) 100-5 CN (Macherey–Nagel, Düren, Germany) as column and a solution of 98% hexane and 2% isopropanol as mobile phase. Retinol was detected at 325 nm (CV 2.7%), \(\alpha\)-tocopherol at 292 nm (CV 4.1%) and \(\beta\)-carotene at 450 nm (CV 3.5%). Vitamin E status is expressed as \(\alpha\)-tocopherol to cholesterol ratio.

Zinc was analyzed in heparinized plasma by photometry (CV 1.9%) and selenium by atom absorption spectrometry (CV 3%). Serum albumin was measured by nephelometry, prealbumin by radial immunodiffusion (CV 0.7%), and glutamine in heparinized plasma by HPLC using the OPA method (CV 1.5%).\(^19\)

Reference ranges published recently for retinol,\(^20\) ascorbic acid,\(^20\) \(\alpha\)-tocopherol,\(^21\) \(\beta\)-carotene,\(^20\) albumin,\(^22\) prealbumin,\(^23\) and glutamine\(^24\) are included in Table 3. The reference ranges for zinc, selenium (Table 3), and uric acid (Table 5) were obtained from the Department of Clinical Chemistry and Clinical Pharmacology, University of Bonn.

2.6. Metabolites and inflammatory markers

Leukocytes (flow cytometry), cholesterol (polychromatic measurement), bone specific alkaline phosphatase (immunoassay), C-reactive protein (CRP; nephelometry), interleukin-6 and interleukin-8 (both immunoassay), transferrin (photometry), and ferritin (immunoassay) were analyzed at the Department of Clinical Chemistry and Clinical Pharmacology and evaluated using actual reference values (for details see Table 3).
were measured by a non-invasive technique combining white light spectroscopy with the measurement of laser-Doppler shift in one flat probe (O2C; Lea Medizintechnik, Giessen, Germany) on days 0, 7, and 14. The laser was placed for 30 s horizontally on the surface of the wound (proximal, distal, 4–6 points lateral/medial). For each parameter, the mean of all measured values was calculated. Investigations were always carried out by the same examiner. Reliability and validity of this method had been reported previously.26,27

As an indicator of angiogenesis, vascular endothelial growth factor-A (VEGF-A) was determined in serum (ELISA; Quantikine, R&D Systems Europe, UK; CV 4.5% according to manufacturer).

Wound temperature was measured on d0, d7 and d14 by digital thermograms using a sterilized-cooled infrared scanning camera (VAIOSCAN 3201 ST, Jenoptic Laser, Jena, Germany; accuracy < ±2 K, resolution 0.03 K) and evaluated by the software IRBIS Plus V 2.2 (Infratec, Dresden, Germany). Clothes and bandages were removed 10 min before measurements. No infections or further cooling solutions were used.

Time to wound closure defined as the period between study entry and wound closure (criteria: completed medical treatment, no secretion, infection, and inflammation) was documented in the patient files or by inquiring from the subsequently medicating physician whether wounds were closed after discharge.

2.9. Statistics

Statistical evaluation was performed with the PASW software, version 17.0 (SPSS Inc., Munich, Germany). Non-parametric tests were used to investigate differences between the groups (Mann–Whitney-U test) and within each group (Wilcoxon test). Nominal and ordinal variables were compared by χ²-test and Fisher’s exact test. Changes in the number of patients with nutrients below the reference range were analyzed with the McNemar test. Statistical significance was assumed for P ≤ 0.05. Metric data are presented as median and quartiles.

2.10. Calculation of sample size

The calculation of the sample size was based on the assumption that the mean ascorbic acid concentration in plasma of 23 μmol/l (own results of a cross-sectional study with trauma patients suffering from disorders in wound healing) would increase to 50 μmol/l by supplementation of 500 mg/d ascorbic acid for 14 days. Considering a standard deviation of 16 μmol/l (own results from a cross-sectional study) and α = 0.05, an expected increase in ascorbic acid of 27 μmol/l would afford nine patients per group to achieve a power of 90%. Assuming a dropout rate of 10%, ten patients should be recruited for each group.

3. Results

Twenty Caucasian trauma patients were included and finished the study per protocol. Demographic, anthropometric and clinical data (Table 1) as well as energy and nutrient intake from the daily hospital diet (Table 2) were comparable in the placebo and the verum group. Patients consumed 27 ± 24–28 sachets in each group (compliance: 96%).

Except for a lower α-tocopherol/cholesterol ratio in the placebo group (P = 0.023), nutrient status was comparable in both groups at baseline (Table 3). In the verum group, only plasma concentrations of α-tocopherol (P = 0.007) and selenium (P = 0.009) increased and led to higher concentrations of α-tocopherol (P = 0.005) and selenium (P = 0.028) in the verum compared to the placebo group on d14. Albumin concentrations increased in both groups (verum:

Table 1

<table>
<thead>
<tr>
<th>Demographic, anthropometric, and clinical data at enrolment.</th>
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<tbody>
<tr>
<td>Sex male/female</td>
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<tr>
<td>Age (years)</td>
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<tr>
<td>BMI (kg/m²)</td>
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<tr>
<td>Triceps skin fold thickness (mm)</td>
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<tr>
<td>Calf circumference (cm)</td>
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<tr>
<td>Upper arm circumference (cm)</td>
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<td>Period between trauma and d0 (days)</td>
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Table 2

<table>
<thead>
<tr>
<th>Daily intake of energy and nutrients from food.</th>
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<tbody>
<tr>
<td>Placebo (n = 10)</td>
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<td>Energy (kJ)</td>
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<tr>
<td>(kcal)</td>
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<tr>
<td>(kJ/kg body weight)</td>
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<tr>
<td>(kcal/kg body weight)</td>
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<tr>
<td>Protein (g)</td>
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<tr>
<td>Protein (g/kg body weight)</td>
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<tr>
<td>Ascorbic acid (mg)</td>
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<tr>
<td>α-Tocopherol (mg)</td>
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<tr>
<td>(mg α-tocopherol equivalents)</td>
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<td>β-Carotene (mg)</td>
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</table>

Data: median [interquartile range]. No significant differences (P ≥ 0.05) between the groups according to Mann–Whitney-U test (metric data) or McNemar test (nominal data).
P = 0.017; placebo: P = 0.05). Glutamine decreased only in the placebo group (P = 0.047) (Table 3). At baseline, 15 out of 20 patients had ascorbic acid concentrations below the reference range (≤ 25 µmol/l), whereas all patients had an adequate plasma status of ω-tocopherol (> 12 µmol/l) with an ω-tocopherol/cholesterol ratio > 2.2 µmol/mmol, and retinol concentrations higher than 0.7 µmol/l. Thirteen patients had low values of β-carotene (< 0.9 µmol/l) on d0 and low zinc and selenium concentrations occurred before intervention in four and two patients, respectively. The number of patients with concentrations below the reference values was not different between the groups at d0. The concentrations of ascorbic acid and β-carotene did not change in both groups. Statistical evaluation for zinc and selenium concentrations occurred before intervention in four and two patients, respectively. The number of patients with concentrations below the reference values was not different between the groups at d0. The concentrations of ascorbic acid and β-carotene did not change in both groups. Statistical evaluation for zinc and selenium were not performed due to the low number of patients concerned.

Metabolites and inflammatory markers (Table 4) did not differ on d0 except for cholesterol which was higher in the placebo group (P = 0.026). In the placebo group, CRP (P = 0.037) and interleukin-8 (P = 0.033) decreased, while transferrin (P = 0.028) increased during the study period. In the verum group, interleukin-6 (P = 0.037) and ferritin (P = 0.013) decreased, while transferrin (P = 0.007) increased. Obviously, all patients of the verum group and 80% of the patients of the placebo group had CRP concentrations above the reference value before supplementation.

TEAC, peroxides, MDA, and 8-isoprostanes did not differ on d0 and d14. 8-isoprostanes decreased after supplementation with verum (P = 0.028) (Table 5).

Parameters on microcirculation of the wound and VEGF-A concentrations are shown in Table 6. At baseline, O₂-saturation (P = 0.013) and blood flow (P = 0.043) significantly differed between the groups. O₂-saturation diminished only in the placebo group (P = 0.043). No further changes occurred. VEGF-A was always comparable in both groups and did not change. Wound temperature (°C) did not differ between groups and was not affected by intervention (d0: 34.2 [31.3; 34.8] vs. 34.4 [32.0; 35.1]; d14: 33.9 [33.3; 34.5] vs. 34.2 [32.9; 34.6]).

Most importantly, time to wound closure was shorter in the verum (29 days [22; 52]) than in the placebo group (58 days [46; 92]) (P = 0.01). However, length of hospital stay was not influenced (placebo: 25 days [8; 35]; verum: 31 days [24; 55]).

### 4. Discussion

In line with our working hypothesis, our study could demonstrate that a high-dose oral supplementation with antioxidant micronutrients together with glutamine is an effective therapeutic measure in hospitalized trauma patients with DWH. After 14 days of intervention, time to wound closure was significantly shorter in the verum compared to the placebo group (Fig. 1). This clinically relevant observation was, however, not associated with a reduction in the length of hospital stay. Since pelvic/hip fractures are commonly associated with a longer hospital stay and a higher injury severity score, the higher percentage of patients with pelvic/hip fractures in the verum group compared to the placebo group may partly explain this result. Anthropometric and clinical characteristics (Table 1) as well as nutrient intake by hospital food (Table 2) and nutritional status (Table 3) did not differ between groups. Due to similar medical treatment, accelerated wound healing, thus, seems to be related to the additional intake of micronutrients and glutamine by the oral nutritional supplement (ONS).

The mechanisms behind this effect are, however, still unknown. Generally, low plasma concentrations of ascorbic acid, β-carotene, zinc, and albumin (Table 3) suggest a poor specific nutritional status, thereby confirming earlier results of a cross-sectional study.
in this patient group.\(^{28,29}\) Only the plasma status for selenium improved during intervention (Table 3); in addition, the number of patients with micronutrient concentrations below the reference range remained unchanged. Thus, changes in plasma concentrations of micronutrients cannot explain the accelerated WH observed.

A common phenomenon in patients with DWH is the acute phase response (APR)\(^{22,30–32}\) resulting in elevated levels of inflammatory markers like CRP, as also measured in our study (Table 4). APR is known to reduce the synthesis of albumin and prealbumin which are transporters of several circulating micronutrients.\(^{23–35}\) Thus, inflammation per se may reduce micronutrients in plasma/serum.\(^{2,16–18}\) In line with this assumption, we observed low levels of albumin and prealbumin which may explain the reduced levels of \(\beta\)-carotene and zinc. As suggested by Wilson et al.\(^{39}\) and Fukushima and Yamazaki, APR may also account for the lacking increase in plasma ascorbic acid (Table 3). Micronutrients ingested may have accumulated in the wound area where the metabolic requirements for cell growth and differentiation are probably increased. Such an effect has previously been reported for zinc.\(^{41}\) Under conditions of inflammation and infection, zinc was redistributed to organs with higher priority.

Plasma glutamine concentrations (Table 3) were within the reference range (655 ± 84 \text{mmol/l})\(^{42}\) throughout the study. Despite normal plasma levels, intracellular deprivation may occur.\(^{43}\) Thus, it can be speculated that supplementation of glutamine by our ONS did not only maintain plasma glutamine level (Table 3), but also increased intracellular glutamine availability, thereby supporting healing processes. Campos et al.\(^{44}\) suggested that glutamine improves the cellular utilization of \(\alpha\)-ascorbate by Escherichia coli. If this also applies to human cells, this mechanism may contribute to accelerated wound healing in our verum group (Fig. 1) indicating a beneficial effect of glutamine supplementation in patients with DWH.

An imbalance between pro- and antioxidants, the so-called oxidative stress, is suggested to favour cell damage\(^{45}\) and to enhance inflammatory processes.\(^{46}\) Indeed, biomarkers of pro-/antioxidant balance (Table 5) indicate a low level of antioxidant protection in our patients. TEAC reached only 60\% of values determined previously for young healthy adults by our group,\(^{47}\) and 8-isoprostane levels were twice as high compared to normal.\(^{47}\) The concentration of MDA in young healthy subjects was 14.3 ± 4.1 \text{mmol/l} (unpublished data from our laboratory), and thus, far lower than in the present study (Table 5). Only a decrease in 8-isoprostanes, a marker of lipid peroxidation, may be explained by the increase in \(\alpha\)-tocopherol (Table 3). The lack of similar changes in MDA (Table 5) is not a contradiction as MDA — in contrast to 8-isoprostane — is not a specific product of lipid peroxidation.\(^{48}\) In the verum group, peroxides did not change (Table 5) despite an increase of selenium (Table 3), the cofactor of glutathione peroxidase. Since correlations between the selenium concentration in serum and the activity of glutathione peroxidase in plasma occur only for selenium concentrations < 0.63 \text{mmol/l},\(^{49}\) an increase in glutathione peroxidase activity in the verum group is unlikely. As oxidative stress is discussed to impair wound healing,\(^{7,4}\) the reduction of oxidative stress indicated by reduced concentrations of 8-isoprostanes (Table 5) may also have favoured wound healing in the verum group.

Parameters of microcirculation measured directly by O2C are suggested as reliable measures predicting the course of wound healing processes.\(^{26,27}\) Unfortunately, O2-saturation and blood flow were different between groups on d0 (Table 6). Thus, the impact of the intervention on microcirculation can not be reliably evaluated. A major problem in this respect is the standardization of the measurement. Wound location, wound size, and micromovements during measurement often lead to non reproducible results.

VEGF-A, mainly produced by macrophages, muscle cells, and fibroblasts in the wounded tissue, is an indicator for angiogenesis and induces post-traumatic formation of new vessels.\(^{50}\) In our patients, the concentration of VEGF-A in serum did not differ between the groups at any time and no changes occurred (Table 6). Since VEGF-A levels in serum and plasma were much lower than in the wound fluid\(^{51}\) and the fracture hematoma,\(^{52}\) serum levels as measured in our study did not correctly mirror the situation in the wounded tissue. Analyses in the wound would have been desirable. However, this was not possible for ethical/medical reasons.

In trauma patients with DWH, inflammation and/or infection are associated with hyperemia. Thus, a decrease in wound

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<th>Table 5</th>
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<td><strong>Markers of pro-/antioxidant balance.</strong></td>
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<td>Placebo (n = 10)</td>
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<tr>
<td>TBARs (mmol TE/l)</td>
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<tr>
<td>Peroxides (mmol/l)</td>
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<tr>
<td>MDA (\text{mmol/l})</td>
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<tr>
<td>8-Isoprostanes (pg/ml)</td>
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<td>Uric acid (\text{mmol/l})</td>
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Data: median [quartiles]. d0: baseline; d14: after 14-day supplementation; TEAC: trolox equivalent antioxidant capacity; TE: trolox equivalents; MDA: malondialdehyde. No significant differences (\(P \leq 0.05\)) between the groups according to Mann–Whitney-U test occurred. Column P represents the results of the Wilcoxon test (changes between d0 and d14).

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<table>
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<th>Table 6</th>
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<td><strong>Parameters on microcirculation and vascular endothelial growth factor-A.</strong></td>
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<td>Placebo (n = 10)</td>
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<td>O2-saturation</td>
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<td>Relative haemoglobin</td>
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<tr>
<td>Blood flow</td>
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<td>VEGF-A (pg/ml)</td>
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</table>

Data: median [quartiles]. Parameters are given in arbitrary units if not indicated otherwise. d0: baseline; d14: after 14-day supplementation; VEGF-A: vascular endothelial growth factor-A. Letters indicate significant differences between the groups according to Mann–Whitney-U test: *\(P < 0.013\), \(\dagger\)\(P < 0.043\). Column P represents the results of the Wilcoxon test (changes within groups).
temperature is expected to occur during wound healing. In our study, wound temperature was measured by thermography which reflects local blood flow. Indeed, the maximum temperature was higher in the wounded area of the injured extremity than in the corresponding area of the non-injured extremity (34 °C and 28–30 °C, respectively). However, the temperature did not change over time in either group. Keeping in mind that the wounds of our study, wound temperature was measured by thermography which is higher in the wounded area of the injured extremity than in the non-wounded area.

In conclusion, our randomized and controlled study demonstrates for the first time that the supplementation of antioxidant micronutrients and glutamine is associated with an accelerated wound closure in patients with DWH. The underlying mechanism(s) remain debatable.

Conflict of interest statement
None declared.

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SCB, HC, PS, and SE contributed to the conception and the design of the study. SCB was responsible for data acquisition and performed statistical analysis. HG recruited the patients together with KK. RHT was responsible for the analysis of malondialdehyde and BSW for the analysis of routine laboratory parameter. RHT introduced SCB to the investigation of microcirculation by the O2C. CB was the clinical advisor. SCB, HG, PS, and SE interpreted the data and drafted the manuscript. All authors read and approved the final manuscript.

References