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Role of arginine in superficial wound healing in man

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ABSTRACT

Arginine supplementation has been identified as advantageous in experimental wound healing. However, the mechanisms underlying this beneficial effect in tissue repair remain unresolved. Animal studies suggest that the beneficial role of arginine supplementation is mediated, at least in part through NO. The latter component mediates processes involved in tissue repair, including angiogenesis, epithelialization and collagen formation. This prospective study is performed to investigate arginine metabolism in acute surgical wounds in man. Expression of enzymes, known to be involved in arginine metabolism, was studied in donor sites of skin grafts of 10 hospitalized patients undergoing skin transplantation. Plasma and wound fluid levels of arginine metabolites (ornithine, citrulline, nitrate and nitrite = NO_x) were measured using High Performance Liquid Chromatography. Expression of iNOS, eNOS, arginase-1 and arginase-2 was studied by immunohistochemistry in paraffin sections of skin tissue. Arginase-1 concentration was measured in plasma and wound fluid using ELISA. Arginase-2 was determined using Western blot analysis. We observed increased levels of citrulline, ornithine, NO_x and arginase-1 in wound fluid when compared with plasma. Arginase-2 was expressed in both plasma and wound fluid and seemed higher in plasma. iNOS was expressed by neutrophils, macrophages, fibroblasts, keratinocytes and endothelial cells upon wounding, whereas eNOS reactivity was observed in endothelial cells and fibroblasts. Arginase-1 was expressed in neutrophils post-wounding, while arginase-2 staining was observed in endothelial cells, keratinocytes, fibroblasts, macrophages and neutrophils. For the first time, human data support previous animal studies suggesting arginine metabolism for an NO- as well as arginase-mediated reparation of injured skin.

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Introduction

Wound healing represents a complex process, initiated to restore tissue damage. The amino acid arginine has been identified as an important mediator in this process [1]. Arginine is the sole precursor of nitric oxide (NO), a signal molecule, among others, involved in immune responses, angiogenesis, epithelialization and formation of granulation tissue, all essential aspects accompanying wound healing [2].

Nitric Oxide Synthase (NOS) converts arginine to NO and citrulline. Three isoforms of NOS exist: neuronal NOS (NOS 1) and endothelial NOS (NOS 3) are constitutively expressed by neuronal and endothelial cells, respectively. Inducible NOS (NOS 2), is expressed in response to inflammatory cytokines and endotoxins, such as seen during wound repair. The beneficial effects of arginine supple-

mentation on wound healing have been attributed to enhanced synthesis of NO by NOS [3–6]. Former studies with rodents showed that arginine-free diets impair wound healing, with decreased breaking strengths of incisions and collagen deposition in granulation tissue [7,8], while supplementation of arginine increased hydroxyproline concentration, a marker of collagen synthesis [9–12]. In addition, to its role as precursor of NO, arginine can be metabolized by arginase [13–17]. Two different isoforms of this enzyme have been identified: arginase-1 (ARG1) and arginase-2 (ARG2). The liver type, ARG1, is the cytosolic isoform. ARG2 is the mitochondrial isoform, located in kidney, prostate, small intestine and the breast. Arginase catalyses the conversion of arginine to ornithine and urea. Ornithine is an essential precursor for collagen and polyamines synthesis [18], both required for wound healing processes [19–25]. Moreover, arginase seems to influence the immune response, another important contributor involved in tissue repair. Activated macrophages and neutrophils (PMN's) show increased NO-production for their anti-bacterial function. This NO-production is subsequently down-regulated by arginase through substrate competition [26–28].

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Although arginine supplementation is considered to promote the wound healing process, little is known of its metabolism during normal human wound healing. This study examines the role of arginine during uncompromized human wound healing. To this end, levels of arginine, its metabolites and arginase were measured in wound fluid and plasma of superficial surgical wounds in man. In addition, we evaluated the immunohistochemical distribution of the enzymes arginase, iNOS and eNOS in these wounds on paraffin-embedded tissue sections.

Materials and methods

A prospective study was performed in ten hospitalized healthy adults undergoing skin transplantation as part of reconstructive surgery at the Department of Plastic Surgery in University Hospital Maastricht. Details of their characteristics are given in Table 1. Exclusion criteria were: age younger than 18 or older than 70 years, kidney or liver failure, pregnancy, use of steroids or diabetes mellitus.

Patients underwent screening evaluation, including medical history and physical examination. Nutritional assessment was obtained measuring body weight and length and expressed as Body Mass Index (=weight/length²). Age and sex of the patients were noted. The Maastricht Hospital Medical Ethical Board approved the investigation protocol. Each patient signed informed consent.

Wound protocol. For the purpose of this study we used the donor site of a skin graft as a model for an acute surgical wound. Under aseptic conditions and general anaesthesia skin grafts were obtained by using an electric dermatome (Aesculaap®) with a thickness of 0.3 mm. Biopsies of normal skin obtained on day 0 (=day of operation) were used as controls. To collect wound fluid donor sites were covered with a layer of Gordasoft®, then a polyvinylalcohol (PVA) sponge was applied to the wound (Coldex®) and on top of that a transparent dressing (Tegaderm®), as previously described [29].

Study protocol. The initial protocol was to sample on day 2, 5 and 10 after surgery. However, on day 10 wound fluid samples could only be harvested in three patients, as in the other patients no wound fluid was present in the sponge. Twenty-four hours before sampling, the dressing was changed by removing the transparent dressing and the PVA-sponge and reapplying a new sponge and transparent dressing. On the day of sampling, biopsies were taken under local anaesthesia (1% lidocaine), using a 3 mm punch biopsy from the central part of the wound. The sponge was removed, and immediately stored on ice. Simultaneously a venous blood sample was drawn from a major vein in the cubital fossa and also put on ice. After sampling a new dressing was applied to the wound.

Table 1
Patient characteristics.

Patient	F/M ^a	Age	BMI ^b	Diagnosis
1	M	52	31.4	Burn wounds of the thorax
2	M	43	26.6	Open fracture leg
3	M	32	24.7	Chronic wound ankle
4	F	35	19.7	Defect face after trauma
5	M	75	24.6	Sarcoma of the leg
6	M	36	40.2	Chronic ulcer leg
7	M	70	23.5	SCC leg
8	F	27	27.9	Trauma of the hand
9	M	39	21.2	Leg amputation
10	M	68	25.8	Defect face after SCC ^c
Mean		47.2	26.5	
SEM		5.3	1.9	
Min/max		27–75	19.7–40.2	

^a Female/male.

^b Body Mass Index.

^c Squamous cell cancer.

Sample collection, processing and analysis

Blood was centrifuged at 4 °C for 10 min (4000 rpm) within 1 h after sampling. After centrifugation, 500 µl of plasma was deproteinized using 20 mg dry sulphosalicylic acid (SSA), vortexed and frozen in liquid nitrogen. Samples were stored at –80 °C until analysis. To obtain wound fluid from acute wounds, sponges were centrifuged at 4 °C for 10 min (11,000 rpm). After centrifugation, 500 µl of wound fluid was treated similar to the plasma.

Amino acid concentrations

Plasma and wound fluid amino acids were determined using a fully automated High Performance Liquid Chromatography system (HPLC) as described previously. Nitrate/nitrite concentrations in plasma and wound fluid were also determined using HPLC [6].

Nitrate/nitrite (NOx) concentrations

To study production of NO by NOS we measured nitrite and nitrate concentrations. The sum of both nitrite and nitrate (NOx) is used as an indirect indicator of NO-production as described in several studies [6,14,30]. NOx concentrations in plasma and wound fluid were determined using HPLC [6,31].

Elisa for arginase-1

Plasma and wound fluid arginase-1 levels were measured using sandwich ELISA [32]. Standard, primary and secondary antibodies were kindly provided by HBT (Uden, The Netherlands). In short, a 96-wells plates were coated overnight at 4 °C with 100 µL of the purified anti-human liver-type arginase monoclonal IgG (first antibody; Mo6G3, 5 mg/L carbonate buffer, pH 9.5). Plasma samples were prediluted 1:2 in dilution buffer (1× PBS–0.1% BSA) and wound fluid samples 1:400. Samples were added to the plate. Bound arginase was detected with a second biotinylated antibody (Mo9C5 in dilution buffer 1:200), followed by peroxidase-conjugated streptavidin and TMB.

Western blot arginase-2

Plasma or wound exudates were centrifuged at 10,000 rpm for 5 min, supernatants were collected and total protein concentrations were determined using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL).

Aliquots with equal amounts of protein were heated at 100 °C for 5 min in Laemmli buffer with β-mercaptoethanol, separated on 10% SDS–polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore, Bedford, MA). After incubation with primary antibody, 1 µg/ml anti-human arginase-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, USA) and washing, membranes were incubated with a horseradish peroxidase-conjugated secondary anti mouse IgG. Positive bands were detected using the chemiluminescent substrate Supersignal West Pico (Pierce Chemical Co.) and transferred onto an X-ray film.

Immunohistochemical staining

The cellular distribution of arginine-metabolizing enzymes was studied in man during the process of wound healing. Sequential biopsies were collected in a period of 10 days post-wounding. Skin tissue samples were stained with arginase-1, arginase-2, iNOS and eNOS and compared them to normal skin. For immunophenotyping a standard staining with haematoxylin and eosin (H&E), alpha-smooth muscle actin (ASMA), CD31 and CD68 was performed (data not shown).

Biopsies were fixed in 4% buffered formaldehyde, processed by routine histological procedures and embedded in paraffin. Four micrometre sections were subsequently obtained from each paraffin block. Specimens were initially stained with H&E. Parallel sections were immunohistochemically stained for ASMA (smooth muscle cell staining, Dako, monoclonal mouse–anti-human, 1:500), CD31 (endothelial cell staining, Dako, monoclonal mouse–anti-human, 1:50, pre-treatment TRIS/EDTA/pH 8.0, blocking 5% BSA) and CD68 (macrophage staining, DAKO, monoclonal antibody, pre-treatment pepsin 1:100, no blocking). Subsequently sections were stained using polyclonal rabbit anti-human-iNOS antibody (Zymed Laboratories, Inc., South San Francisco, USA), polyclonal goat anti-human eNOS antibody (R&D systems, Las Vegas, USA), rabbit-anti-arginase-1 (Sigma–Aldrich, St. Louis, USA) and rabbit anti-human type-2 Arginase (kindly provided by Tomomi Gotoh, Kumamoto University, Japan). Briefly, slides were dewaxed and rehydrated in decreasing concentrations of alcohol. For arginase-1, slides were pre-heated in citrate buffer. Sections were then incubated for 30 min with iNOS, eNOS, arginase-1 and arginase-2 antibodies (resp. diluted 1:50, 1:10, 1:750, and 1:500 in 1% BSA/TBS/0.1% Tween-20 solution). Then slides were treated with biotinylated anti-rabbit (diluted 1:200, Dako) or biotinylated anti-goat (diluted 1:400, Dako) and treated with avidin–biotin–peroxidase–complex (Dako). Positive staining was visualized by applying alkaline phosphatase (Vector Laboratories, Inc., Burlingame) and slides were counter-stained with Mayer's haematoxylin. Negative controls were treated in the same manner but with the omission of the primary antibodies. The slides were independently reviewed in a blinded fashion by two observers, by light microscope. For determination of the wound score, slides were rated semi-quantitatively on a score of 0–4 for cells with cytoplasmic staining. 0 (negative staining); 1 (0–25% of the cells stained positive); 2 (26–50% of the cells stained positive), 3 (51–75% of the cells stained positive) and 4 (76–100% of the cells stained positive).

Statistical analysis

Data are expressed as mean \pm SEM. Amino acid concentrations were expressed as μ M. Differences of plasma and wound fluid parameters were analysed using ANOVA repeat measurements tests from SPSS, this statistical package for social sciences (SPSS) software was used for statistical analysis. In all cases, $p < 0.05$ was considered statistically significant.

Results

Patient characteristics

Patient characteristics are shown in Table 1. Eight males and two females entered the study, mean age was 47 years. Patients were in a general good condition. Mean Body Mass Index was 26.5 and none of the patients had suffered from weight loss $> 10\%$ of bodyweight prior to our study (data not shown). Patients needed reconstruction of soft tissue defects for different reasons (Table 1). Seven patients underwent a free vascularized tissue transfer in combination with skin transplantation, three patients received only skin transplantation. All donor sites healed without complications.

Plasma and wound fluid amino acid concentrations

Wound fluid samples were collected on day 2, 5 and 10. At day 10, wound fluid was only harvested from three out of ten patients, and therefore these data were excluded from analysis.

Plasma arginine levels did not differ over time and were similar to healthy humans [31]. Wound fluid arginine levels were lower

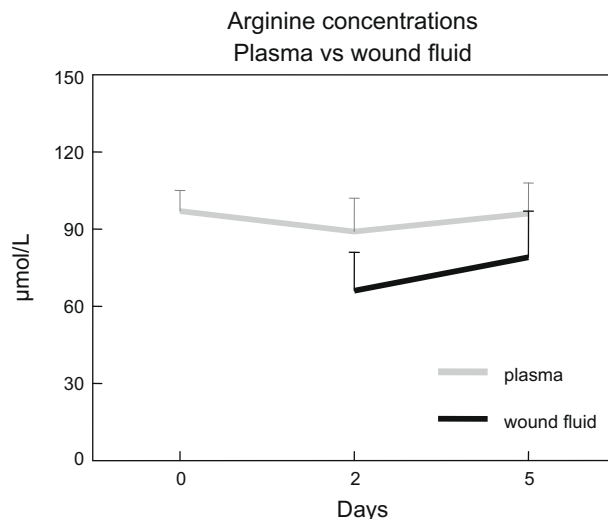


Fig. 1. Concentrations of arginine in plasma and wound fluid (μ M). Plasma samples are obtained pre-operatively, and days 2–5 post-operative. Wound fluid samples are obtained days 2–5 post-operative. Data are expressed as mean \pm SEM, $n = 10$, $*p < 0.05$.

compared to plasma, however this difference was not significant (Fig. 1).

Plasma citrulline levels showed no significant difference over time and were similar to healthy humans [31]. In contrast to arginine, wound fluid citrulline levels were significantly higher than plasma levels (Fig. 2).

Plasma ornithine levels were higher than normal healthy subjects (normal value: $57 \pm 1.0 \mu$ M [33]). Although an incline was observed from day 2 to 5, no significance was reached over time. Wound fluid ornithine levels were higher compared with plasma. A significant difference was seen on day 2 (Fig. 3).

Plasma and wound fluid NOx

Comparison of plasma and wound fluid nitrite/nitrate levels shows significantly higher NO concentration in wound fluid compared with plasma (Fig. 4).

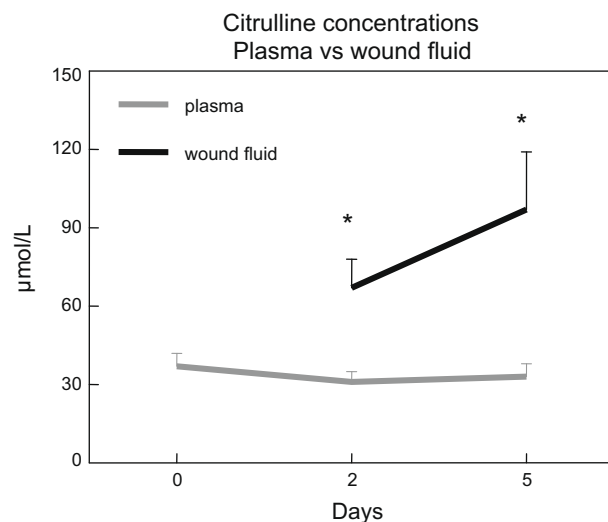


Fig. 2. Concentrations of citrulline in plasma and wound fluid (μ M). Plasma samples are obtained pre-operatively, and days 2–5 post-operative. Wound fluid samples are obtained days 2–5 post-operative. Data are expressed as mean \pm SEM, $n = 10$, $*p < 0.05$.

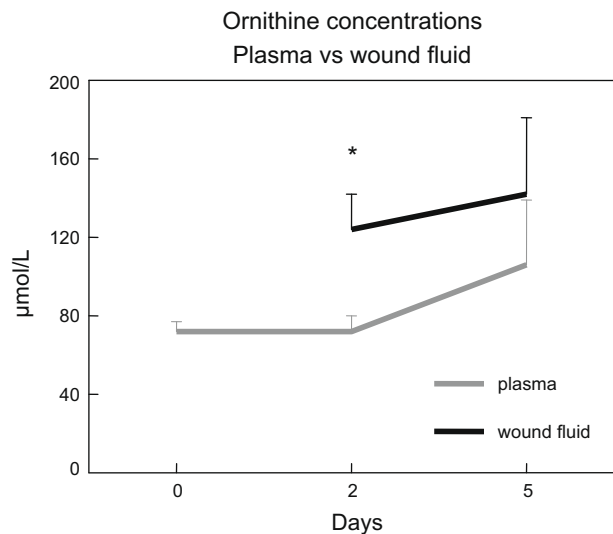


Fig. 3. Concentrations of ornithine in plasma and wound fluid (μM). Plasma samples are obtained pre-operatively, and days 2–5 post-operative. Wound fluid samples are obtained days 2–5 post-operative. Data are expressed as mean \pm SEM, $n = 10$, * $p < 0.05$.

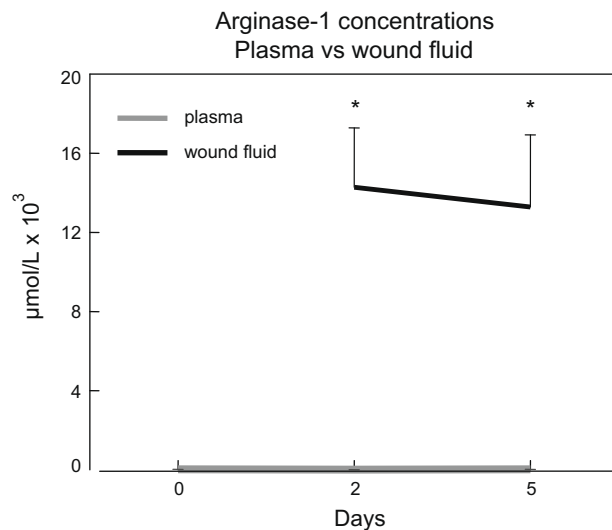


Fig. 5. Arginase-1 concentrations in plasma and wound fluid (μM). Measurements were performed in plasma on the day of operation (day 0), day 2 and 5 post-operatively. Wound fluid samples were only measured on day 2 and 5 post-operatively. Data are expressed as mean \pm SEM, $n = 10$, * $p < 0.05$.

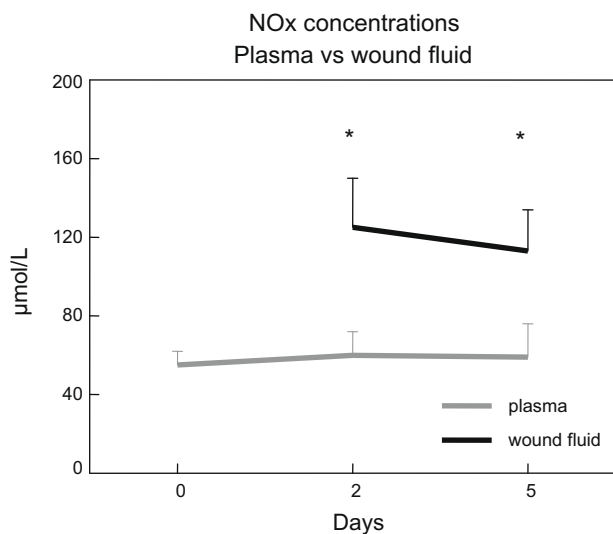


Fig. 4. Concentrations of NOx (=nitrate + nitrite) in plasma and wound fluid (μM). Plasma samples are obtained pre-operatively, and days 2–5 post-operative. Wound fluid samples are obtained days 2–5 post-operative. Data are expressed as mean \pm SEM, $n = 10$, * $p < 0.05$.

Plasma and wound fluid arginase-1

Comparison of plasma and wound fluid Arginase-1 concentrations revealed significantly higher concentrations in wound fluid compared with plasma (Fig. 5).

Plasma and wound fluid arginase-2

Arginase-2 expression was investigated by Western blot analysis. Using densitometric analysis with LeicaQwin software (Leica Microsystems Switzerland, Ltd.), we could observe increased levels of ARG2 in plasma (Fig. 6A and B).

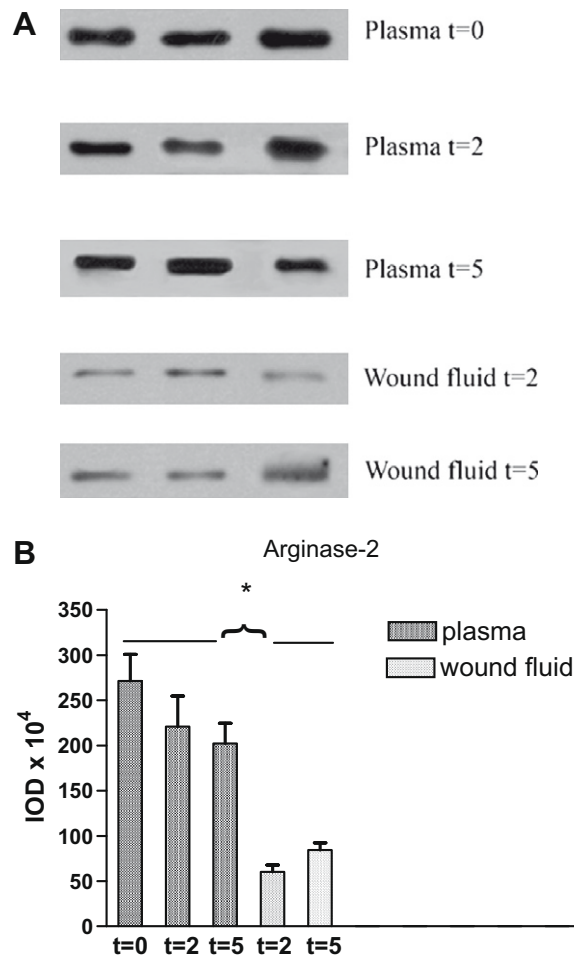


Fig. 6. (A and B) Arginase-2 levels in plasma and wound fluid. Enzyme detection by Western blot (A). Densitometric quantification of levels, expressed as IOD; mean \pm SEM, $n = 10$, * $p < 0.05$ (B).

Immunohistochemical distribution of iNOS, eNOS and arginase-2 in wounds

The basic histological pattern was similar for all wound sections. Haematoxylin and eosin-stained wound tissue showed a connective tissue matrix filled with fibrin, inflammatory cells, fibroblasts, glandular cells, vessels, smooth muscle cells.

iNOS (Fig. 7)

In unwounded skin, some constitutive expression was detected in epithelial cells, smooth muscle and endothelial cells. This was in contrast with the abundant staining in wounds (Fig. 7A, magnification 20 \times), where iNOS was additionally expressed by macrophages, fibroblasts and PMN's (Fig. 7B, magnification 40 \times). Semi-quantitative analysis showed significantly more positive cells and cell types in wounds compared to normal skin (Table 2). Negative control was negative (Fig. 7C, magnification 20 \times).

eNOS (Fig. 8)

In unwounded skin, moderate eNOS expression was observed in some of the endothelial cells. In addition, mild eNOS staining was found in keratinocytes and smooth muscle (Fig. 8A, magnification 20 \times). Semi-quantitative analysis showed equal expression of eNOS in vessels in wounds at all time points $p < 0.05$ (Table 2). However, in wounded skin the number of blood vessels was significantly increased from day 5 to 10 compared with normal skin (16.1 ± 1.7 to 28.1 ± 3.8 vessels/ μm^2 , $p < 0.05$) (Fig. 8B, magnification 40 \times). Negative control was negative (Fig. 8C, magnification 20 \times).

Arginase-1 (Fig. 9)

In unwounded skin no arginase-1 staining is observed (Fig. 9A, magnification 20 \times). In wounded tissue arginase-1 staining is only

Table 2

Cell types expressing iNOS, eNOS, arginase-1 and arginase-2, normal (unwounded) versus wounded skin. Mean score of enzyme expression on a four point scale; 0: no expression, 1: 1–25% expression, 2: 26–50% expression, 3: 51–75% expression and 4: 76–100% expression. Mean percentage \pm SD is shown.

Cell type	Unwounded	Day 2	Day 5	Day 10
iNOS				
Epithelial cells	0*	0.75 \pm 0.35	0.4 \pm 0.5	0.6 \pm 0.3
Fibroblasts	0*	0.7 \pm 0.3	0.5 \pm 0.3	0.7 \pm 0.4
PMN's	0*	0.7 \pm 0.2	0.35 \pm 0.1	0.25 \pm 0.0
Macrophages	0*	0.5 \pm 0.1	0.7 \pm 0.3	0.7 \pm 0.2
Endothelial cells	0*	0.8 \pm 0.4	0.7 \pm 0.3	0.8 \pm 0.4
Glandular cells	0*	0.9 \pm 0.2	1.0 \pm 0.0	0.65 \pm 0.5
eNOS				
Epithelial cells	1.0 \pm 0.0	1.0 \pm 0	0.5 \pm 0.5	1.0 \pm 0
Fibroblasts	0	0.3 \pm 0.2	0.2 \pm 0.1	0.3 \pm 0.2
PMN's	0	0.2 \pm 0.2	0.1 \pm 0.1	0.25 \pm 0.2
Macrophages	0	0	0	0
Endothelial cells	1.0 \pm 0.0	1.0 \pm 0	1.0 \pm 0	1.0 \pm 0
Glandular cells	0	0	0	0
ARG2				
Epithelial cells	0.5, 0 \pm 0.1*	1.0 \pm 0.2	1.0 \pm 0.1	1.0 \pm 0.2
Fibroblasts	0*	1.0 \pm 0	1.0 \pm 0	1.0 \pm 0
PMN's	0*	0.9 \pm 0.4	0.9 \pm 0.25	0.3 \pm 0.4
Macrophages	0*	0.6 \pm 0.2	0.5 \pm 0.1	0.7 \pm 0.3
Endothelial cells	0*	1.0 \pm 0	1.0 \pm 0	1.0 \pm 0
Glandular cells	0.5 \pm 0.3*	1.0 \pm 0	1.0 \pm 0	1.0 \pm 0
ARG1				
Epithelial cells	0	0	0	0
Fibroblasts	0	0	0	0
PMN's	0*	1.0 \pm 0	1.0 \pm 0	1.0 \pm 0
Macrophages	0	0	0	0
Endothelial cells	0	0	0	0
Glandular cells	0	0	0	0

PMN's: polymorphonuclear cells.

* $p < 0.05$ versus days 2, 5 and 10 post-wounding.

seen in PMN's (Fig. 9B, magnification 40 \times). Macrophages do not show positive staining. Semi-quantitative scoring shows signifi-

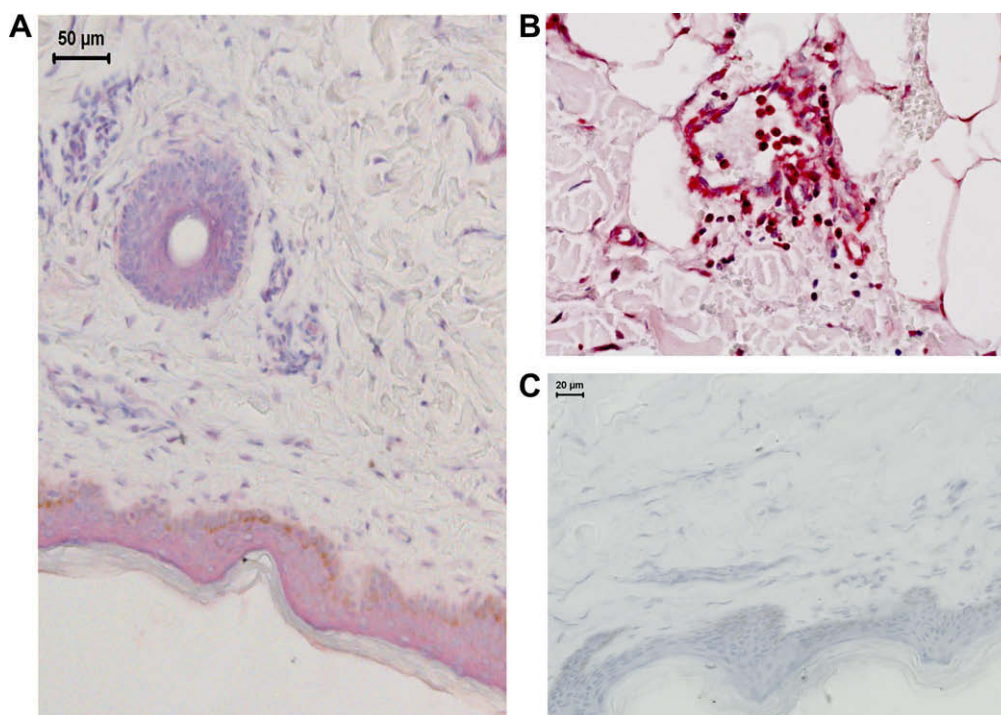


Fig. 7. (A–C) iNOS-staining in normal human skin and wounds 10 days post-wounding. Normal skin shows constitutive iNOS-staining in epithelial cells, smooth muscle and endothelial cells (A, magnification 20 \times). Wounded skin shows similar findings, in addition fibroblasts, PMN's and macrophages stained positive during wound healing (B, magnification 40 \times). Negative control (C, magnification 20 \times).

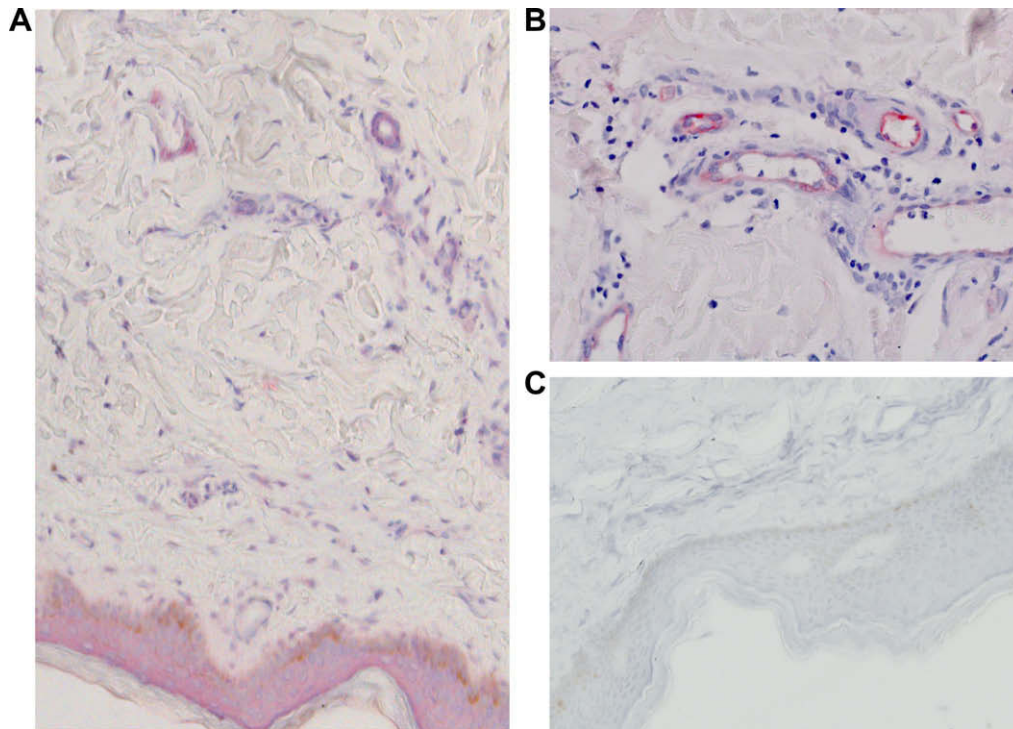


Fig. 8. (A–C) eNOS-staining in normal human skin and wounds 10 days post-wounding. Normal skin shows constitutive eNOS-staining in few endothelial cells and keratinocytes (A, magnification 20 \times). Wound tissue shows positive eNOS-staining predominantly in vessels and some in fibroblasts (B, magnification 40 \times). Negative control (C, magnification 20 \times).

cant more ARG1 expression in PMN's post-wounding compared to normal skin and equal expression of positive PMN's at different days post-wounding (Table 2). Negative control was negative (Fig. 9C, magnification 20 \times).

Arginase-2 (Fig. 10)

In unwounded skin constitutive staining was observed in keratinocytes, smooth muscle and endothelial cells (Fig. 10A, magnifica-

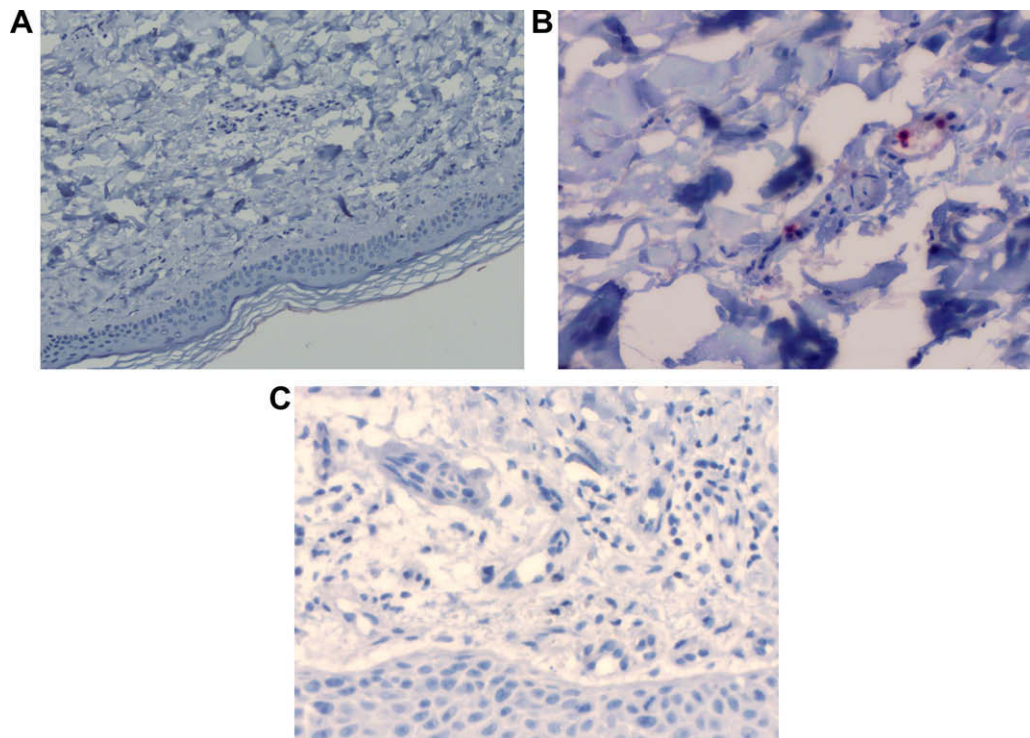


Fig. 9. (A–C) Arginase-1 staining in normal skin and wounds 10 days post-wounding. Normal skin shows no arginase-1 staining (A, magnification 20 \times). In wounded skin arginase-1 staining is only seen in PMN's. Macrophages are not stained (B, magnification 40 \times). Semi-quantitative scoring shows significant more ARG1 positively stained PMN's in wounds compared to normal skin and equal expression of positive PMN's at different days post-wounding (Table 2). Negative control (C, magnification 20 \times).

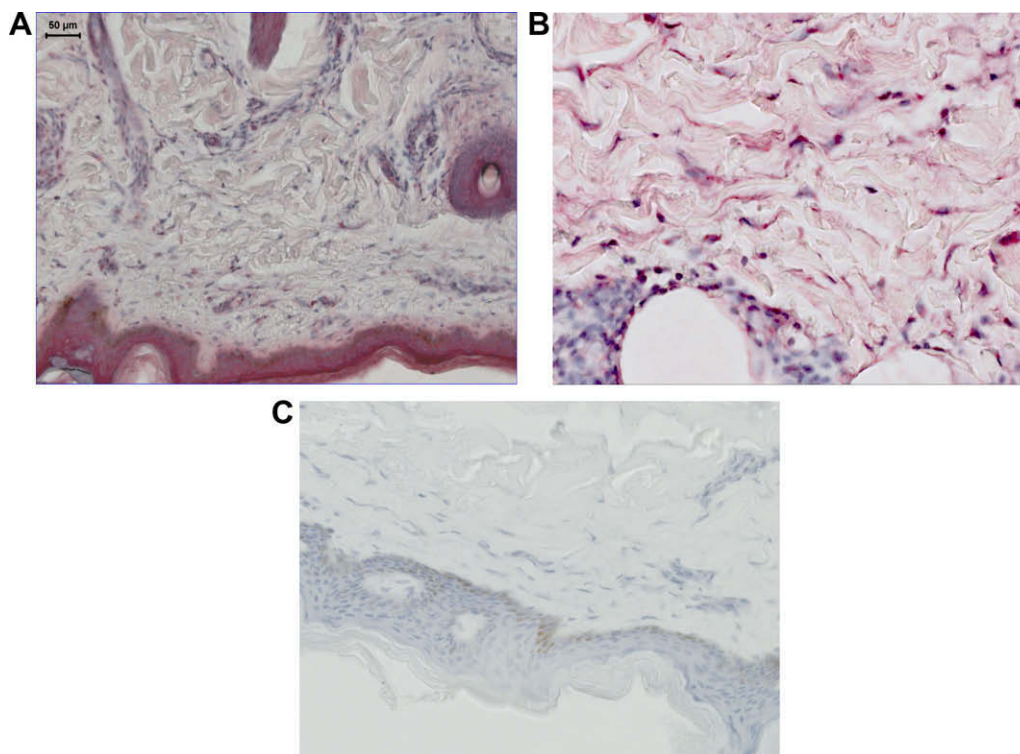


Fig. 10. (A–C) arginase-2-staining in normal human skin and wounds 10 days post-wounding. Constitutive staining was observed in keratinocytes, smooth muscle and endothelial cells of normal skin (A, magnification 20 \times). In wounds predominantly fibroblasts, macrophages and PMN's stained positive (B, magnification 40 \times). Negative control was negative (C, magnification 20 \times).

tion 20 \times). All cells showed milder expression of arginase-2 in unwounded skin. In wounded tissue (Fig. 10B, magnification 40 \times) additional staining of macrophages, fibroblasts and PMN's was observed when compared to healthy skin. Semi-quantitative analysis showed significant more cells expressing ARG2 compared to normal skin (Table 2). No difference in ARG2-expression was observed at sequential time points during healing. Negative control was negative (Fig. 10C, magnification 20 \times).

Discussion

Data from animal studies suggested that arginine metabolism occurs during wound healing. In addition, arginine supplementation in animals and humans appears to improve skin repair. However the mechanisms underlying this effect remain unidentified. To our opinion, this is the first study supporting the role of arginine metabolism in normal wound healing in man. It provides insight in the mechanisms by showing expression of arginine-metabolites and arginine-metabolizing enzymes at different time points during normal healing of surgical wounds, compared with normal skin.

The supporting evidence is shown by increased levels of wound fluid citrulline and NO $_x$ when compared with plasma, indicating arginine conversion by NOS [14,34,35]. Moreover, a significant rise of ornithine detected in wound fluid compared with plasma suggests that L-arginine is also metabolized by arginase. Since citrulline and ornithine are not released by protein breakdown, these findings are indicative for arginine metabolism both through NOS and arginase-pathways. This suggestion is also supported by levels of ARG1 and ARG2 detected in wound fluid. Although not significant, arginine levels in wound fluid tend to be lower compared to plasma. This is in line with animal studies [35,36,14], which showed decreased arginine levels in wound fluid when compared

with plasma during healing, suggesting arginine consumption at the wound site [34,37–39].

We observed simultaneous increase levels of citrulline and ornithine in wound fluid. Experimental studies show temporal elevation of NO $_x$ and ornithine in wound fluid when compared to plasma, suggesting sequential activation of the different arginine metabolic pathways [35,39]. Moreover, others like Finnen et al. suggest a time course requirement for different arginine metabolites, as increased healing upon NO-application in the early healing phase was observed [40,41]. Since the number of sequential samples was limited, we cannot draw conclusions about the time course activation or requirements of arginine metabolism. Unfortunately, more extensive sampling was not ethical for our patients.

The mechanisms by which arginine stimulates cutaneous wound repair is still not completely understood. In the present study, we observed iNOS expression in macrophages, PMN's, fibroblasts, epithelial and endothelial cells upon wounding. In contrast, these cells did not express iNOS in normal skin, indicating wounding activates arginine metabolism. All these cells have specific functions during the complex process of healing and NO has been implicated as a mediator. PMN's and macrophages are needed to debride the wound area and attract other cells important for wound healing. Human neutrophils generate NO [42–44] which modulates their migration [45]. In addition, activated macrophages and neutrophils need arginine for adequate NO-synthesis [46–48]. Moreover, after arginine supplementation, an increased myeloperoxidase-activity in PMN's is observed, that was accompanied by increased NO levels and wound tensile strength [49–51]. Fibroblasts are responsible for collagen formation in granulation tissue, a process essential in tissue repair. In vitro and animal studies show that NO regulates collagen synthesis in wound fibroblasts [25,38,52,39,53]. Wang et al. showed that fibroblasts derived from human skin express eNOS and iNOS upon stimulation with LPS and INF- γ [54,55]. This is in line with our findings, as eNOS and iNOS were ex-

pressed in fibroblasts of wounds at all time points, suggesting NO-mediated collagen formation in wound healing in man (Table 2). Neovascularization is of central importance in wound healing. In vitro studies showed that VEGF, the most potent known angiogenic protein, is induced by NO [56,57]. Inhibition of NO-synthesis impairs VEGF expression and angiogenesis. Moreover, it delays wound healing [58–61]. We observed constitutive expression of eNOS in endothelial cells, which did not alter upon wounding, whereas iNOS was expressed in endothelial cells only after injury. Comparable findings were observed in keratinocytes in present study. They showed a constitutive expression of eNOS and iNOS in normal skin, whereas after injury a more abundant expression of iNOS was noticed. Keratinocytes in normal human skin express all NOS isoforms [61] and iNOS is induced in proliferating keratinocytes upon cutaneous injury in rodents [62]. As all our wounds healed without complications, we speculate that the up-regulation of iNOS/eNOS as seen in the present study implicates that normal human wound healing is NO-mediated, and therefore arginine is required.

Arginase, the other major L-arginine-consuming enzyme, regulates polyamines and proline synthesis through the production of ornithine [1,63]. In addition, arginase regulates NO-formation through substrate competition [64–66,67]. Previous animal and in vitro studies showed exclusive up regulation of ARG1 during normal wound healing, which was required for collagen formation [25]. Human studies that addressed the role of arginases during wound healing are limited. They described overexpression of arginase in pathological wound states (e.g. diabetes, psoriasis and venous ulcers) [13,16,68]. We detected ARG1 in both plasma and wound fluid. Surgery and sepsis are known to induce elevated plasma levels of ARG1. It has been suggested that ARG1 is released by inflammatory cells, hepatocytes and erythrocytes in order to decrease T-cell immunity [69–72]. In this study, ARG1 levels were significantly higher wound fluid than in plasma, supporting our hypothesis of arginine metabolism through ARG1 in the wound environment. In contrast, we measured higher plasma ARG2 levels compared with wound fluid (Fig. 6B). ARG2 has a wide tissue distribution, with the highest expression in kidney, prostate and vasculature. In vessels it appears to be involved in the regulation of vascular tone and atherosclerosis [66]. Only Corraliza et al. measured ARG2 in joint fluid from arthritic joints and suggested that this contributed to the disturbed healing process [15]. At present, we do not have an explanation for the elevated levels of ARG2 in plasma and wound fluid observed in this study.

Interestingly we observed coexpression of ARG1 and ARG2 in PMN's, at all time points post-wounding, while no expression was seen in PMN's of normal skin. Coexpression of ARG1 and ARG2 has not yet been reported in PMN's [69]. However, it has been observed in endothelial cells, where intracellular ARG1 and ARG2 compete for arginine, leading to decreased NO-production. A specific role for arginase isotypes has been suggested; ARG1: ornithine synthesis and ARG2: polyamines production [73]. Jacobsen suggested that the function of release of arginase from PMN's is to reduce NO-formation by macrophages, endothelial and T-cells [74]. All, these previous findings could apply to PMN function in human wound healing, but the complexity of wound healing in vivo warrants more research. Another interesting finding is that we only observed ARG2 expression in macrophages after injury. Arginase expression by macrophages seems to be species and cell specific as murine macrophages express ARG1 and ARG2, rat macrophages ARG1 [25,75,76] and human myeloid cells express ARG1 [27]. The role of arginase in activated macrophages seems to be down regulation of NO-production through substrate competition which is more pronounced when L-arginine availability is reduced [76,77]. Gotoh et al. suggested early and late arginase expression in macrophages: first ARG2 to produce polyamines for macrophage growth and differentiation and later on ARG1 to produce ornithine

for collagen production [77]. It is possible that assessment of ARG in this study was only conducted in the early ARG-expressing phase. Alternatively, different arginase isotypes may play different roles during normal and pathological healing.

Comparable findings were observed in fibroblasts, only expressing ARG2 upon injury. In rats, up-regulation of only ARG1 is detected in fibroblasts after wounding to provide substrate for collagen-synthesis and cell proliferation [25,78].

Furthermore, our study shows ARG2 expression in endothelial cells. Non-human endothelial cells express ARG1. Unstimulated human endothelial cells express some ARG2, which increases upon stimulation [79,80]. Arginase seems to be involved in vascular function [65]. In addition, Li et al. suggested ARG1 is needed for ornithine and ARG2 for polyamines production by bovine endothelial cells [73,24]. Which makes both pathways important for the formation of new blood vessels, an essential process for wound healing.

Finally, expression of ARG2 in epithelial cells of healthy skin was seen, and increased in wounds. The expression of ARG2 in normal skin was already seen by Wohlrab et al. [81]. Present study is the first to show that expression of ARG2 is increased in normal wound healing in man.

In summary, our findings support previous experimental studies suggesting arginine metabolism for an NO-mediated as well as arginase-mediated repair of injured skin. Our findings suggest a more prominent role of ARG2 in normal healing. As it is known that ARG1 is more activated in pathological skin healing our finding may have important therapeutic consequences. Inhibition of ARG1 and stimulation of ARG2 during pathological wound healing might be considered in the future. More work remains to be done to translate the needs of injured skin into clinically useful agents.

Conflict of interest

The authors state no conflict of interest.

References

- [1] J.N. Curran, D.C. Winter, D. Bouchier-Hayes, Biological fate and clinical implications of arginine metabolism in tissue healing, *Wound Repair Regen.* 14 (4) (2006) 376–386.
- [2] M.B. Witte, A. Barbul, Role of nitric oxide in wound repair, *Am. J. Surg.* 183 (4) (2002) 406–412.
- [3] J.S. Pollock et al., Nitric oxide synthase isoform expression in a porcine model of granulation tissue formation, *Surgery* 129 (3) (2001) 341–350.
- [4] M.R. Schaffer et al., Nitric oxide metabolism in wounds, *J. Surg. Res.* 71 (1) (1997) 25–31.
- [5] K.A. Norris et al., Enhancement of macrophage microbicidal activity: supplemental arginine and citrulline augment nitric oxide production in murine peritoneal macrophages and promote intracellular killing of *Trypanosoma cruzi*, *Infect. Immun.* 63 (7) (1995) 2793–2796.
- [6] M.J. Bruins et al., L-Arginine supplementation in hyperdynamic endotoxemic pigs: effect on nitric oxide synthesis by the different organs, *Crit. Care Med.* 30 (3) (2002) 508–517.
- [7] M.R. Schaffer et al., Acute protein-calorie malnutrition impairs wound healing: a possible role of decreased wound nitric oxide synthesis, *J. Coll. Surg.* 184 (1) (1997) 37–43.
- [8] J.G. Nirgiotis, P.J. Hennessey, R.J. Andrassy, The effects of an arginine-free enteral diet on wound healing and immune function in the postsurgical rat, *J. Pediatr. Surg.* 26 (8) (1991) 936–941.
- [9] A. Barbul et al., Arginine enhances wound healing and lymphocyte immune responses in humans, *Surgery* 108 (2) (1990) 331–336 (discussion 336–337).
- [10] S.J. Kirk et al., Arginine stimulates wound healing and immune function in elderly human beings, *Surgery* 114 (2) (1993) 155–159 (discussion 160).
- [11] M. Shashidharan et al., Influence of arginine dietary supplementation on healing colonic anastomosis in the rat, *Dis. Colon Rectum* 42 (12) (1999) 1613–1617.
- [12] M.A. Arbs et al., Early effects of exogenous arginine after the implantation of prosthetic material into the rat abdominal wall, *Life Sci.* 67 (20) (2000) 2493–2512.
- [13] S.A. Abd-El-Aleem et al., Expression of nitric oxide synthase isoforms and arginase in normal human skin and chronic venous leg ulcers, *J. Pathol.* 191 (4) (2000) 434–442.

- [14] J.E. Albina et al., Temporal expression of different pathways of L-arginine metabolism in healing wounds, *J. Immunol.* 144 (10) (1990) 3877–3880.
- [15] I. Corraliza, S. Moncada, Increased expression of arginase II in patients with different forms of arthritis. Implications of the regulation of nitric oxide, *J. Rheumatol.* 29 (11) (2002) 2261–2265.
- [16] E.B. Jude et al., The role of nitric oxide synthase isoforms, arginase in the pathogenesis of diabetic foot ulcers: possible modulatory effects by transforming growth factor beta 1, *Diabetologia* 42 (6) (1999) 748–757.
- [17] H. Kampfer, J. Pfeilschifter, S. Frank, Expression and activity of arginase isoenzymes during normal and diabetes-impaired skin repair, *J. Invest. Dermatol.* 121 (6) (2003) 1544–1551.
- [18] S.M. Morris Jr., Regulation of enzymes of urea and arginine synthesis, *Annu. Rev. Nutr.* 12 (1992) 81–101.
- [19] E. Adams, L. Frank, Metabolism of proline and the hydroxyprolines, *Annu. Rev. Biochem.* 49 (1980) 1005–1061.
- [20] J.E. Albina, J.A. Abate, B. Mastrofrancesco, Role of ornithine as a proline precursor in healing wounds, *J. Surg. Res.* 55 (1) (1993) 97–102.
- [21] K.N. Dolynchuk, R. Bendor-Samuel, J.M. Bowness, Effect of putrescine on tissue isoenzymes activity in wounds: decreased breaking strength and increased matrix fucoprotein solubility, *Plast. Reconstr. Surg.* 93 (3) (1994) 567–573.
- [22] J. Lesiewicz, L.A. Goldsmith, Inhibition of rat skin ornithine decarboxylase by nitrofurazone, *Arch. Dermatol.* 116 (11) (1980) 1225–1226.
- [23] Y. Maeno et al., A study on the vital reaction in wounded skin: simultaneous determination of histamine and polyamines in injured rat skin by high-performance liquid chromatography, *Forensic Sci. Int.* 46 (3) (1990) 255–268.
- [24] G. Wu, S.M. Morris Jr., Arginine metabolism: nitric oxide and beyond, *Biochem. J.* 336 (Pt. 1) (1998) 1–17.
- [25] M.B. Witte et al., Upregulation of arginase expression in wound-derived fibroblasts, *J. Surg. Res.* 105 (1) (2002) 35–42.
- [26] V. Bansal, J.B. Ochoa, Arginine availability, arginase, and the immune response, *Curr. Opin. Clin. Nutr. Metab. Care* 6 (2) (2003) 223–228.
- [27] V. Bronte et al., L-Arginine metabolism in myeloid cells controls T-lymphocyte functions, *Trends Immunol.* 24 (6) (2003) 302–306.
- [28] M. Mori, Regulation of nitric oxide synthesis and apoptosis by arginase and arginine recycling, *J. Nutr.* 137 (6 Suppl. 2) (2007) 1616S–1620S.
- [29] I.B. Debats et al., Oral arginine supplementation and the effect on skin graft donor sites: a randomized clinical pilot study, *J. Burn Care Res.* 30 (3) (2009) 417–426.
- [30] G. Giovannoni, Adaptation of the nitrate reductase and Griess reaction methods for the measurement of serum nitrate plus nitrite levels, *Ann. Clin. Biochem.* 34 (Pt. 2) (1997) 193–198.
- [31] H.M.H. van Eijk, C.H.C. Dejong, N.E.P. Deutz, P.B. Soeters, Influence of storage conditions on normal plasma amino-acid concentrations, *Clin. Nutr.* 13 (1994) 374–380.
- [32] M. Ikemoto et al., A useful ELISA system for human liver-type arginase, and its utility in diagnosis of liver diseases, *Clin. Biochem.* 34 (6) (2001) 455–461.
- [33] J.Z. Williams, N. Abumrad, A. Barbul, Effect of a specialized amino acid mixture on human collagen deposition, *Ann. Surg.* 236 (3) (2002) 369–374 (discussion 374–375).
- [34] J.E. Albina et al., Arginine metabolism in wounds, *Am. J. Physiol.* 254 (4 Pt. 1) (1988) E459–E467.
- [35] R.H. Lee et al., Nitric oxide in the healing wound: a time-course study, *J. Surg. Res.* 101 (1) (2001) 104–108.
- [36] E. Seifter et al., Arginine: an essential amino acid for injured rats, *Surgery* 84 (2) (1978) 224–230.
- [37] D.T. Efron et al., A novel method of studying wound healing, *J. Surg. Res.* 98 (1) (2001) 16–20.
- [38] M.R. Schaffer et al., Nitric oxide regulates wound healing, *J. Surg. Res.* 63 (1) (1996) 237–240.
- [39] G. Zunic et al., Increased nitric oxide formation followed by increased arginase activity induces relative lack of arginine at the wound site and alters whole nutritional status in rats almost within the early healing period, *Nitric Oxide* 20 (4) (2009) 253–258.
- [40] R. Weller, M.J. Finnen, The effects of topical treatment with acidified nitrite on wound healing in normal and diabetic mice, *Nitric Oxide* 15 (4) (2006) 395–399.
- [41] T.P. Amadeu et al., Nitric oxide donor improves healing if applied on inflammatory and proliferative phase, *J. Surg. Res.* 149 (1) (2008) 84–93.
- [42] C.D. Wright et al., Generation of nitric oxide by human neutrophils, *Biochem. Biophys. Res. Commun.* 160 (2) (1989) 813–819.
- [43] M. Markert, B. Carnal, J. Mael, Nitric oxide production by activated human neutrophils exposed to sodium azide and hydroxylamine: the role of oxygen radicals, *Biochem. Biophys. Res. Commun.* 199 (3) (1994) 1245–1249.
- [44] E. Jablonska et al., iNOS expression and NO production by neutrophils in cancer patients, *Arch. Immunol. Ther. Exp. (Warsz)* 53 (2) (2005) 175–179.
- [45] S. Nolan et al., Nitric oxide regulates neutrophil migration through microparticle formation, *Am. J. Pathol.* (2007).
- [46] V. Bronte, P. Zanovello, Regulation of immune responses by L-arginine metabolism, *Nat. Rev. Immunol.* 5 (8) (2005) 641–654.
- [47] J.S. Reichner et al., Molecular and metabolic evidence for the restricted expression of inducible nitric oxide synthase in healing wounds, *Am. J. Pathol.* 154 (4) (1999) 1097–1104.
- [48] N. Mullner, A. Lazar, A. Hrabak, Enhanced utilization and altered metabolism of arginine in inflammatory macrophages caused by raised nitric oxide synthesis, *Int. J. Biochem. Cell Biol.* 34 (9) (2002) 1080–1090.
- [49] S. Moncada, R.M. Palmer, E.A. Higgs, Nitric oxide: physiology, pathophysiology, and pharmacology, *Pharmacol. Rev.* 43 (2) (1991) 109–142.
- [50] J. Muhling et al., Effects of arginine, L-alanyl-L-glutamine or taurine on neutrophil (PMN) free amino acid profiles and immune functions in vitro, *Amino Acids* 22 (1) (2002) 39–53.
- [51] N.Z. Canturk et al., The role of L-arginine and neutrophils on incisional wound healing, *Eur. J. Emerg. Med.* 8 (4) (2001) 311–315.
- [52] M.R. Schaffer et al., Inhibition of nitric oxide synthesis in wounds: pharmacology and effect on accumulation of collagen in wounds in mice, *Eur. J. Surg.* 165 (3) (1999) 262–267.
- [53] F.J. Thornton et al., Enhanced collagen accumulation following direct transfection of the inducible nitric oxide synthase gene in cutaneous wounds, *Biochem. Biophys. Res. Commun.* 246 (3) (1998) 654–659.
- [54] H.P. Shi et al., The role of iNOS in wound healing, *Surgery* 130 (2) (2001) 225–229.
- [55] R. Wang et al., Human dermal fibroblasts produce nitric oxide and express both constitutive and inducible nitric oxide synthase isoforms, *J. Invest. Dermatol.* 106 (3) (1996) 419–427.
- [56] M. Kapoor et al., Effects of epicatechin gallate on wound healing and scar formation in a full thickness incisional wound healing model in rats, *Am. J. Pathol.* 165 (1) (2004) 299–307.
- [57] D. Fukumura et al., Predominant role of endothelial nitric oxide synthase in vascular endothelial growth factor-induced angiogenesis and vascular permeability, *Proc. Natl. Acad. Sci. USA* 98 (5) (2001) 2604–2609.
- [58] E. Noiri et al., Podokinesis in endothelial cell migration: role of nitric oxide, *Am. J. Physiol.* 274 (1 Pt. 1) (1998) C236–C244.
- [59] M.S. Goligorsky et al., Nitric oxide modulation of focal adhesions in endothelial cells, *Am. J. Physiol.* 276 (6 Pt. 1) (1999) C1271–C1281.
- [60] D. Most et al., Characterization of incisional wound healing in inducible nitric oxide synthase knockout mice, *Surgery* 132 (5) (2002) 866–876.
- [61] Y. Shimizu et al., Immunohistochemical localization of nitric oxide synthase in normal human skin: expression of endothelial-type and inducible-type nitric oxide synthase in keratinocytes, *J. Dermatol.* 24 (2) (1997) 80–87.
- [62] S. Frank et al., Induction of inducible nitric oxide synthase and its corresponding tetrahydrobiopterin-cofactor-synthesizing enzyme GTP-cyclohydrolase I during cutaneous wound repair, *J. Invest. Dermatol.* 111 (6) (1998) 1058–1064.
- [63] A. Barbul, Proline precursors to sustain Mammalian collagen synthesis, *J. Nutr.* 138 (10) (2008) 2021S–2024S.
- [64] C.I. Chang, J.C. Liao, L. Kuo, Arginase modulates nitric oxide production in activated macrophages, *Am. J. Physiol.* 274 (1 Pt. 2) (1998) H342–H348.
- [65] C. Zhang et al., Constitutive expression of arginase in microvascular endothelial cells counteracts nitric oxide-mediated vasodilatory function, *FASEB J.* 15 (7) (2001) 1264–1266.
- [66] W. Durante, F.K. Johnson, R.A. Johnson, Arginase: a critical regulator of nitric oxide synthesis and vascular function, *Clin. Exp. Pharmacol. Physiol.* 34 (9) (2007) 906–911.
- [67] V. Holan et al., Production of nitric oxide during graft rejection is regulated by the Th1/Th2 balance, the arginase activity, and L-arginine metabolism, *Transplantation* 81 (12) (2006) 1708–1715.
- [68] D. Bruch-Gerharz et al., Arginase 1 overexpression in psoriasis: limitation of inducible nitric oxide synthase activity as a molecular mechanism for keratinocyte hyperproliferation, *Am. J. Pathol.* 162 (1) (2003) 203–211.
- [69] M. Munder et al., Suppression of T-cell functions by human granulocyte arginase, *Blood* 108 (5) (2006) 1627–1634.
- [70] R. Rotondo et al., IL-8 induces exocytosis of arginase 1 by neutrophil polymorphonuclears in nonsmall cell lung cancer, *Int. J. Cancer.* 1 (2009) 1–2.
- [71] B.J. Tsuei et al., Surgery induces human mononuclear cell arginase I expression, *J. Trauma* 51 (3) (2001) 497–502.
- [72] J.B. Ochoa et al., Arginase I expression and activity in human mononuclear cells after injury, *Ann. Surg.* 233 (3) (2001) 393–399.
- [73] H. Li et al., Regulatory role of arginase I and II in nitric oxide, polyamine, and proline syntheses in endothelial cells, *Am. J. Physiol. Endocrinol. Metab.* 280 (1) (2001) E75–E82.
- [74] L.C. Jacobsen et al., Arginase 1 is expressed in myelocytes/metamyelocytes and localized in gelatinase granules of human neutrophils, *Blood* 109 (7) (2007) 3084–3087.
- [75] D. Kepka-Lenhart et al., Arginase I: a limiting factor for nitric oxide and polyamine synthesis by activated macrophages?, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 279 (6) (2000) R2237–R2242.
- [76] S.M. Morris Jr., D. Kepka-Lenhart, L.C. Chen, Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells, *Am. J. Physiol.* 275 (5 Pt. 1) (1998) E740–E747.
- [77] T. Gotoh, M. Mori, Arginase II downregulates nitric oxide (NO) production and prevents NO-mediated apoptosis in murine macrophage-derived RAW 264.7 cells, *J. Cell Biol.* 144 (3) (1999) 427–434.
- [78] M.B. Witte et al., Arginase acts as an alternative pathway of L-arginine metabolism in experimental colon anastomosis, *J. Gastrointest. Surg.* 7 (3) (2003) 378–385.
- [79] W. Xu et al., Increased arginase II and decreased NO synthesis in endothelial cells of patients with pulmonary arterial hypertension, *FASEB J.* 18 (14) (2004) 1746–1748.
- [80] T. Bachetti et al., Arginase pathway in human endothelial cells in pathophysiological conditions, *J. Mol. Cell. Cardiol.* 37 (2) (2004) 515–523.
- [81] J. Wohlrab, C. Siemes, W.C. Marsch, The influence of L-arginine on the regulation of epidermal arginase, *Skin Pharmacol. Appl. Skin Physiol.* 15 (1) (2002) 44–54.