LIPUS vs. reaming in non-union treatment: Cytokine expression course as a tool for evaluation and differentiation of non-union therapy

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1. Introduction

Previous studies have demonstrated the expression of specific cytokines (e.g. TGF- β , IGF-1, etc.) in normal fracture healing, delayed union, non-union, after non-union therapy^{1–3} and after LIPUS (low intensity pulsed ultrasound). We were able to show typical cytokine expression patterns which could be linked to the biochemical processes in fracture healing and bone regeneration and which led to a successful outcome.^{4–6} The results of studies on LIPUS have shown that this is not a proper treatment for long bone unions and that typical cytokine expression patterns are missing. We know from previous studies that there is an inflammatory response in proper bone healing.^{7,8}

In the present study, we aimed at comparing the cytokine process of angiogenic and inflammatory factors of LIPUS with the standard non-union reaming treatment. To our knowledge, to date there have been no systemic measurements of bone-specific growth factors and cytokines within a human reaming collective, and especially with no reference to a collective treated with LIPUS. Therefore, this study assessed the expression of cytokine serum levels after non-union treatment in order to decode the bone regeneration process and to explain the differentiation between the surgical therapy with reaming and the conservative treatment with LIPUS.

One of the major problems in trauma surgery is the treatment of non-unions, which make up between 5 and 10% of all fractures. This rate increases up to 30% in high-risk patients.^{9,10} The consequences of this worst-case scenario are a therapeutic challenge to the surgeon, with severe socioeconomic burden and the incurrence of above-average health care-associated costs. Even in the early phase, with sufficient osseous regeneration capacity and mechanical stability,⁷ non-invasive treatment options are established e.g. mechanical stimulation, physiotherapy, extracorporeal shockwave therapy¹¹ or LIPUS. If these initial

treatment options prove to be inefficient, then surgery is often needed.

In this study, the aim was to identify differences in the cytokine expression between patients who were treated using the reaming technique and using LIPUS within a half year follow-up.

Intramedullary nailing is one of the well-established treatment options in non-union therapy. Furthermore, soft tissue damage is marginal, hence cytokine levels almost entirely reflect bone metabolism.¹² Clinical data were used to evaluate the intramedullary reaming technique as a valid therapeutic option,² and for this we included a group of patients in our study who had undergone this treatment as an example of an approved non-union therapy.

The non-invasive non-union treatment option in this study is the application of LIPUS to the fracture or non-union site. In pre-clinical studies, an up-regulation of anabolic genes was demonstrated in osteoblasts under the influence of LIPUS.¹³ Cytokine expression was observed with our established protocol of venous blood sample collection.

Various molecules are involved in fracture healing and bone regeneration.^{4,14–20} Furthermore, multiple studies showed that an early inflammatory response, characterized by several cytokines, e.g. IL-6, IL-8, TNF- α , is responsible for the stimulation of bone healing.^{21,22} For the present study, seven cytokines for bone healing and inflammatory response were monitored: Interleukin-10 (IL-10), Interleukin-1b (IL-1b), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interferon-gamma (IFN- γ), Tumor Necrosis Factor-alpha (TNF- α), and Vascular Endothelial Growth Factor (VEGF).

Our aim was, on the one hand, to observe and evaluate characteristic serum cytokine expression patterns during non-union therapy of long bones by intramedullary reaming and LIPUS, and on the other hand to compare the two techniques in order to reveal the considerable differences between the angiogenic and inflammatory factors of both treatment options.

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Our hypothesis was that LIPUS does not increase the cytokine factors, which are necessary for a successful non-union treatment and only reaming imitates physiological bone healing.

2. Materials and methods

2.1. Patients

This study was conducted in accordance with the declaration of Helsinki in its current form. All individuals agreed with the study protocol and written informed consent was obtained from all patients. The study was approved by all local ethics committees (157/2002, S-636/2011 and 837.422.12).

Out of our fracture and non-union blood sample patient register at the trauma center 1 and the trauma center 2, we made a retrospective selection of 15 patients with non-unions treated by intramedullary reaming and nailing and 13 patients, who had undergone LIPUS of long bone non-unions.

An experienced orthopedic and trauma surgeon determined the indication for the reaming with intramedullary nailing as well as the use of LIPUS according to the EXOGEN® guidelines, which include stability of osteosynthesis, lack of bone consolidation and a defect gap < 1 cm. The intervention-free interval was at least three months in all patients in order to avoid a possible influence of the surgical treatment. The patients were instructed to apply LIPUS 20 min/day to the non-union site every day at home within the whole follow-up.

Inclusion criteria of the selected patients were a complete follow-up over at least six months, an age over 18, diaphyseal fractures of long bones (humerus, radius, ulna, femur, tibia) and the formal agreement to the study before treatment.

Exclusion criteria were advanced liver disease, chronic inflammatory diseases, malignancy as well as long-term medication with immunosuppressive drugs, which can influence normal cytokine levels.

The follow-up included routine lab tests at the standardized intervals of day before treatment, second day after treatment, 1, 4, 6, 12, and 25 weeks after treatment in accordance with previously published studies⁹ (Fig. 1).

We included two different groups in order to analyze changes and differences in cytokine expression in each group and to compare between the two groups (Fig. 2): G1 with non-union and radiologic consolidation six months after intramedullary reaming and nailing (responders, 15 patients, Figs. 3–5), G2 patients with non-union, who underwent low-intensity pulsed ultrasound treatment (LIPUS, 13 patients) over a period of at least six months. We only selected patients with a complete set of blood samples (according to our protocol) which were stored at our research facility.

2.2. Clinical and radiologic outcome of group 1 and group 2

Successful clinical outcome included mechanical stability, i.e. ability to bear full weight and radiologic signs of consolidation after six to twelve months. Outcomes were evaluated by experienced consultant trauma and orthopedic surgeons from the trauma center 1 and trauma center 2.

Fig. 2. Patient overview: G1: study group - intramedullary reaming and nailing, all 15 patients consolidated 6–12 months after reaming - Responders, G2: control group - LIPUS patients, 5 patients did not consolidate after LIPUS - Non-Responders, 8 patients consolidate after LIPUS - Responders.

Fig. 3. Atrophic tibial non-union, right tibia and fibula. PreOp X-ray.

2.3. Patient demographics study group “responders - G1”

A total number of 15 patients (eleven males, four females) were included in our study group. The mean age was 47.8 years, (46; 26–76 years). Average BMI was 25.77 (median 25.93) with a range from 18.75 to 35.49. The fracture location was only the tibia. Three cases were combined fractures of tibia and fibula. The mean defect length of the non-unions was 1.02 cm (0.95, 0.6–1.93 cm). Nine of fifteen fractures were on the right side and six were on the left side. Four of fifteen fractures were open fractures. The initial accidents were between 2001 and 2017. On average, G1 patients underwent 2.5 (range 1–8) surgical treatments before first admission in our clinic. The mean time period from the time of the initial treatment until the reaming treatment in our clinic was 3.1 years (1, 1–14 years). The tibia non-unions were mainly

Fig. 1. Standardized blood sample collection protocol. Stages of blood sample acquisition. No blood samples were taken on the day of operation.

Fig. 4. X-ray four days after intramedullary reaming and nailing with an ETN-PROtect™ nail.

Fig. 5. Six months after treatment with intramedullary reaming. Union; fibula non-union was not treated.

treated (thirteen of fifteen) with ETN-PROtect™ nails (Synthes, Umkirch, Germany). Four of fifteen patients (26.7%) were smokers. Approximately six months after intramedullary reaming, every non-union in study group G1 led to union (Table 1).

2.4. Patient demographics control group “LIPUS - G2”

A total number of 13 patients (12 males, one female) were included in the LIPUS study group. The mean age was 42.08 years (48, 17–62 years). Average BMI was 29.22 (median 25.88) with a range from 21.60 to 44.19. The fracture location was mostly the lower extremity. Six cases were fractures of the femur and four were fractures of the tibia. On the upper extremity there were two fractures of the humerus and one fracture of the radius. Eight of thirteen fractures were on the right side and seven were on the left side. The mean defect length of the non-unions was 0.52 cm (0.4, 0.1–1 cm). Seven of thirteen patients (53.8%) were smokers, one patient was a former smoker.

The mean time period from the time of the accident until the LIPUS treatment was 7.4 months (7, 3–13 months). The mean time period from the time of last surgery until the start of LIPUS treatment (4, 3–7 months) was 4.5 months. Five of thirteen patients did not consolidate after 6 months.

2.5. Sample acquisition

Venous blood was drawn from all participating patients (7.5 ml monovette, Sarstedt, Germany) according to the determined time pattern (Fig. 1) from patients on an empty stomach to avoid intraday measurement bias.^{4,9} Blood samples were centrifuged at 3000 rpm for 10 min at a temperature of 15 °C within 2 h after collection. The supernatant obtained from the serum samples was aliquoted and stored at –80 °C until analysis of the cytokines which was performed due to our patient selection and in the same laboratory.

2.6. Measurement of serum levels

Commercially available Luminex Performance Human High Sensitivity Assays (Quantikine®, R&D Systems, Inc., Minneapolis, USA) were used for the quantitative determination of serum IL-10, IL-1b, IL-6, IL-8, IFN-γ, TNF-α and VEGF levels. Before analysis, serum samples were thawed and equilibrated to room temperature for 2 h before analysis. The tests were performed in strict accordance with the guidelines given by the manufacturer. The lab technician who performed the Luminex assays was blinded to all patients and clinical information.

2.7. Data analysis

The analyses were carried out using SPSS for Windows (Norusis SPSS GmbH Inc™) and GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). We conducted standard paired and unpaired Student's *t*-test and ANOVA test to detect significant changes over time and between the groups.

Serum levels of each group are expressed as absolute mean concentrations ± SEM (standard error of the mean). The figures are illustrated in terms of means and standard mean error. Statistical significance was determined for $p < 0.05^*$. Cytokine levels of the G2 control group were compared to the levels of G1.

3. Results

3.1. IL-10

At the beginning the preop value of G1 (1.02 pg/ml) was significantly higher than in G2 (0.25 pg/ml; $p = 0.003$) and increased extraordinarily on the second day (2.17 pg/ml). In sum, IL-10 mean concentration in chronological sequence was always lower in G2 than in G1 with a range from 0.25 pg/ml to 0.38 pg/ml. At week 12, IL-10 concentrations of G1 (0.86 pg/ml) and G2 (0.83 pg/ml) approach each other. Additionally, we assessed significant differences between G1 and

Table 1

Patient demographics: responders after reaming and nailing - G1; LIPUS patients - G2; M - male, F - female, R - right side, L - left side, y - yes, n - no, BMI - body mass index, Nail, reamed nailing.

	Patient	Sex	Age	Localisation	Fixation	BMI	Previous surgeries	Smoker
G1	G1/1	F	45	Tibia	Nail	35.5	1	no
	G1/2	F	48	Tibia	Nail	–	3	no
	G1/3	F	43	Tibia	Nail	18.8	3	no
	G1/4	M	26	Tibia	Nail	22.3	3	no
	G1/5	M	33	Tibia/Fibula	Nail	21.6	3	no
	G1/6	M	47	Tibia	Nail	35.1	1	yes
	G1/7	M	44	Tibia	Nail	20.3	8	no
	G1/8	M	30	Tibia	Nail	28.1	2	yes
	G1/9	M	55	Tibia	Nail	30.0	2	yes
	G1/10	M	55	Tibia	Nail	27.2	2	no
	G1/11	M	34	Tibia	Nail	26.7	2	no
	G1/12	M	72	Tibia	Nail	25.9	2	no
	G1/13	F	61	Tibia	Nail	20.2	2	no
	G1/14	M	76	Tibia	Nail	23.4	1	yes
	G1/15	M	44	Tibia	Nail	20.3	8	no
G2	G2/1	F	24	Femur	Nail	23.1	–	no
	G2/2	M	59	Femur	–	24.9	–	no
	G2/3	M	59	Radius	Plate	22.8	2	yes
	G2/4	M	18	Femur	Nail	44.2	2	yes
	G2/5	M	30	Humerus	Plate	25.0	2	no
	G2/6	M	42	Tibia	Plate	34.0	2	yes
	G2/7	M	18	Tibia	Plate	25.9	6	no
	G2/8	M	56	Tibia	Plate	30.3	1	previous
	G2/9	M	51	Femur	Nail	28.4	3	yes
	G2/10	M	48	Tibia	Nail	25.3	5	yes
	G2/11	M	62	Humerus	Plate	40.5	2	no
	G2/12	M	35	Femur	Plate	21.6	3	yes
	G2/13	M	49	Femur	ext. Fixation	34.0	1	yes

Fig. 6. IL-10 concentrations as per study protocol (* $p < 0.05$; ** $p < 0.01$). IL 10 levels in G1 were consistently higher than G2 and had a great but non-significant peak in G1 two days after treatment.

G2 at week 1 ($p = 0.032$), at week 4 ($p = 0.001$) and at week 6 ($p = 0.050$) (Fig. 6).

3.2. IL-1b

The IL-1b mean concentration of G2 showed a lower overall concentration over time when compared to G1 with a range from 0.36 pg/ml to 0.62 pg/ml. In G1, we observed a steady increase of the serum levels to a maximum level on week 6 after surgery (0.81 pg/ml). The first week value and fourth week value of G2 (0.14 pg/ml, 0.16 pg/ml) was significantly lower than in G1 (0.73 pg/ml; $p = 0.007$, 0.65 pg/ml; $p = 0.005$). At week 12, IL-1b concentrations showed overlaps between G1 0.41 pg/ml and G2 (0.39 pg/ml) and on week 25 G1 concentrations were below G2 concentrations (0.52 pg/ml) (Fig. 7).

Fig. 7. IL-1b concentrations as per study protocol (* $p < 0.05$; ** $p < 0.01$). From preop until 12. week IL-1b levels in G1 were always higher than in G2 and showed significant differences in weeks 1 and 4.

3.3. IL-6

Comparing group G1 to group G2, IL-6 was notably elevated within the first week only. Explicitly, the IL-6 levels of G1 increased to a peak mean level concentration two days after treatment (31.96 pg/ml). This increase was significant ($p = 0.014$). Afterwards, the cytokine levels of the reaming patients decreased significantly ($p = 0.019$) to similar levels at admission within the first (5.46 pg/ml) and fourth weeks (2.28 pg/ml) after treatment. Compared to this, the IL-6 levels of G2 at the beginning were similar to those in G1 group and showed over the whole time period no major differences in the cytokine expression. However, in contrast, the particular increase on day 2 of G2 (3.92 pg/ml) was absent (Fig. 8).

3.4. IL-8

The IL-8 levels of G1 showed a higher overall concentration over

Fig. 8. IL-6 concentrations as per study protocol (* $p < 0.05$; ** $p < 0.01$). IL-6 levels in G1 showed a significantly increase on day 2 after treatment.

time when compared to G2. We found a significant increase in G1 from preop day (15.55 pg/ml) to first week value (120.82 pg/ml; $p = 0.0038$). After that, a steady decrease of the serum levels from G1 to a minimum on week 12 after treatment could be observed (10.45 pg/ml).

In summary, G2 did not show remarkable differences in cytokine expression over the whole follow-up (Fig. 9).

3.5. IFN- γ

In contrast to the other cytokines, an initial increase from 4.81 pg/ml to the maximum level (7.17 pg/ml) of G2 two days after beginning of treatment was observed. Interestingly, the highest value of G1 could be found at another time point six weeks after surgery (6.82 pg/ml). Overall, no significant differences could be found in comparing both groups at each time point (Fig. 10).

3.6. TNF- α

The cytokine course of both groups over the whole follow-up were similar. We did not detect any significant differences when comparing both groups at every measured time point. It is noteworthy that the cytokine expression levels of G1 were below G2 at the end of our follow-up (week 12 G1: 10.43 pg/ml $\leftrightarrow$ G2: 12.58 pg/ml, week 25 G1: 10.30 pg/ml $\leftrightarrow$ G2: 12.97 pg/ml) (Fig. 11).

3.7. VEGF

The VEGF mean concentrations in chronological sequence in both groups were always similar with a peak at week 1 (G1: 537.76 pg/ml,

Fig. 9. IL-8 concentrations as per study protocol (* $p < 0.05$; ** $p < 0.01$). IL-8 level had a significant increase in G1 1 week after treatment. This peak was missing in G2.

Fig. 10. IFN- γ concentrations as per study protocol (* $p < 0.05$; ** $p < 0.01$). No significant differences between G1 and G2 could be detected.

Fig. 11. TNF- α concentrations as per study protocol (* $p < 0.05$; ** $p < 0.01$). No significant differences were detectable between G1 and G2.

G2: 532.49 pg/ml) and a higher value of G2 at preop day (399.51 pg/ml). Only in group 1 (G1) could we observe a significant increase from admission (275.30 pg/ml) to week 1 ($p = 0.030$). Following this, a constant decrease in both groups to levels that were comparable to those at admission were detectable and they continued until twelve weeks after treatment (G1: 304.50 pg/ml, G2: 349.08 pg/ml). No statistical significances could be found when comparing both groups at each point of measure (Fig. 12).

4. Discussion

This observer, clinical trial was aimed at investigating the serum cytokine levels of the proinflammatory and angiogenesis cytokines IL-10, IL-1b, IL-6, IL-8, IFN- γ , TNF- α and VEGF levels in patients with

Fig. 12. VEGF concentrations as per study protocol (* $p < 0.05$; ** $p < 0.01$). VEGF level had a significant increase in G1 1 week after treatment.

long-bone non-unions who were treated with reaming (G1) or with LIPUS (G2), which were compared with each other.

The strengths of this study include a well-established design and protocol,^{2,4,9,23} a prospective approach, clear inclusion and exclusion criteria, and the comparison of two different bone regeneration groups.

The low soft tissue damage and the minimal invasive approach of the reaming technique allowed us to observe cytokine expression of bone metabolism alone²⁴ and gave us a better possibility to compare this with LIPUS, where no soft tissue damage is needed.

It became apparent, as in previous studies,²¹ that the typical cytokine expression increase of the proinflammatory cytokines was missing in non-union patients, who were treated with LIPUS and was much lower than in non-union patients, who were treated with reaming. Only VEGF showed similar cytokine expression courses in both groups.

These high levels of the proinflammatory cytokines in G1 imply that they need a stronger stimulus, such as an operation, to be raised and then lead to a successful outcome and that LIPUS is insufficient to stimulate the early proinflammatory process in non-unions. The VEGF serum levels after successful reaming treatment were similar to the cytokine expression of G2. Only in G1 did VEGF show a significant increase in the first week and a lesser decrease over the whole follow-up. It can be concluded that VEGF might play a major role after fresh fracture treatment but not in bone regeneration of manifest non-unions.¹² As a result, LIPUS seems to be a method with no utility in treating manifest non-unions, as has been previously reported.⁷

4.1. Inflammation

An early inflammatory response, characterized by several cytokines of this study, as well as a sufficient angiogenesis, are required for physiological bone healing.²¹ The proinflammatory cytokines IL-6, TNF- α and IL-8 are important for the initiation of the acute phase response.²² Shortly after a fracture event or an operation an increase in the serum cytokine levels can be observed.²⁵ Triggered by these proinflammatory cytokines, inflammatory cells, such as leukocytes and macrophages migrate into the fracture/operation hematoma caused by injuries of vessels due to the fracture or operation.²⁶ The interfragmentary hematoma arranges a cellular basis for the following bone regeneration.²⁷ During this early inflammatory response, various proinflammatory molecules, signal pathways and growth factors are expressed. IL-6, IL-8 and TNF- α play especially important roles.

These previous findings were confirmed by our results, which showed only a significant increase of IL-6 expression in G1 from preop day to the second day after treatment and represented the early inflammatory response after reaming. This relevant response was missing in G2 and could be indicative that an operative stimulus is needed for reactivating the bone healing process within the non-union gap.

Previous studies of IL-8 confirmed our observation of the typical significant increase up to one to two weeks after treatment²¹ with a higher level in G1 compared to G2 over the whole time period. In our study, the increase of IL-8 levels during the initial inflammatory response after reaming strengthened the role of IL-8 as a proinflammatory cytokine in bone regeneration but not in LIPUS. As noted in the above, IL-6 and TNF- α are co-factors for IL-8. Our results show an earlier increase of IL-6 in G1 than IL-8 in G1. It is assumed that IL-6 triggers the expression of IL-8. In contrast, TNF- α showed no dependence on both these cytokines in G1 as well as in G2.

IL-10 in G1 showed an early peak in our study at the second day after treatment and was always higher over the whole follow-up with significant higher values in week 1, 4 and 6 compared to patients who were treated with LIPUS. This suggests that IL-10, with its increase during the early inflammatory response, plays a major role for bone regeneration after reaming as a proinflammatory cytokine.

IL-1b concentrations of G1 were always higher than in G2 with a steady rise from preop day to sixth week after treatment. We can suppose that these high concentrations of IL-1b in G1 represent a strong

and early proinflammatory response after reaming treatment, which apparently leads to successful bone healing.

No significant differences were detectable between G1 and G2 in the TNF- α and IFN- γ cytokine expression. That implies that a stronger stimulus, such as the additional application of BMP-7, is needed. Therefore, further studies are necessary to prove this assumption.

4.2. Angiogenesis

Only a sufficient angiogenesis offers the precondition for a successful healing and this is needed shortly after fracture occurrence to secure the following bone regeneration process.²⁸ VEGF, the most important mediator of angiogenesis,²⁹ promotes the new formation of blood vessels and induces the inflammatory response. Its inhibition causes a failure in fracture healing.³⁰

Our study confirmed the research of Sahrarudi et al.³¹ with the typical increase of VEGF levels up to 2 weeks after fracture/treatment. However, subsequent VEGF levels showed a continual and steeper decrease in G2 compared to G1.

Hence, our results indicate a strong modulation of VEGF expression immediately after treatment beginning, but only in G1, where we recognized a higher overall level.

In sum, our data strengthen the assumption that VEGF is important for angiogenesis and is highly expressed after fresh fracture or surgical treatment, but it seems that VEGF does not play a major role in manifest non-unions. We presume, that LIPUS could be a treatment option only for fresh fractures to support the healing process.

The limitations of this study were the small sample size despite our extensive patient register, deviations of the matching criteria and ambiguity about unknown side effects which might possibly influence bone regeneration and fracture healing.³² Because of the limited number of included patients, we were not able to focus on comorbidities such as smoking,^{10,33} diabetes mellitus, anemia, malnutrition, peripheral arterial occlusive disease, chronic intake of non-steroidal antiphlogistics and other medications³² affecting the final outcome. Our patient demographics show proportionally more smokers in G2 than in G1.³⁴ This difference is significant ($p = 0.046$) and could be one of the reasons for therapy failure in this group. We know from previous studies, that smoking reduces vascularity and bone density.³²

Finally, our hypothesis that LIPUS does not increase the cytokine serum levels could not be confirmed. Although the study clearly demonstrates differences in the cytokine expression between both groups, the clinical effectiveness of LIPUS could not be validated. Patients, who were treated with reaming or LIPUS differ in their serum cytokine expression because of discrepancies in their bone regeneration metabolism, especially in the early proinflammatory response.

In summary, it can be stated that non-union treatment via LIPUS is not a proper non-union treatment.

In the future, a cytokine protocol might be established as a standard method to track changes in bone metabolism and regeneration. Therefore, cytokine expressions in the first two weeks after surgery require special attention in order to analyze the process of bone metabolism and the effectiveness of non-union treatment. There should be further investigation on the systemic appearance of these factors at specific time points in larger surveys with more participants in order to validate our findings.

In this study, significant differences of cytokine expression between patients, who were treated with intramedullary reaming and LIPUS could be demonstrated.

LIPUS provides an insufficient stimulus to the early proinflammatory process in non-unions, but stimulates the rise of VEGF, which might be especially important for recent fracture healing. These results suggest, as in previous studies, that LIPUS is not qualified for non-union therapy but rather is potentially qualified for the process of recent fracture healing.

Conflicts of interest

The authors have revealed all financial and personal relationships to other people or organizations that could inappropriately influence (bias) this work.

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