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Nutritional Status of Trauma Patients with

Disorders in Wound Healing –

Assessment and Effects of Nutrient Supplementation

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Disorders in wound healing (DWH) are severe post-surgical complications in trauma patients, unfortunately, the reasons being not completely understood. Wound related factors like infections, wound depth and localisation, as well as diabetes mellitus and vascular diseases may impair the tissue reformation. However, nutrition is hypothesised as an important factor for proper wound healing as micronutrients are directly involved in the healing process. The hypothesis of this thesis is that an inadequate status of antioxidant micronutrients may favour DWH. Reliable data on causal relationships, however, are lacking.

Within a cross-sectional study (CHAPTER ONE) the plasma/serum status of vitamins A, C, D, E, and β-carotene, albumin, prealbumin were determined in 44 trauma patients with DWH (wound not closed or persisting secretion within 10 days post surgery or trauma) and in 45 trauma patients with regular wound healing (RWH). Moreover, markers indicating an imbalance between pro- and antioxidant status (malondialdehyde (MDA), peroxides, trolox equivalent antioxidant capacity (TEAC) as well as C-reactive protein (CRP) were assessed. Interestingly, Vitamin C, vitamin E/cholesterol-ratio, beta-carotene, prealbumin, TEAC and peroxides were higher in patients with DWH than in patients with RWH. Most patients showed deficiencies in vitamin C, vitamin D, and β-carotene without differences between DWH and RWH. Only four patients with DWH and only one person with RWH had CRP concentrations within the reference range. Obviously, low values of several micronutrients are common in trauma patients with and without DWH and may be triggered by inflammation.

In a placebo-controlled randomized trial (CHAPTER TWO), 20 trauma patients with DWH were recruited. The verum group (V) received an oral nutritional supplement (Glutamine Plus, Fresenius Kabi, Bad Homburg, Germany) for 14 days providing 500 mg/d ascorbic acid, 166 mg/d α-tocopherol, 3.2 mg/d β-carotene, 100 µg/d selenium, 6.6 mg/d zinc and 20 g/d glutamine; the placebo group (P) received isoenergetic amounts of maltodextrine instead. Before (d0) and after (d14) supplementation, plasma/serum levels of the ingested micronutrients, CRP and vascular endothelial growth factor-A (VEGF-A) were analysed and the microcirculation (O_2-saturation, relative Hb, blood flow and velocity) of the wound was measured in 2 mm depth. The time to wound closing was recorded. At d0, the plasma status of micro-nutrients was comparable between the groups. No changes were observed in V and P, except for selenium which increased in V. Prevalence for hypovitaminosis did not change in any group. Glutamine decreased only in P. VEGF-A did not change in any group whereas CRP decreased in P. The O_2-saturation was lower in V than in P at d0. Except for the decrease in O_2-saturation in P, the microcirculation did not change. The time to wound closing (no secretion/inflammation/infection) was shorter in V than in P. The supplementation of antioxidant micronutrients and glutamine accelerates wound closing in trauma patients with DWH. Thus, the supplementation of antioxidative micronutrients and glutamine seems to be an effective dietary tool to improve wound healing.

In conclusion, the results of the cross-sectional and interventional study do not show associations between the micronutrient concentrations in plasma/serum, parameters of oxidative stress and the wound healing of trauma patients with DWH. However, marker of the nutritional status in plasma/serum have to be evaluated cautiously due to post-traumatic inflammation which lowers micronutrient concentrations directly or indirectly by reduced availability of transporter proteins. Since the supplementation of micronutrients and glutamine accelerated WH, tailored nutritional measures contribute effectively to wound treatment and, thus, should be implemented in daily clinical routine.

Wundheilungsstörungen (WHS) sind schwere postoperative Komplikationen bei unfallchirurgischen Patienten (UP), wobei die Ursachen noch nicht vollständig bekannt sind. Infektionen, Wundtiefe und -lokalisation betreffen die Wunde direkt und können ebenso wie Diabetes mellitus und vaskuläre Erkrankungen die Gewebeneubildung im Wundgebiet behindern. Es wird angenommen, dass die Ernährung ein wichtiger Faktor für eine regelgerechte Wundheilung (WH) ist, da Mikronährstoffe direkt am Heilungsprozess beteiligt sind. Die Hypothese dieser Dissertation ist, dass ein inadäquater Mikronährstoffstatus Störungen der Wundheilung begünstigen kann. Verlässliche Daten bezüglich der kausalen Zusammenhänge existieren bisher nicht.

In einer Querschnittstudie (CHAPTER ONE) wurden bei 44 UP mit WHS (offene Wunde oder persistierende Sekretion 10 Tage nach Unfall oder Operation) und bei 45 UP mit einer regelgerechten Wundheilung (RWH) die Plasma/Serum Konzentrationen (Konz.) von Vitamin A, C, D, E, β-Carotin, Albumin und Prealbumin bestimmt. Weiterhin wurden Malondialdehyd (MDA), Peroxide und trolox equivalent antioxidant capacity (TEAC), die ein Ungleichgewicht zwischen Pro- und Antioxidantien angeben, sowie C-reaktives Protein (CRP) analysiert. Die Konzentration von Vitamin C, Vitamin E/ Cholesterol Ratio, β-Carotin, Prealbumin, TEAC aber auch Peroxide waren höher bei Patienten mit WHS als mit RWH. Die meisten Patienten hatten einen Mangel an Vitamin C, Vitamin D und β-Carotin, wobei keine Unterschiede zwischen den Gruppen vorlagen. Bei nur vier Patienten mit WHS und einem mit RWH lagen die CRP Werte im Referenzbereich. Niedrige Mikronährstoffkonz. kommen bei UP mit WHS oder RWH offenbar häufig vor, wobei diese durch die Inflammation ausgelöst werden können.

In einer Placebo-kontrollierten und randomisierten Studie (CHAPTER TWO) wurden 20 UP mit WHS eingeschlossen. Patienten in der Verumgruppe (V) erhielten für 14 Tage ein orales Supplement (Glutamine Plus, Fresenius Kabi, Bad Homburg, Deutschland), das 500 mg/d Ascorbinsäure, 166 mg/d α-Tocopherol, 3,2 mg/d β-Carotin, 100 μg/d Selen, 6,6 mg/d Zink and 20 g/d Glutamin enthielt. Patienten der Plazebogruppe (P) wurden isoenergetische Mengen an Maltodextrin verabreicht. Vor (d0) und nach (d14) der Supplementation wurden die Plasma/Serum Konzentrationen der verabreichten Mikronährstoffe, CRP sowie vascular endothelial growth factor-A (VEGF-A) analysiert und die lokale Mikrozirkulation der Wunde (O_2-Sättigung, relatives Hämoglobin, Blutfluss und Blutgeschwindigkeit) in 2 mm Gewebetiefe gemessen. Die Zeit bis zum Wundverschluss wurde erfasst. Zum Zeitpunkt d0 waren die Plasmakonzentrationen der Mikronährstoffe zwischen den Gruppen nicht verschieden und bis auf Selen (gestiegen) in V änderten sie sich nicht. Ebenfall blieb die Anzahl der Patienten mit einer Hypovitaminose sowie VEGF-A in beiden Gruppen unverändert. Allerdings sanken die Glutamin- und CRP Spiegel in P. Die O_2-Sättigung war zum Zeitpunkt d0 in V geringer als in P, wobei diese in P später sank. Andere Parameter der Mikrozirkulation veränderten sich nicht. Die Zeit bis zum Wundverschluss (keine Sekretion/ Inflammation/ Infektion) war kürzer in V als in P. Demnach scheint die Supplementation von antioxidativen Mikronährstoffen und Glutamin eine effektive Ernährungsmaßnahme zur Verbesserung der Wundheilung zu sein.

Zusammenfassend kann gesagt werden, dass die Ergebnisse der Querschnitt- und Interventionsstudie keine Assoziation zwischen der Mikronährstoffkonzentration im Plasma/Serum, den Parametern des oxidativen Stresses und der Wundheilung bei UP mit WHS zeigen. Marker des Ernährungsstatus im Plasma/Serum müssen vorsichtig bewertet werden, da die posttraumatische Inflammation die Mikronährstoffkonzentrationen direkt oder indirekt über eine verringerte Verfügbarkeit an Transportproteinen erniedrigt. Da die Supplementation von Mikronährstoffen und Glutamin die WH beschleunigen, trägt eine zugeschnittene ernährungstherapeutische Maßnahme effektiv zur Wundbehandlung bei und sollte demnach in die tägliche klinische Routine aufgenommen werden.

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APR	Acute phase response
BMI	Body mass index
CV	Coefficient of variation
CRP	C-reactive protein
DNA	Desoxyribonucleic acid
DWH	Disorders in wound healing
ELISA	Enzyme linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
f	Female
m	Male
MDA	Malondialdehyde
NHANES	National health and nutrition examination survey
n.s.	Not significant
n	Sample size
NRS	Nutritional risk screening
ONS	Oral nutritional supplement
O_2	Oxygen
O2C	Oxygen to see
P	Placebo (group)
ROS	Reactive oxygen species
RWH	Regular wound healing
SGA	Subjective global assessment
TEAC	Trolox equivalent antioxidant capacity
VEGF	Vascular endothelial growth factor
V	Verum (group)
VAS	Visual analogue scale
WH	Wound healing
$25(OH)D_3$	25-Hydroxycholecalciferol

GENERAL INTRODUCTION

Disorders in wound healing (DWH) are severe post-surgical complications in trauma patients [1] and favour wound infections (approximately 130,000 per year in Germany) which essentially contribute to high morbidity and mortality rates, and prolong the length of hospital stay [2]. Thus, DWH represent a significant individual burden and a challenge for the health care system.

Wound healing (WH) is generally divided into four phases with their specific physiological functions which overlap in time: '1) hemostasis (vascular constriction, platelet aggregation, degranulation, and fibrin formation), 2) inflammation (leucocyte infiltration and the differentiation to macrophages), 3) tissue formation (reepithelialization, angiogenesis, collagen synthesis, formation of the extracellular matrix), and finally 4) tissue remodelling (collagen remodelling and vascular maturation) [3, 4]'. For proper WH, this complex process must elapse precisely timed and in a regulated progression with an optimal intensity in each stage [4]. Several local and systemic factors are identified to impair WH, activate one another and thus, can lead to DWH *(Table 1)*. In general, local factors characterize the wound directly (eg. infection, wound type, depth or localisation) whereas systemic factors address health/disease/nutritional status, age, and lifestyle of the patients.

Table 1: **Factors affecting wound healing (modified according to Guo and DiPietro 2010[4])**

Local factors	Systemic factors
Type of wound/grade of soft tissue trauma	Main diagnosis/severity of disease
Wound depth	Diseases/cormorbidities: diabetes mellitus, keloids, fibrosis, hereditary healing disorders, skin diseases (e.g. atopic dermatitis), jaundice, uremia
Wound localisation	Medications: glucocorticoid steroids, non-steroidal anti-inflammatory drugs, chemotherapy
Wound contamination/infection	Immune-compromised conditions: cancer, radiation therapy, AIDS
Necroses	Nutritional status: mal-/undernutrition, overweight/obesity
Vascular diseases	Oxidative stress
Tissue oxygenation	Smoking, drug and alcohol abuse
pH-value of the wound	Age

Below, commonly seen factors which impair WH are shortly introduced. A major challenge in the therapy of wounds is to inhibit and avoid tissue necrosis as it leads to infections. The higher the grade of the soft tissue trauma, depth of the wound, and the location of the injury in peripheral areas, the higher is the risk for infections. Local bacterial growth is favoured by local hypoxia and diminished supply

of needed nutrients and vice versa in terms of a vicious circle [1]. In general, vascularisation and proliferation of new cells are impaired by decreased microcirculation in the wound area, diminished local perfusion [5], and oxygenation of the tissue. Thus, local tissue hypoxia plays a major role in the development of DWH. Smoking and diabetes mellitus are both associated with tissue hypoxia that lead to impaired proliferation and tissue remodelling by reducing the synthesis of collagen and hydroxyproline in case of smoking. As a consequence, smoking post-surgery is accompanied with DWH [6], limited elasticity of the wound [7] and formation of necroses [8, 9].

Major complications in patients with diabetes mellitus are severe diabetic foot ulcers which may lead to minor amputations of the foot or even major amputation of the leg [10]. Constant hyperglycemia damages vessel epithelia and may finally induce local hypoxia by capillary stenosis and lead to neuropathy and an impaired function of leukocytes [11, 12]. Thus, the adjustment of blood glucose levels [13] and HbA1c-level [10] are crucial in the treatment of chronic wounds of patients with diabetes mellitus. In general, patients with a compromised or deranged immune system due to cancer, radiation therapy [14], HIV/AIDS [15], or drug abusus [16] are at high risk for DWH since leukocytes function and the whole immune system are severely impaired. However, acetylsalicylic acid and ibuprofen as nonsteroidal anti-inflammatory drugs decrease collagen production probably by impaired prosta-glandin metabolism [11, 14]. Indeed, cortisone is a very potent drug against excessive inflammation but may inhibit severely WH by impairing fibrogenesis, angiogenesis, collagen synthesis, wound contraction and migration of epithelial cells [14, 17, 18].

As a further factor for DWH, age is commonly discussed since chronic wounds mainly occur in elderly patients. It is known that WH is altered in the elderly [19] but the recent consensus is that the wound closure is retarded 'but the final result is qualitatively similar to that in young people' [19]. Thomas [20] concluded that DWH in the elderly are more often related to age-related comorbidities like diabetes mellitus rather than to age alone. Thus, an effective treatment of DWH requires a detailed insight of the patients´ anamnesis, a comprehensive training of the physicians and a multilateral approach.

Integral parts of the modern wound therapy are: 1) radical debridement as the most common surgical measurement to eliminate wound necroses and debris, 2)

wound adapted care giving with different types of dressings and disinfection, and 3) systemic and/or local antibiosis [21]. Recently, the negative pressure wound therapy and skin grafting are implemented frequently to treat severe and chronic wounds [22, 23]. These techniques target local factors to revitalize the injured tissue and to minimize contamination and infections. In general, the treatment of diseases and comorbidities as well as the education about a detrimental life style is implemented in the therapy.

As previously addressed, severe illness, comorbidities, medication and metabolic stress have a huge impact on WH but also on the nutritional status of the patients. Since side effects of trauma and therapy like vomiting, nausea and decreased appetite significantly affect the nutritional intake of inpatients. Also high weight loss due to related illnesses leads frequently to under- and malnutrition. In consequence, low energy, protein and micronutrient intake favour protein-energy malnutrition and micronutrient deprivation which are discussed to impair cellular repair function and reduce plasma concentrations of transport proteins for micronutrients. Thus, adequate supply of energy, protein and selected micronutrients such as ascorbic acid, retinol, zinc, selenium is discussed to support proper WH. This leads to the question whether specific dietary measures may improve the WH process. The first hints that inadequate nutrition impairs WH originated from sailors in the 18th and 19th century. In 1740, a ship's surgeon observed how old wounds "in the progress of the disease broke out afresh" and hypothesized that fresh fruits and vegetables would cure scurvy [24]. Sailors who obtained the fruits and vegetables recovered quickly from scurvy and previously unhealed sores, in contrast to those who did not [25]. Nowadays, scurvy is known to result from a severe deficiency of ascorbic acid which is essential for fibroblast maturation, angiogenesis, and collagen synthesis [26]. WH is a complex process involving blood cells, extracellular matrix, and parenchymal cells [3, 27]. This process is orchestrated by enzymes and soluble mediators that usually depend on cofactors like vitamins or trace elements [28]. Retinol maintains the integrity of epithelial and mucosal surfaces and enhances fibroplasia that increases collagen synthesis and epithelialization [18, 29]. Zinc, a cofactor for enzymes like matrix metalloproteinases, is required for cell division, differentiation, growth as well as cross-linking of collagen fibres [30, 31]. Furthermore, zinc is essential for retinol transport in blood and for the oxidation of retinol to retinal [29]. Glutamine, an essential amino acid in stress conditions, appears to play an important role in WH as

a major metabolic fuel for rapidly proliferating cells such as enterocytes, fibroblasts, lymphocytes, epithelial cells, and macrophages [32]. Macrophages release nitric oxide and oxygen radicals which are antimicrobial and exert oxidative stress. Reactive oxygen species (ROS) play an outstanding role in coagulation, inflammation, and reepitheliazation as second messengers for inducing vascular endothelial growth factors (VEGF) [33]. However, ROS may also impair WH. An overproduction of ROS may inactivate epidermal enzymatic antioxidants which prolongs the inflammatory phase and delays or even inhibits the transition to tissue formation. Thus, oxidative stress is assumed to contribute to DWH [33-35]. Micronutrients with antioxidant properties (e.g., ascorbic acid, α-tocopherol, β-carotene; zinc and selenium as cofactors for antioxidant enzymes) may reduce oxidative stress. Hence, they are discussed to enhance WH [36-39] in addition to their physiological role in WH.

Although nutrition in general seems to be crucial for proper WH, an adequate nutritional therapy is often not implemented in the treatment of non-healing wounds in trauma patients. This situation may be due to a missing nutritional education of the physicians in the medical professional training and certainly a lack of well-designed studies in trauma patients with DWH and, thus, evidence for efficacy. Hence, knowledge about specific nutritional deficiencies is a prerequisite for investigating the efficacy of a tailored nutritional supplementation in these patients.

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PURPOSE OF THE THESIS

As a consequence of the lacking data, this thesis tries to answer the following questions:

1) Is there a difference in nutritional status and parameters of pro-/ antioxidant balance between patients with regular and delayed wound healing?

2) Is the prevalence of deficiencies in specific micronutrients between patients with regular and delayed wound healing different?

3) Is it possible to prevent trauma patients with DWH from hypovitaminoses by administration of a supplement rich in antioxidant micronutrients (vitamin C, E, β-carotene, zinc, selenium) and glutamine?

4) Does the ingestion of this supplement improve the wound healing in these patients?

5) Does the ingestion of this supplement influence parameters of oxidative stress in these patients?

To approach these goals, two clinical studies were performed:

The cross-sectional study (CHAPTER ONE) aims to answer question 1) and 2) by investigating the nutritional status as well as parameters of pro-/ antioxidant balance in adult trauma patients with regular and delayed wound healing, respectively.

In a randomised, placebo controlled and double-blinded clinical trial (CHAPTER TWO), the questions 3) to 5) are tried to be answered by investigating the effect of a daily ingestion of an oral supplement rich in antioxidant micronutrients (vitamin C, E, β-carotene, zinc, selenium) and glutamine for 14 days in adult trauma patients with DWH.

CHAPTER ONE

Extracellular micronutrient levels and markers of pro-/antioxidant status in trauma patients with wound healing disorders: a cross-sectional study

Abstract

Background: Disorders in wound healing (DWH) are common in trauma patients, the reasons being not completely understood. Inadequate nutritional status may favour DWH. Reliable data, however, are lacking.

Methods: Within a cross-sectional study, the plasma/serum status of vitamin A, C, D, E, β-carotene, albumin, prealbumin, C-reactive protein, and cholesterol was determined in 44 trauma patients with DWH and in 45 trauma patients with regular wound healing (RWH).

Results: Vitamin C (23.1±15.9 vs. 16.3±12.8 µmol/l), vitamin E/cholesterol ratio (9.8±3.2 vs. 5.6±1.9 µmol/mmol), β-carotene (0.6±0.4 vs. 0.4±0.3 µmol/l), and prealbumin (24.8±8.2 vs. 21.3±8.1 mg/dl) were significantly higher in patients with DWH than in patients with RWH. Most patients showed levels of vitamin C (<25 µmol/l; 64 vs. 83%), vitamin D (<50 µmol/l; 59 vs. 70%), and β-carotene (<0.9 µmol/l; 86 vs. 93%) below the reference range without differences between DWH and RWH. Only four patients with DWH and only one person with RWH had C-reactive protein concentrations within the reference range (0 to <3 mg/l).

Conclusions: Trauma patients with RWH and DWH suffer frequently from protein malnutrition and reduced plasma concentrations of several micronutrients probably due to inflammation, increased requirement, and oxidative burden. Thus, adequate nutritional measures are recommended to trauma patients.

Introduction

Disorders in wound healing (DWH) are frequently observed in post-surgical patients with vascular diseases and soft tissue trauma [1]. DWH are associated with a prolonged hospital stay and essentially contribute to high morbidity and mortality rates [2]. Thus, apart from the individual burden, DWH generate enormous costs in the health care system.

The pathophysiological mechanisms leading to DWH are not completely understood. Recent studies in patients with pressure ulcers [3] now support the hypothesis that general protein/energy malnutrition can considerably increase the risk for DWH by several mechanisms. The lack of energy and nitrogen containing metabolites like amino acids may hamper wound healing by diminishing the body's capacity for cell repair. Low food/energy intake is often related to an insufficient provision of essential micronutrients. It is well-known that several vitamins and trace elements like retinol, ascorbic acid, 25-hydroxycholecalciferol (25(OH)D$_3$), and zinc are involved in collagen synthesis, cell division, and in epithelialization [4-7]. A nutritive intake below actual recommendations published for healthy subjects may, thus, increase the risk to develop DWH or even aggravate DWH already existing. Moreover, it is still under debate whether in trauma the metabolic needs for micronutrients are considerably higher compared to physiological conditions [3]. An insufficient intake of micronutrients may lead to intra-/extracellular deficiencies resulting in an imbalance between pro-/ and antioxidants which exerts cytotoxic effects and, consequently, may impair wound healing as shown in a small patient group for selenium [8]. Representative cross-sectional studies focusing on the assessment and evaluation of general and specific nutritional status in a variety of patients with DWH are, however, scarce.

The aim of this cross-sectional study was, thus, to assess the general nutritional status, the micronutrient profile and the concentration of selected biomarkers of pro-/antioxidative balance in trauma patients with or without DWH within a routine clinical setting.

Materials and Methods

Patients

Following a mono-centre cross-sectional design, adult trauma patients were recruited within two time periods (May - December 2006 and October 2008 - January 2009) at the Department of Orthopaedics and Trauma Surgery, University of Bonn. Exclusion criteria were defined as follows: parenteral and enteral nutrition, exclusive implant removal, pressure ulcers as primary diagnosis, HIV infection, chronic inflammatory bowel diseases, liver diseases, drug abuse, pregnancy, lactation, stay in the intensive care unit, and sepsis. After enrolment, data on main diagnosis, comorbidities, and on medication were obtained and the individual injury severity score [9] was determined. The time between trauma/surgery and enrolment was documented. Wound healing progress was then evaluated using clinical criteria: DWH was diagnosed if the wound was not closed or secretion persisted within ten days after surgery or trauma. All patients received hospital food provided by an external caterer. Patients were asked whether they supplemented vitamins, zinc and/or selenium.

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 local Ethics Committee (No. 029/06).

Blood sampling

At the day after enrolment, blood samples (EDTA-, lithium heparinized tubes and tubes without anticoagulant) were collected after an overnight fast. Plasma and serum were obtained within two hours by centrifugation at 2,000 x g, 4 °C for 10 min. EDTA-plasma for ascorbic acid analysis was stabilized with 0.75 M metaphosphoric acid, centrifuged at 13,000 x g and the supernatant was discarded. Heparinized plasma for malondialdehyde (MDA) was protected against lipid peroxidation by addition of 0.05% butylhydroxytoluol. All samples were stored at -80 °C until analysis. Laboratory parameters, except for those investigated routinely, were analyzed in duplicate.

Clinical chemistry

Leukocytes (flow cytometry, Sysmex, Norderstedt, Germany), C-reactive protein (CRP; nephelometry, Siemens Healthcare Diagnostics, Eschborn, Germany), and

cholesterol (polychromatic measurement, Siemens Healthcare Diagnostics, Eschborn, Germany) were analyzed by routine clinical chemistry. The reference value for CRP was obtained from the Department of Clinical Chemistry and Clinical Pharmacology, University of Bonn.

Anthropometric data and nutritional status

Body mass index (BMI, kg/m^2) was determined by weighing the patients and asking for their height and was evaluated according to the criteria of the International Obesity Task Force 1998 (underweight: <18.5; normal weight: 18.5 – 24.9; overweight: 25.0 – 29.9; obesity: ≥ 30.0). The calf and upper arm circumferences (cm) were measured in duplicate. Reference values for calf and upper arm circumferences (sex- and age-dependent) were taken from the NHANES study [10]; patients with values below the 10th percentile were categorized as malnourished (*Table 2*). The general nutritional status and the disease-associated weight loss were determined by the Subjective Global Assessment (SGA) [11]. Patients were classified as well-nourished (SGA A), moderately malnourished or suspected to be malnourished (SGA B) or severely malnourished (SGA C).

Plasma concentrations of retinol (CV 4.3%), ascorbic acid (CV 3.2%), α-tocopherol (CV 4.1%), and β-carotene (CV 3.2%) were measured in EDTA-plasma by HPLC [12, 13]. 25(OH)D$_3$ was analyzed by ELISA (CV 5.6% according to manufacturer; IDS, Frankfurt/Main, Germany). Vitamin E status was determined as α-tocopherol to cholesterol ratio. Zinc was measured in heparinized plasma by photometry (CV 1.9%; Wako Chemicals, Neuss, Germany) and selenium by atom absorption spectrometry (CV 3%; biosyn, Fellbach, Germany). Albumin was analyzed by routine clinical chemistry (Department of Clinical Chemistry and Clinical Pharmacology, University of Bonn). Prealbumin was determined by radial immunodiffusion (CV 0.7%; The Binding Site, Schwetzingen, Germany). Reference ranges published recently for retinol [14], ascorbic acid [14], α-tocopherol [15], β-carotene [14], albumin [16], and prealbumin [17] are included in *Table 3* The reference ranges for zinc and selenium (*Table 3*) were obtained from the Department of Clinical Chemistry and Clinical Pharmacology, University of Bonn.

Markers of pro-/antioxidant balance

The trolox equivalent antioxidant capacity (TEAC) assay [18] was used to determine antioxidant capacity (CV 1.2%) in EDTA-plasma. Malondialdehyde (MDA) [19] was measured in plasma by photometry (CV 7.4%). An ELISA kit was used to determine the concentration of peroxides (Dr. Franz Tatzber KEG, Bisamberg, Austria; CV 4.3% according to the manufacturer) in EDTA-plasma. Uric acid was analyzed in serum by photometry (Siemens Healthcare Diagnostics, Eschborn, Germany). Normal values for TEAC, peroxides, and MDA were obtained by analyzing plasma/serum of healthy subjects from the staff (*n* = 19 - 89) (*Table 4*).

Evaluation strategy and statistics

The Student´s t-test was used to compare metric variables between patient groups with regular wound healing (RWH) and with DWH if data were normally distributed. The Mann-Whitney-U test was used instead if normal-distribution was either lacking or uncertain. For the comparison of nominal and ordinal variables between the groups, the χ^2-test and Fisher´s Exakt test were used. Correlations between CRP, albumin and prealbumin, and selected micronutrients were analyzed by the Pearson`s test. Statistical significance was assumed for *P*<0.05. PASW software, version 17.0 (SPSS Inc., Munich, Germany), was used for statistical evaluation. Results are presented as means ± SD and as median and quartiles, respectively.

Results

Patients

Within the two time periods, out of 89 patients fulfilling the inclusion criteria, 44 showed DWH based on the predefined clinical criteria. The demographic data in DWH and RWH patients were comparable *(Table 1)*. Compared with DWH, patients in the RWH group had more often soft tissue traumata ($P<0.001$). The time between trauma and study entry ($P=0.003$) and the length of hospital stay ($P<0.001$) was longer, and the prevalence for infections ($P<0.001$) and vascular diseases ($P<0.001$) was higher in patients with DWH than RWH *(Table 1)*. Infections of osteosynthesis material occurred in five and methicillin-resistant *Staphylococcus aureus* infection in three patients with DWH, respectively. Two patients with DWH suffered from osteomyelitis.

Clinical chemistry

Leukocyte counts (DWH: 8.1 ± 2.2 G/l vs. RWH: 7.3 ± 2.4 G/l) and CRP concentrations (DWH: 21.2 [6.4; 88.7] mg/l vs. RWH: 42.4 [17.7; 83.1] mg/l) did not differ between the groups. Only four patients with DWH and one patient with RWH had CRP concentrations within the reference range (0 to <3 mg/l). Uric acid concentration was higher in patients with DWH (280 ± 85 µmol/l) than in patients with RWH (240 ± 91µmol/l; $P=0.045$).

Body composition and nutritional status

As shown in *Table 2*, BMI as well as calf and upper arm circumference were not different between the two groups. Five patients of both groups were underweight. Overweight was observed in 31% of the patients with DWH and in 48% with RWH, respectively, and 21% and 14% of the patients with DWH and RWH were obese. Values for calf circumference below the 10th percentile were noticed in 24% of the patients with DWH and in 14% of the patients with RWH, respectively. An upper arm circumference below the 10th percentile was identified in 19% of the patients with DWH and in 4% of those with RWH. In patients with DWH and RWH, the prevalence of general malnutrition (SGA B/C) was 55% and 49%, respectively. Significant weight loss (> 10% of weight within six months) was observed in 18% of the patients with DWH and in 2% of those with RWH ($P=0.001$). According to SGA classification,

15 (DWH) and 21 (RWH) patients were at risk for malnutrition; 9 patients with DWH were judged as severely malnourished (SGA C), and only one in RWH group.

Patients with RWH had lower plasma concentrations of ascorbic acid (P=0.035), a lower α-tocopherol/cholesterol ratio (P<0.001), and lower levels of β-carotene (P=0.005) but higher concentrations of selenium (P<0.001) *(Table 3)*. Prealbumin (P=0.047) was higher in patients with DWH than in RWH. The concentrations of retinol, 25(OH)D_3, zinc and albumin were not different between the groups. However, as shown in *Figure 1*, the prevalence of micronutrient concentrations below the actual reference ranges was not different between patients with DWH and RWH except for selenium (P<0.001) where low values occurred more often in patients with DWH than RWH.

The prevalence of low plasma values of pro-/vitamins, selenium, zinc, as well as albumin and prealbumin was not different between generally well-nourished (SGA A) and malnourished (SGA B/C) DWH and RWH patients (data not shown).

As expected, there was an inverse correlation between CRP and albumin (r = -0.44, *P*<0.01) as well as between CRP and prealbumin (r = -0.486, *P*<0.01). Retinol (r = -0.533, *P*<0.001) ascorbic acid (r = -0.208, *P*<0.005), zinc (r = -0.567, *P*<0.001), and selenium (r = -0.371, *P*<0.01) were negatively correlated with CRP.

Markers of pro-/antioxidant balance

Results on TEAC, peroxides, and on MDA are shown in *Table 4*. TEAC and peroxides were lower in patients with RWH than with DWH (for both parameters, *P*<0.001). Due to technical problems, data on MDA of patients with RWH are lacking. Uric acid was even higher in patients with DWH (4.4 [3.8; 5.5]) than with RWH (3.7 [2.9; 4.7], *P*=0.017). In patients with either DWH (*P*=0.005) or RWH (*P*<0.001), TEAC as well as the concentration of peroxides (DWH: *P*<0.001; RWH: *P*<0.001) were lower than values known for healthy adults. MDA was higher in patients with DWH (*P*<0.001) compared to reference values.

Table 1: **Demographic and clinical data**

	DWH (*n* = 44)	RWH (*n* = 45)	*P*
Sex (male/female) [*n*]	29/15	23/22	n.s.*
Age [years]	60 ± 21	62 ± 17	n.s.[#]
Age (<65/≥65 years) [*n*]	22/22	22/23	n.s.*
Period between trauma and study entry [d] [a]	26 [15; 68]	15 [12; 20]	0.003˜
Main diagnosis [*n*]			<0.001*
Spinal trauma	–	5	
Long bone fracture	10	22	
Joint fracture	14	9	
Hip/Pelvic fracture	6	3	
Miscellaneous injuries	14	8	
Injury severity score	14 ± 8	10 ± 9	n.s.[#]
Soft tissue trauma [*n*]			
None	29	11	<0.001*
Grade I°/ II°/ III°	5/4/2	20/10/1	
Infections [*n*]	30	–	<0.001*
Comorbidities [*n*]			
Vascular diseases	13	–	<0.001*
Diabetes mellitus	7	4	n.s.*
Atopic dermatitis	3	1	n.s.*
Cortisone intake [*n*]	7	2	n.s.*
Current intake of supplements [*n*] (vitamins, minerals, combinations) [b]	4	29	<0.001*
Smoker [*n*]	12	9	n.s.*
Length of hospital stay [d] [a]	29 [21; 67]	15 [10; 21]	<0.001˜

DWH: disorders in wound healing; RWH: regular wound healing; data: means ± SD; [a] median [quartiles]; [b] ingested by own decision; Group comparison: * χ^2-test, [#] Student´s t-test; ˜ Mann-Whitney-U test; n.s.: not significant

Table 2: **Anthropometric data**

	DWH (*n* = 44)	RWH (*n* = 45)	Reference range
BMI [kg/m^2]	25.5 ± 5.3	25.7 ± 6.6	18.5 – 24.9
Calf circumference [cm]	34.8 ± 5.1	36.0 ± 4.7	> 10th percentile
Upper arm circumference [cm]	30.5 ± 4.9	31.6 ± 3.7	> 10th percentile

DWH: disorders in wound healing; RWH: regular wound healing; data: means ± SD
Differences between the groups were not detected (Student's t-test).

Table 3: **Plasma protein and micronutrient concentrations**

	DWH (*n* = 44)	RWH (*n* = 45)	*P*[#]	Reference range
Retinol [µmol/l]	1.4 ± 0.7	1.2 ± 0.5	n.s.	0.7 – 1.75
Ascorbic acid [µmol/l]	23.1 ± 15.9	16.3 ± 12.8	0.035	25 – 85
25(OH)D$_3$ [nmol/l]	46.2 ± 30.6	44.6 ± 18.7	n.s.	50 – 175
α-Tocopherol/cholesterol [µmol/mmol]	9.8 ± 3.2	5.6 ± 1.9	<0.001	> 2.2
β-Carotene [µmol/l]	0.6 ± 0.4	0.4 ± 0.3	0.005	0.9 – 4.6
Zinc [µmol/l]	12.4 ± 3.2	12.6 ± 3.7	n.s.	11.5 – 19.4
Selenium [µmol/l]	0.79 ± 0.19	1.10 ± 0.24	<0.001	> 0.94
Albumin [g/dl]	32.8 ± 8.5	32.1 ± 5.7	n.s.	age-related [$]
Prealbumin [mg/dl]	24.8 ± 8.2	21.3 ± 8.1	0.047	15 – 36

DWH: disorders in wound healing; RWH: regular wound healing; data: means ± SD; 25(OH)D$_3$: 25-hydroxycholecalciferol; Reference range: [$] albumin [g/l]: 35–53 (≤ 60 yrs), 34–48 (> 60 yrs), 33–47 (> 70 yrs), 31–45 (> 80 yrs); Group comparison: [#] Student`s test; n.s.: not significant
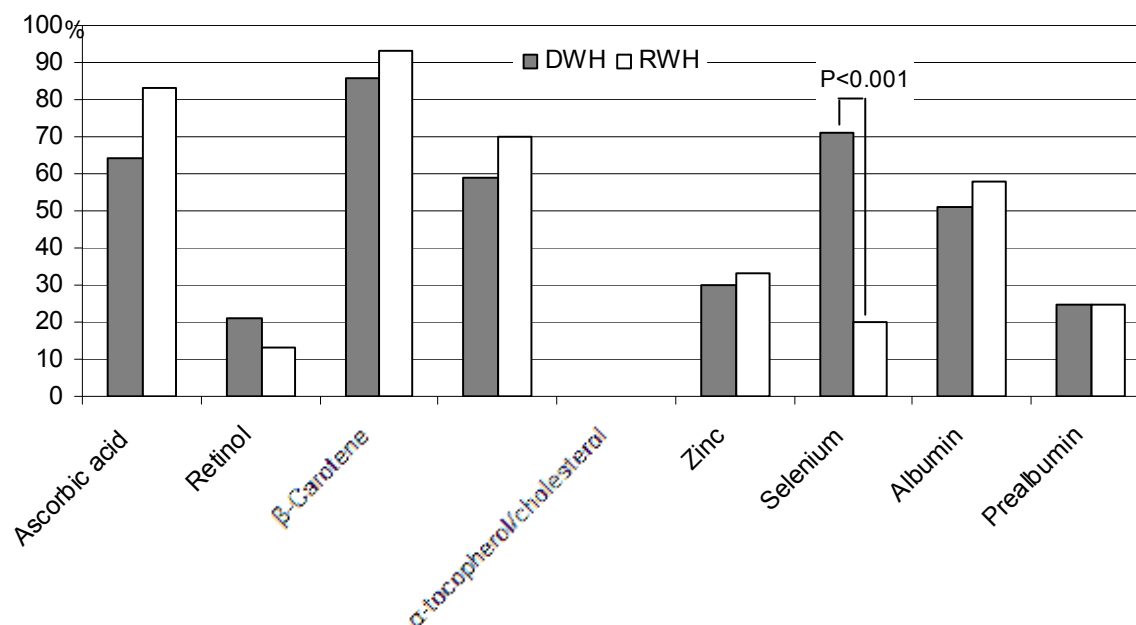
Figure 1: Prevalence of micronutrients and plasma proteins concentrations below the reference range

DWH (*n* = 44): disorders in wound healing; RWH (*n* = 45): regular wound healing; y: years; 25(OH)D$_3$: 25-hydroxycholecalciferol; Reference values: ascorbic acid: >28 µmol/l, retinol: 0.7 – 1.75 µmol/l, β-carotene: >0.9 µmol/l, 25(OH)$_2$D$_3$: >50 nmol/l, α-tocopherol/cholesterol >2.2 µmol/mmol, zinc: >11.2 µmol/l, selenium: >0.94 µmol/l, albumin [g/l]: 35–53 (≤ 60 yrs), 34–48 (> 60 yrs), 33–47 (> 70 yrs), 31–45 (> 80 yrs), prealbumin: >15 mg/dl; Group comparison: χ^2-test. Nobody had an α-tocopherol status below the reference range.

Table 4: **Markers of pro-/antioxidant balance**

	DWH (*n*=44)	**RWH** (*n*=45)	***P***[#]	**Reference range**[$]
TEAC [mmol TE/l]	1.24 ± 0.16	1.06 ± 0.19	<0.001	1.60 ± 0.16
Peroxides [mmol/l]	0.90 ± 0.27	0.48 ± 0.12	<0.001	0.72 ± 0.10
MDA [µmol/l]	83.7 ± 33.5	–	–	14.3 ± 4.1

DWH: disorders in wound healing; RWH: regular wound healing; data: means ± SD; TEAC: trolox equivalent antioxidant capacity; TE: trolox equivalents; MDA: malondialdehyde; [$]healthy controls (see text); Group comparison (DWH vs. RWH): [#]Student`s t-test

Discussion

To the best of our knowledge, this is the first cross-sectional study collecting various anthropometric, biochemical, and clinical data to evaluate the nutritional status and body composition in a great variety of post-surgical patients with vascular diseases and soft tissue trauma. The mono-centre study was performed within clinical routine ensuring that all patients received comparable medical therapy, care, and dietetic measures. Thus, a comparative evaluation of selected clinical and biochemical markers monitored in patients with and without DWH may provide hints for the failure of wound healing (WH).

Interestingly, anthropometric data were not different between the groups; moreover, BMI as well as calf and upper arm circumferences were within the normal range *(Table 2)*. Parameters of long-term (albumin) and short-term (prealbumin) protein status *(Table 3, Figure 1)* were in the lower normal range, but they were also comparable between the groups. Consequently, general protein/energy malnutrition which was proposed to favour DWH [3] cannot be seen as the decisive factor whether our post-surgical patients develop DWH or not.

Although all patients exhibited an acceptable general nutrition status *(Table 2),* mean plasma concentrations of ascorbic acid, $25(OH)D_3$, and β-carotene *(Table 3)* were below the reference values and 60 to 90% of the patients had a deficiency in these micronutrients *(Figure 1)*. Post-traumatic and post-surgical metabolic events like inflammation [20, 21] and oxidative stress [22] may generally contribute to the lower plasma levels of ascorbic acid and β-carotene. Since mean plasma concentrations of ascorbic acid and β-carotene were even slightly higher in patients with DWH compared with RWH, low plasma status of these nutrients may not be the reason to develop DWH. It should be, however, kept in mind that plasma levels provide no information with respect to substrate fluxes to the wound area. Independent whether DWH has developed or not, 30% of the patients exhibited an insufficient serum zinc status *(Figure 1)*. Comparably low concentrations of zinc transporters like albumin and prealbumin *(Table 3)* can at least partly explain this observation. Another line of reasoning is the acute phase response (APR) itself [23, 24]. Elevated concentrations of APR proteins like CRP and interleukin-6 are known to increase the expression of the zinc importer Zip14 [25] which leads to a fast zinc redistribution to organs [26]. Zorilla et al. [5, 27] showed that the serum zinc level is predictive for the WH process. Zorilla et al. [5, 27] determined the serum zinc level in

preoperative patients before elective hip replacement which may explain the different results obtained in our study (post-surgical analysis). In contrast to other micronutrients like retinol, ascorbic acid, and zinc, selenium has not a direct physiological function in WH. However, as a cofactor of glutathione peroxidase, selenium may reduce oxidative stress in patients with DWH [28]. Therefore, higher selenium concentrations in patients with RWH than in patients with DWH (*Table 3*) may partly explain beneficial WH. Insufficient serum $25(OH)D_3$ concentrations were observed in most of our patients in both groups (*Figure 1*). Since Miller et al. [29] observed an inverse association between serum vitamin D levels and inflammatory response following hip fracture, low $25(OH)D_3$ concentrations in our patients may result from APR-mediated inflammation. Moreover, immobilization of the patients may contribute to the low vitamin D status due to the lack of UV-induced endogenous synthesis.

Disturbances in the redox state are discussed to be risk factors for delayed WH [8]. Since direct measurements of the short-lived reactive oxygen species require laborious and expensive techniques like electron paramagnetic resonance, indirect methods are used in routine clinical setting to detect an imbalance between pro- and antioxidants [30]. This includes the analysis of peroxidation products like MDA and peroxides, the analysis of single antioxidants (e.g., ascorbic acid, α-tocopherol, β-carotene) [31], and of total antioxidant capacity which reflects the synergistic action between endogenous (albumin, uric acid) and nutritive (ascorbic acid, α-tocopherol, and β-carotene) antioxidants [32]. Oxidative stress in patients with DWH was indicated by increased concentrations of peroxides and of MDA compared to the reference values for healthy adults. TEAC was higher in DWH than in RWH (*Table 4*) probably due to higher concentrations of uric acid.

The strength of this study is the broad variety of biochemical, anthropometric, and clinical parameters which provides a detailed picture on the micronutrient status of trauma patients with DWH and RWH. Unfortunately, data on the quantity and quality of the diet considering energy, protein, and micronutrients of interest and data on the kind and dose of supplemented micronutrients were not collected. Hence, the impact of the nutritional intake on the nutritional status of our patients cannot be assessed. Moreover, vascular diseases and wound infections, known risk factors for DWH [1], were more prevalent in patients with DWH than with RWH (*Table 1*). As the period between trauma and study entry was shorter in RWH than in DWH

(*Table 1*) it cannot be completely excluded that the DWH and RWH patients were in different stages of inflammation even if CRP concentrations were comparable.

In conclusion, trauma patients with RWH and DWH suffer frequently from protein malnutrition and reduced plasma concentrations of several micronutrients probably due to inflammation, increased requirement, and oxidative burden. Thus, tailored nutritional measures (fresh fruits, vegetables, and high quality protein) and/or an early supplementation with selected micronutrients are strongly recommended to all hospitalised trauma patients.

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CHAPTER TWO

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

Abstract

Background & aims: We hypothesize that wound closure in trauma patients with disorders in wound healing is accelerated by supplementation of antioxidant micronutrients and glutamine.

Methods: In a randomized, double-blind, placebo-controlled trial, 20 trauma patients with disorders in wound healing were orally supplemented with antioxidant micronutrients (ascorbic acid, α-tocopherol, β-carotene, zinc, selenium) and glutamine (verum) or they received isoenergetic amounts of maltodextrine (placebo) for 14 days. Plasma/serum levels of micronutrients, glutamine, and vascular endothelial growth factor-A (VEGF-A) were determined before and after supplementation. In the wound, several parameters of microcirculation were measured. Time from study entry to wound closure was recorded.

Results: Micronutrients in plasma/serum did not change except for selenium which increased in the verum group (1.1 ± 0.2 vs. 1.4 ± 0.2 µmol/l; $P=0.009$). Glutamine decreased only in the placebo group (562 ± 68 vs. 526 ± 55 µmol/l; $P=0.047$). The prevalence of hypovitaminoses and the concentration of VEGF-A did not change. Considering microcirculation, only O_2-saturation decreased in the placebo group (56.7 ± 23.4 vs. 44.0 ± 24.0 [arbitrary units]; $P=0.043$). Wound closure occurred more rapidly in the verum than in the placebo group (29 [22; 52] vs. 58 d [46; 92]; $P=0.01$).

Conclusions: Time to wound closure can be shortened by oral antioxidant and glutamine containing supplements in trauma patients with disorders in wound healing.

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 [2]. Retinol maintains the integrity of epithelial and mucosal surfaces and plays a role in fibroplasia [2,5]. Zinc is needed for the synthesis of the retinol binding protein which is required for retinol mobilization from hepatic stores [2,6] 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 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 [8].

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 [9]. 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.

Materials and Methods

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).

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® granulate (Fresenius Kabi, Bad Homburg, Germany) twice daily (2 x 22.4 g) providing 500 mg ascorbic acid, 166 mg α-tocopherol, 3.2 mg β-carotene, 100 µg selenium, 6.6 mg zinc, and 20 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, α-tocopherol, β-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 (Bundeslebens-mittelschluessel, BLS) version II.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].

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]. Heparinized plasma samples for malondialdehyde (MDA) and 8-isoprostane measurements were protected against lipid peroxidation by addition of 0.05% butylhydroxytoluol. All laboratory parameters, except for those investigated routinely, were analyzed in duplicate.

Nutrient status
The concentration of ascorbic acid in EDTA plasma was measured according to Steffan [17] (CV 3.2%). Retinol, α-tocopherol, and β-carotene were also determined by HPLC. The protocol of Erhardt et al. [18] was modified by using apocarotenal as internal standard, Nucleosil® 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%), α-tocopherol at 292 nm (CV 4.1%) and β-carotene at 450 nm (CV 3.5%). Vitamin E status is expressed as α-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], α-tocopherol [21], β-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.

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).

Markers of pro-/antioxidant balance

Total antioxidant capacity of EDTA plasma was measured using the trolox equivalent antioxidant capacity (TEAC) assay [25] (CV 1.2%). Plasma malondialdehyde (MDA) was analyzed photometrically (CV 7.4%). ELISA kits were used to follow plasma concentrations of peroxides (Dr. Franz Tatzber KEG, Bisamberg, Austria; CV 4.3% according to the manufacturer) and total 8-isoprostanes (sum of free plus esterified 8-isoprostanes) according to the procedure described previously by our group [16] (CV 10%). Uric acid was analysed photometrically within clinical routine and evaluated according to the reference range of the Department of Clinical Chemistry and Clinical Pharmacology, University of Bonn.

Wound healing

Parameters of microcirculation at a tissue depth of 2 mm (oxygen saturation, relative haemoglobin, blood flow and velocity) 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 seconds 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 sterling-cooled infrared scanning camera (VAIOSCAN 3201 ST, Jenoptic Laser, Jena, Germany; accuracy < ± 2K, 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 disinfectants 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 if wounds were closed after discharge.

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 χ^2-test and Fisher's exakt test. Changes in the number of patients with nutrients below the reference range were analyzed with the McNemar test. Statistical significance was assumed for $P \leq 0.05$. Metric data are presented as median and quartiles.

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 $\alpha = 0.05$, 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.

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: *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 α-tocopherol (>12 µmol/l, with an α-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 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 on VEGF-A concentrations are shown in Table 6. At baseline, O_2-saturation (*P*=0.013) and blood flow (*P*=0.043)

significantly differed between the groups. O_2-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 [9; 35]; verum: 31 days [24; 55]).

Table 1: **Demographic data, anthropometric, and clinical data at enrolment**

	Placebo (n=10)	Verum (n=10)
Sex male/female	6/4	8/2
Age [yrs]	45 [36; 76]	46 [27; 56]
Age ≥ 65 years [n]	3	1
Anthropometric data		
BMI (kg/m^2)	27.6 [23.4; 30.7]	25.8 [23.4; 31.7]
Triceps skin fold thickness (mm)	19.2 [11.8; 24.8]	16.1 [11.3; 21.9]
Calf circumference (cm)	38.9 [33.8; 41.6]	37.6 [33.9; 42.1]
Upper arm circumference (cm)	32.5 [29.2; 35.4]	31.3 [29.1; 36.4]
Period between trauma and d0 [d]	30 [15; 70]	26 [12; 34]
Main diagnosis [n]		
Proximal tibia/ankle joint/foot trauma	9	6
Pelvic/hip trauma	1	4
Injury severity score	9 [9; 21]	16 [9; 21]
Soft tissue trauma [n]		
None/ grade I°/ II°- III°	3/2/5	5/3/2
Infection [n]	4	4
Comorbidities [n]		
Vascular diseases	-	1
Diabetes mellitus	1	1
Hypertension	2	3
Dementia	1	1
Decubitus ulcer	1	3
Smoker [n]	5	4
Length of hospital stay [d]	25 [9; 35]	31 [24; 55]
SGA A/B/C (n)	6/2/2	3/5/2
NRS no risk/risk/high risk (n)	6/2/2	3/4/3

Data: median [quartiles]; significant differences (*P*≤0.05) between the groups according to χ^2-Test (nominal/ordinal data) and Mann-Whitney-U test (metric data) did not occur. BMI: body mass index; SGA: subjective global assessment; SGA A: well-nourished; SGA B: at risk for malnutrition or moderately malnourished; SGA C: severely malnourished; NRS: Nutritional Risk Screening.

Table 2: **Daily intake of energy and nutrients from food**

	Placebo (n=10)	Verum (n=10)
Energy		
(kJ)	7477 [5498; 8259]	6799 [6335; 8506]
(kcal)	1787 [1314; 1974]	1625 [1514; 2033]
(kJ/kg body weight)	79.8 [67.3; 113.8]	75.9 [61.6; 94.5]
(kcal/kg body weight)	19.1 [16.1; 27.2]	18.1 [14.7; 22.6]
Protein [g]	67 [49; 83]	62 [47; 92]
Protein [g/kg body weight]	0.7 [0.6; 1.1]	0.6 [0.5; 1.2]
Ascorbic acid [mg]	45 [32; 60]	78 [48, 153]
α-Tocopherol [mg α-tocopherol equivalent]	6 [5; 10]	8 [4; 9]
β-Carotene [mg]	2 [1; 3]	2 [1; 3]
Zinc [mg]	11 [7; 12]	8 [6; 12]

Data: median [quartiles]; significant differences ($P \leq 0.05$) between the groups according to Mann-Whitney-U test did not occur

Table 3: **Nutrient status**

	Placebo (n=10)			Verum (n=10)			Reference range
	d0	**d14**	*P*	**d0**	**d14**	*P*	
Ascorbic acid [µmol/l]	16.7 [9.5; 21.9]	40.8 [18.2; 50.9]	n.s.	27.1 [13.1; 37.7]	35.8 [28.4; 50.1]	n.s.	25 - 85
α-Tocopherol [µmol/l]	33.6 [28.6; 34.8]	34.1 [27.9; 41.8][a]	n.s.	33.2 [25.8; 38.5]	46.8 [42.2; 65.0][a]	0.007	12 - 50
α-Tocopherol/cholesterol [µmol/mmol]	6.3 [5.8; 6.6][b]	6.4 [5.5; 6.9]	n.s.	7.2 [6.5; 9.7][b]	8.7 [8.2; 12.1]	n.s.	> 2.2
β-Carotene [µmol/l]	0.6 [0.3; 0.9]	0.5 [0.3; 0.9]	n.s.	0.5 [0.3; 1.3]	0.9 [0.5; 1.4]	n.s.	0.9 - 4.6
Retinol [µmol/l]	1.48 [1.29; 1.89]	1.82 [1.33; 2.12]	n.s.	1.39 [0.99; 1.97]	1.52 [1.25; 1.89]	n.s.	0.70 – 1.75
Zinc [µmol/l]	13.2 [11.2; 14.6]	11.8 [10.0; 15.3]	n.s.	13.4 [11.9; 17.5]	15.4 [13.2; 16.5]	n.s.	11.5 - 19.4
Selenium [µmol/l]	1.1 [0.9; 1.3]	1.1 [0.9; 1.3][c]	n.s.	1.0 [0.9; 1.1]	1.4 [1.2; 1.5][c]	0.009	> 0.63
Albumin [g/l]	33.3 [32.0; 36.1]	37.3 [32.1; 38.9]	0.05	32.3 [25.2; 34.9]	34.1 [31.4; 37.5]	0.017	age related [$]
Prealbumin [mg/dl]	23.9 [18.1; 30.8]	23.2 [19.7; 28.4]	n.s.	23.2 [14.1; 27.5]	26.0 [21.2; 28.8]	n.s.	15 - 36
Glutamine [µmol/l]	551 [495; 636]	535 [468; 567]	0.047	536 [459; 593]	590 [505; 719]	n.s.	655 ± 84

Data: median [quartiles]; d0: baseline; d14: after 14-day supplementation; n.s.: not significant; [$] Reference ranges for albumin [g/l]: 35-53 (≤ 60 yrs), 34-48 (> 60 yrs), 33-47 (> 70 yrs), 31-45 (> 80 yrs); letters indicate significant differences (*P*≤0.05) between the groups according to Mann-Whitney-U test: [a] *P*=0.005, [b] *P*=0.023, [c] *P*=0.028; *P*: significant changes (*P*≤0.05) within d0 and d14 according to Wilcoxon-test

Table 4: **Metabolites and inflammatory markers**

	Placebo (n=10)			Verum (n=10)			Reference range
	d0	**d14**	*P*	**d0**	**d14**	*P*	
Leukocytes [G/l]	7.9 [6.4; 9.4]	7.4 [6.4; 8.6]	n.s.	7.1 [6.4; 8.8]	6.8 [5.3; 7.3]	n.s.	3.5 - 9.8
Cholesterol [mmol/l]	5.4 [4.7; 6.0][a]	5.4 [6.9; 5.4]	n.s.	4.4 [3.7; 5.1][a]	5.3 [4.8; 5.5]	0.005	< 5.2
Uric acid [µmol/l]	280 [196; 375]	292 [220; 386]	n.s.	274 [161; 309]	321 [220; 369]	n.s.	214-446 (m) 137-157 (f)
Bone specific alkaline phosphatase [µg/l]	16.1 [12.1; 22.6]	15.9 [13.5; 24.6]	n.s.	11.9 [7.9; 25.5]	13.3 [9.4; 31.4]	n.s.	7 - 20
C-reactive protein [mg/l]	9.8 [5.2; 29.0]	6.4 [3.5; 8.5]	0.037	25.3 [9.7; 73.6]	10.7 [5.9; 21.9]	n.s.	< 3
Interleukin-6 [pg/ml]	3.3 [2.0; 8.6]	3.6 [2.2; 4.9]	n.s.	10.2 [2.5; 19.6]	4.9 [2.0; 7.9]	0.037	< 5
Interleukin-8 [pg/ml]	9.5 [6.2; 12.9]	6.8 [5.5; 11.7]	0.033	10.4 [5.1; 18.6]	6.9 [5.1; 15.2]	n.s.	< 15
Transferrin [g/l]	2.7 [1.9; 3.3]	2.9 [2.2; 3.2]	0.028	2.2 [1.9; 2.5]	2.4 [2.2; 2.8]	0.007	2 – 3.6
Ferritin [µg/l]	98.4 [27.1; 168.4]	56.1 [22.2; 193.8]	n.s.	167.8 [117.3; 444.0]	92.2 [55.7; 368.3]	0.013	26 - 388 (m) 13 - 252 (f)

Data: median [quartiles]; d0: baseline; d14: after 14-day supplementation; n.s.: not significant; letters indicate significant differences (*P*≤0.05) between the groups according to Mann-Whitney-U test: [a] *P*=0.026; *P*: significant changes within the groups between d0 and d14 according to Wilcoxon-test

***Table 5*: Markers of pro-/antioxidant balance**

	Placebo (n=10)			Verum (n=10)		
	d0	**d14**	*P*	**d0**	**d14**	*P*
TEAC [mmol TE/l]	1.02 [0.96; 1.07]	1.04 [0.95; 1.07]	n.s.	1.04 [1.01; 1.06]	1.00 [0.96; 1.12]	n.s.
Peroxides [mmol/l]	0.42 [0.37; 0.56]	0.41 [0.34; 0.55]	n.s.	0.49 [0.29; 0.64]	0.46 [0.36; 0.53]	n.s.
MDA [µmol/l]	199.3 [163.0; 222.3]	174.6 [155.7; 199.3]	n.s.	174.3 [147.6; 194.2]	170.3 [138.6; 187.7]	n.s.
8-Isoprostane [pg/ml]	14.9 [9.8; 21.5]	19.8 [11.4; 26.3]	n.s.	20.6 [18.3; 23.5]	13.3 [9.6; 16.9]	0.028

Data: median [quartiles]; d0: baseline; d14: after 14-day supplementation; TEAC: trolox equivalent antioxidant capacity; TE: trolox equivalents; MDA: malondialdehyde; significant differences (*P*≤0.05) between the groups according to Mann-Whitney-U test did not occur; *P*: changes within the groups analysed by Wilcoxon-test

***Table 6:* Parameters on microcirculation and vascular endothelial growth factor**

	Placebo (n=10)			Verum (n=10)		
	d0	**d14**	*P*	**d0**	**d14**	*P*
O$_2$-saturation	67 [28; 73][a]	53 [19; 65]	0.043	19 [14; 35][a]	44 [23; 65]	n.s.
Relative haemoglobin	48 [24; 56]	55 [33; 91]	n.s.	51 [28; 62]	43 [12; 64]	n.s.
Blood flow	80 [24; 93][b]	40 [30; 78]	n.s.	32 [20; 43][b]	44 [28; 104]	n.s.
Blood velocity	17 [14; 19]	16 [14; 17]	n.s.	14 [12; 17]	14 [13; 17]	n.s.
VEGF-A [pg/ml]	695 [415; 879]	553 [289; 749]	n.s.	551 [318; 1019]	539 [290; 910]	n.s.

Data: median [quartiles] 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 (*P*≤0.05) between the groups according to Mann-Whitney-U test: [a] *P*=0.013, [b] *P*=0.043; P: significant changes within d0 and d14 according to Wilcoxon test.
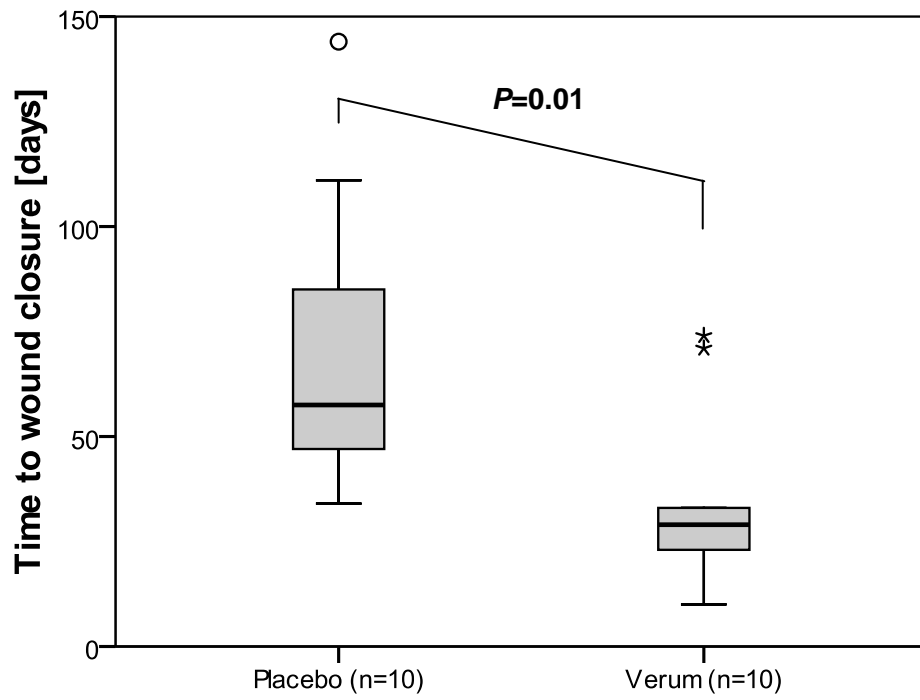
Figure 1: **Time to wound closure**

Group comparison: done according to Mann-Whitney-U test

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 (*Figure 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) [2,30-32] 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 [2,33-35]. Thus, inflammation *per se* may reduce micronutrients in plasma/serum [2, 36-38]. In line with this assumption, we observed low levels of albumin and prealbumin which may explain the reduced levels of β-carotene and zinc. As suggested by Wilson et al. [39] and Fukushima and Yamazaki [40], 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 µmol/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 L-ascorbate by *Escherichia coli.* If this also applies to human cells, this mechanism may contribute to accelerated wound healing in our verum group (*Figure 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 µmol/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 α-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 µmol/l [49], an increase in glutathione peroxidase activity in the verum group is unlikely. As oxidative stress is discussed to impair wound healing [7], 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, O_2 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 micro-movements 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 haematoma [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 temperature is expected to occur during wound healing. In our study, wound temperature was measured by thermography which reflects local blood flow [53]. 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 patients were closed 29 and 58 days after onset of intervention, no changes in wound temperature on d14 may be expected.

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.

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GENERAL DISCUSSION

The aim of this thesis was the assessment and evaluation of the nutritional status of trauma patients with DWH and its relation to WH. To clarify the impact of the nutritional status on WH, two studies were conducted. The cross-sectional study (CHAPTER ONE) addressed the question if the nutritional status is different compared to trauma patients with RWH. Then, the aim of the interventional study (CHAPTER TWO) was to investigate if the status of specific micronutrients and the wound healing can be enhanced in trauma patients with DWH by an oral supplementation with these micronutrients and glutamine.

Surprisingly, the general nutritional status and the prevalence of micronutrient concentrations in plasma below the reference range were not different between patients with RWH and DWH. The concentrations of ascorbic acid, β-carotene as well as the α-tocopherol/cholesterol ratio were even lower in patients with RWH (see CHAPTER ONE *Table 3* and *Figure 1*). Nevertheless, it is hypothesised that the poor status of several micronutrients in acute trauma patients with DWH is associated with DWH. Consequently, meeting the increased requirement by metabolic changes as well as loss of blood and wound fluid may probably not be achieved by a normal diet. Hence, we assumed that an oral supplementation with antioxidant micronutrients and glutamine enhances the nutritional status and, thus, WH in trauma patients with DWH. In fact, wound closure was faster in patients of the verum group even if the micronutrients, except for selenium, and glutamine in plasma did not change and were, thus, not different between the verum and the placebo group after intervention (see CHAPTER TWO *Table 3*, *Table 4* and *Figure 1*). Hence, an association between the status of selected micronutrients in plasma/serum and WH was not verifiable in both studies (CHAPTER ONE and CHAPTER TWO).

To explain this phenomenon, the inflammatory status of the trauma patients has to be considered. Nearly all patients, regardless the progress of WH, suffered from inflammation as concentrations of CRP, a reliable marker for the inflammatory status [1] were elevated (see CHAPTER ONE: results section and CHAPTER TWO: *Table 5*). Maximum CRP concentrations are usually achieved two to five days after trauma or surgery and normalize within 30 days. However, in patients with post-surgical complications (e.g. infections or failed WH), CRP levels are permanently increased [2]. As discussed in CHAPTER ONE and TWO, inflammation has a major impact on the plasma nutrient status in injured patients e.g. by lowering the nutrients *per se* or reducing the transportation-proteins for several micronutrients. This relationship was

underlined by the inverse correlation between CRP and several micronutrients (ascorbic acid retinol, zinc, and selenium) as well as albumin and prealbumin (CHAPTER ONE, result section). This result is in line with findings of other studies [3, 4]. An association between elevated CRP concentrations and plasma nutrients was observed by Gariballa et al. [4] in 445 elderly hospitalized patients with several diagnoses. Hereby, plasma vitamin C was 6.5 µmol/l lower in patients with elevated CRP levels (CRP concentrations > 10 mg/l) compared to patients with normal CRP concentrations. Inflammation explained most of the variance (P=0.011) for vitamin C concentrations in plasma. After adjusting for confounders (comorbidities, age, medication), inflammation was identified as an independent risk factor for poor nutritional status in plasma/serum which corroborates our observations in DWH patients (see CHAPTER ONE: *Table 3, Figure 1* and CHAPTER TWO: *Table 3*, *Table 4*). Craig et al. [3] observed in geriatric patients with an acute illness a negative correlation (r=0.33, P<0.001) between serum zinc and CRP. This may be caused by acute infections, tissue injury and the elevated synthesis of CRP and IL-6 that increased the gene expression of the zinc importer Zip14 on hepatic cells [5]. Thus, the micronutrient und protein concentrations in plasma/serum depend on the inflammatory status.

In the interventional study (CHAPTER TWO), trauma patients with DWH were supplemented with moderate doses of micronutrients but an increase in plasma concentrations of micronutrients (except for selenium, P=0.009) was not observed. Nevertheless, wounds were closed faster in the verum than in the placebo group. Similar results were obtained by Desneves et al. [6] who investigated patients with pressure ulcers stage II-IV and non-acute wounds. The pressure ulcer scale for healing score [7] decreased in the verum group after three weeks of intervention but not in the control group even if the plasma concentrations of zinc and vitamin C did not change after a daily intake of an oral nutritional supplement providing 500 mg ascorbic acid, 7.5 mg zinc, 21 g protein, and 9 g arginine. In the study of Raffoul et al. [8] patients with lower limb and pressure ulcers received a tablet with several vitamins as well as minerals and additionally a tablet with 500 mg vitamin C for 15 days. The time to wound closure in all supplemented patients was comparable to our patients (CHAPTER TWO) as well as the observation that the concentrations of ascorbic acid and further micronutrients in serum/plasma were not affected by intervention. In other studies [9-11], complete wound closure was also achieved after

a supplementation of micronutrients and arginine. However, two studies [10, 11] were uncontrolled and the micronutrient status in plasma/serum was not investigated.

Besides inflammation, the relatively short period of the supplementation in our study (CHAPTER TWO) may be the reason for stable nutrient concentration in plasma/serum. In a recent study, van Anholt et al. [9] investigated the effect of a daily high-dose supplementation of several micronutrients (714 mg vitamin A, 750 mg vitamin C, 114 mg α-tocopherol equivalents, 27 mg zinc, 192 µg selenium, 4.05 mg copper and 600 µg folic acid), 60 g protein and 9 g arginine for eight weeks versus placebo in non-malnourished patients with pressure ulcers stage III and IV. Interestingly, after intervention, an increase in plasma concentration of micro-nutrients was only observed for ascorbic acid (P=0.015) in the verum group. In contrast to the interventional study (CHAPTER TWO), a higher dose of vitamin C (750 mg vs. 500 mg/d) and a longer period of intervention (eights vs. two weeks) were chosen. Since CRP concentrations remained unchanged in both groups, the nutrient concentration-lowering impact of the inflammation seems to dominate influence of dose and length of the supplementation. Nevertheless, the wound closed faster in the verum compared to the placebo group. Thus, beneficial effects on wound closure by supplementation of nutrients as observed in CHAPTER TWO without changes in plasma concentration of these nutrients are not a contradiction. Unfortunately, the mechanisms remain unclear due to our study design. An intracellular accumulation of micronutrients in the wounded area may provide an explanation for the accelerated WH. Thus, the analysis of intra-cellular concentrations may elucidate the destination of the ingested micronutrients which is only possible in animal studies due to ethical reasons.

It is well-known that post-traumatic inflammation favours an imbalance between oxidants and antioxidants [12, 13], the so called oxidative stress. After injury, neutrophils and macrophages invade the wounded tissue to phagocytize debris and to kill microorganisms by respiratory burst and releasing reactive oxygen species (ROS) [14-16]. Furthermore, ROS act as a second messenger to gene expression of various growth factors that induce VEGF expressions in keratinocytes, and activate the gene expression of collagenase that helps in the degradation of extracellular matrix [17]. Macrophages, fibroblasts and endothelial cells also express inducible nitric oxide synthase that produces nitric oxide to kill microbials and to

support collagen synthesis and angiogenesis, respectively. Thus, oxidative stress is important for proper WH. However, ROS may also impair WH [18]. Non-healing wounds remain in the inflammatory phase without transition to tissue formation. The reason may be inactivation of epidermal enzymatic antioxidants due to oxidative stress that prolongs the inflammatory phase [16]. Thus, excessive oxidative stress is assumed to delay wound healing [16, 18, 19]. Unfortunately, the threshold between physiologic and pathologic oxidative stress is still unknown. Since direct measurements of ROS were impossible, indirect parameters like peroxides, MDA and 8-isoprostanes were used to determine oxidative stress in the present studies (see CHAPTER ONE: *Table 4*, discussion section, and CHAPTER TWO: *Table 6*). Interestingly, these parameters were elevated in patients with DWH and TEAC was lower in all patients compared to healthy volunteers in both present studies (CHAPTER ONE: *Table 4,* and CHAPTER TWO: discussion section and *Table 6*). Hence, an imbalance between antioxidants and oxidants can strongly be assumed in our patients and may favour DWH. 8-isoprostanes decreased after intervention that indicated a reduction of oxidative stress and may have favoured the WH in the verum group (CHAPTER TWO: *Table 6*). However, MDA, peroxides, and TEAC did not change. This effect may be due to the still present inflammation and the relatively short period between the analyses of these parameters.

Interestingly, none of the patients with RWH had vascular diseases and infections compared to patients with DWH (CHAPTER ONE (*Table 1*)) whereas the prevalence for patients without soft tissue trauma were even higher in patients with DWH. Infections and vascular diseases are linked to each other since a decreased microcirculation in the wound area limits the wound healing by diminished local perfusion [20] and oxygenation of the tissue. Thus, local bacterial growth is favoured by local hypoxia and diminished supply of nutrients and vice versa in terms of a vicious circle [21] and occur obviously in all types of wound irrespective from the severity of (soft tissue) trauma (CHAPTER ONE and TWO: *Table1*, ever). Oxygenation is also connected to the formation of new vessels [22] as an increased oxygenation enhanced the VEGF concentration in the wound fluid [23]. Unfortunately, no changes of the O_2 saturation and VEGF concentrations were observed even though time to wound closure was shorter in patients with DWH who received micronutrients and glutamine (CHAPTER TWO, *Figure 1*). As discussed in CHAPTER TWO, these

findings are not a contradiction as the measurement of the microcirculation by O2C and the analysis of VEGF-A in serum did not necessarily mirror local angiogenesis.

Although the general nutritional status was not associated with WH in our study patients probably due to a small proportion of undernourished patients and short observation time, anthropometric parameters should be evaluated regularly. As a consequence of our and former study results in patients with DWH and pressure ulcers [24], a tailored nutritional therapy with several micronutrients and a diet with adequate amounts of protein and energy should be implemented regularly in the medical treatment Furthermore, to ensure the comprehension of clinical nutrition and to create awareness for nutrition-associated illnesses and comorbidities, seminars in nutritional physiology and clinical nutrition should be integrated in the physicians´ education. Shang et al. [25] demonstrated that nutrition support teams in hospitals consisting of physicians, nutritional scientist, dieticians, nurses, and pharmacologists are an excellent mechanism for identifying patients in need of nutrition support. Thus, the formation and establishing of these teams is highly desirable for improving the efficacy of nutrition support in a clinical setting.

In conclusion, the results of the cross-sectional and interventional study (CHAPTER ONE and TWO) do not show associations between the micronutrient concentrations in plasma/serum, parameters of oxidative stress and the wound healing of trauma patients with DWH. However, the nutritional status in plasma/serum has to be evaluated cautiously due to post-traumatic inflammation which lowers micronutrient concentrations directly or indirectly by reduced availability of transporter proteins. Moreover, the micronutrient concentrations in the wound may be more decisive for WH than those concentrations in plasma/serum. Since the supplementation of micronutrients and glutamine accelerated WH, tailored nutritional measures contribute effectively to wound treatment and, thus, should be implemented in daily clinical routine.

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ANNEX A: Questionnaire of the studies

Studie „Ernährungsstatus u. Wundheilung"   **Proband Nr.** | | |

Datum Trauma/OP   _ _ . _ _ . 20_ _

Datum der Untersuchung   _ _ . _ _ . 20_ _

Geschlecht   ○ männlich   ○ weiblich

Geburtsdatum   _ _ . _ _ . _ _ _ _

Alter (Jahre)   _ _

Raucher   ○ ja   ○ nein

Supplementierung (Vitamine u.a.)   ○ ja   ○ nein

Wenn ja, welche? (evtl. Präparat, Dosis)

_____

Medikamente:

Cortison   ○ ja   ○ nein

Zytostatika   ○ ja   ○ nein

Erkrankungen/Beschwerden

1) Diabetes mellitus: Typ 1   ○ ja   ○ nein

                  Typ 2   ○ ja   ○ nein

   Insulinpflicht: ○ ja   ○ nein

   Genetische Disposition für Diab. mellitus Typ II ?   ○ ja   ○ nein

2) Gastrointestinale Erkrankungen   ○ ja   ○ nein

Wenn ja, welche: _____

Gastrointestinale Beschwerden (nach Eingriff)   ○ ja   ○ nein
(Appetitlosigkeit, Durchfall, Schmerzen)

3) Atopische Dermatitis   ○ ja   ○ nein

4) Hypertonie   ○ ja   ○ nein

5) Patient mobil   ○ ja   ○ nein

6) Gefäßerkrankungen   ○ ja   ○ nein

ggf. pAVK nach Fontaine   I   II   III   IV

7) Patient dement: ○ ja   ○ nein

8) Karnofsky-Index: _____

<u>Sonstige Erkrankungen</u>

Dekubitus   ○ ja   ○ nein
(nicht als Primärdiagnose)

Anthropometrische Daten

Gewicht   _____,___ kg
Größe   _____,___ cm
BMI (kg/m^2)   _____

<u>Umfangmessungen</u>
Umfang Hüfte (HU)   ____,__ cm   ____,__ cm
Umfang Taille (TU)   ____,__ cm   ____,__ cm
Waist-to-hip-Ratio (TU [cm]:HU [cm])   _____
Wadenumfang   ____,__ cm   ____,__ cm
Oberarmumfang   ____,__ cm   ____,__ cm
Trizepshautfaltendicke   ____,__ cm   ____,__ cm   ____,__ cm

Bioelektrische Impedanzanalyse

Frequenz (kHz)	R (Ohm)	Xc (Ohm)	s	R tot (Ohm)	alpha (Grad)	R Hand (Ohm)	R Fuß (Ohm)
50							
5							

ANNEX B: Nutritional Risk Screening

Screening auf Mangelernährung im Krankenhaus

Nutritional Risk Screening (NRS 2002)

nach Kondrup J et al., Clinical Nutrition 2003; 22: 415-421

Empfohlen von der Europäischen Gesellschaft für Klinische Ernährung und Stoffwechsel (ESPEN)

Vorscreening:

• Ist der Body Mass Index < 20,5 kg/m² ?	ja	nein
• Hat der Patient in den vergangenen 3 Monaten an Gewicht verloren?	ja	nein
• War die Nahrungszufuhr in der vergangenen Woche vermindert?	ja	nein
• Ist der Patient schwer erkrankt? (z.B. Intensivtherapie)	ja	nein

⇒ Wird eine dieser Fragen mit „Ja" beantwortet, wird mit dem Hauptscreening fortgefahren

⇒ Werden alle Fragen mit „Nein" beantwortet, wird der Patient wöchentlich neu gescreent.

⇒ Wenn für den Patienten z.B. eine große Operation geplant ist, sollte ein präventiver Ernährungsplan verfolgt werden, um dem assoziierte Risiko vorzubeugen.

Hauptscreening:

Störung des Ernährungszustands	Punkte
Keine	**0**
Mild	**1**
Gewichtsverlust > 5%/ 3 Mo. oder Nahrungszufuhr < 50-75% des Bedarfes in der vergangenen Woche	
Mäßig	**2**
Gewichtsverlust > 5%/ 2 Mo. oder BMI 18,5-20,5 kg/m² und reduzierter Allgemeinzustand (AZ) oder Nahrungszufuhr 25-50% des Bedarfes in der vergangenen Woche	
Schwer	**3**
Gewichtsverlust> 5% /1 Mo. (>15% / 3 Mo.) oder BMI <18,5 kg/m² und reduzierter Allgemeinzustand oder Nahrungszufuhr 0-25% des Bedarfes in der vergangenen Woche	

+

Krankheitsschwere	Punkte
Keine	**0**
Mild	**1**
z.B. Schenkelhalsfraktur, chronische Erkrankungen besonders mit Komplikationen: Leberzirrhose, chronisch obstruktive Lungenerkrankung, chronische Hämodialyse, Diabetes, Krebsleiden	
Mäßig	**2**
z.B. große Bauchchirurgie, Schlaganfall, schwere Pneumonie, hämatologische Krebserkrankung	
Schwer	**3**
z.B. Kopfverletzung, Knochenmarkstransplantation, intensivpflichtige Patienten (APACHE-II >10)	

+ | 1 Punkt, wenn Alter ≥ 70 Jahre |

≥ 3 Punkte	Ernährungsrisiko liegt vor, Erstellung eines Ernährungsplanes
< 3 Punkte	wöchentlich wiederholtes Screening. Wenn für den Patienten z.B. eine große Operation geplant ist, sollte ein präventiver Ernährungsplan verfolgt werden, um das assoziierte Risiko zu vermeiden

T. Schütz, L. Valentini, M. Plauth. Screening auf Mangelernährung nach den ESPEN-Leitlinien 2002. Aktuel Ernaehr Med 2005; 30: 99-103.

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ANNEX C: Subjective Global Assessment

AKE

Subjective Global Assessment (SGA) – Einschätzung des Ernährungszustandes
(Nach Detsky et al., JPEN 1987; 11: 8–13)

Name, Vorname:_____

Geburtsdatum: _____

Station: _____

Untersuchungsdatum: _____

bitte ankreuzen ( ) oder ausfüllen (__)

A. Anamnese

1. Gewichtsveränderung

Gewichtsverlust in den vergangenen 6 Monaten: _____kg (____%)

Veränderung in den vergangenen 2 Wochen: Zunahme

keine Veränderung

Abnahme

2. Veränderung in der Nahrungszufuhr (im Vergleich zur gewöhnlichen Zufuhr)

keine Veränderung   Veränderung: Dauer _____Wochen

Art: suboptimale feste Kost

ausschließlich Flüssigkost

hypokalorische Flüssigkeiten

keine Nahrungsaufnahme

3. Gastrointestinale Symptome (die >2 Wochen bestehen)

keine   Erbrechen   Appetitlosigkeit

Übelkeit   Durchfall

4. Leistungsfähigkeit

voll leistungsfähig   eingeschränkt leistungsfähig: Dauer _____Wochen

Art: eingeschränkt arbeitsfähig

gehfähig

bettlägerig

5. Auswirkung der Erkrankung auf den Nährstoffbedarf

Hauptdiagnose: _____

_____

_____

Metabolischer Bedarf: kein Streß   niedriger Streß

mäßiger Streß   hoher Streß

B. Untersuchung (0 = normal; 1+ = gering; 2+ = mäßig; 3+ = ausgeprägt)

_____ Verlust von subkutanem Fettgewebe

_____ Muskelatrophie (Quadrizeps, Deltoideus)

_____ Knöchelödem

_____ präsacrale Ödeme (Anasarka)

_____ Aszites

C. Subjektive Einschätzung des Ernährungszustandes (bitte auswählen)

A = gut ernährt

B = mäßig mangelernährt oder mit Verdacht auf Mangelernährung

C = schwer mangelernährt