Functional responses of dermal fibroblasts to low nutrition and pro-inflammatory stimuli mimicking a wound environment in vitro

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Abstract
Dermal fibroblasts (DF) constitute one of key cells involved in wound healing. However, the functions they perform in wound conditions remain poorly understood. This study involved exposing DF to low nutrition and to low nutrition + LPS for 5 d as conditions representing the wound. Although DF exhibited increasing metabolic activity in time under all conditions including control, the proliferation did not change in both low nutrition and low nutrition + LPS. Only the low nutrition + LPS was found to potentiate the migration and pro-inflammatory phenotype (IL6 release) of DF. The potential of DF to contract collagen hydrogel declined only under low nutrition as a consequence of low cell number. The expression of α-SMA was reduced under both conditions independently of the cell number. The remodeling capability of DF was affected under both conditions as documented by the enhanced MMP2 activity. Finally, the production of collagen type I was not affected by either condition. The study shows that low nutrition as the single factor is able to delay the healing process. Moreover, the addition of the mild pro-inflammatory stimulus represented by LPS may amplify the cell response in case of decreased α-SMA expression or excite DF to produce IL6 impairing the healing process.

Keywords Dermal fibroblasts · Inflammation · Lipopolysaccharide · Low nutrition · Wound healing

Introduction
Dermal fibroblasts constitute a heterogeneous population of mesenchymal cells that are present in the dermis of the skin. They play a major role in terms of tissue structural support due to their producing an extracellular matrix (ECM). In addition, they are able to secrete and respond to cytokines and growth factors. In this way, they communicate both with each other and with other cell types. As a result, dermal fibroblasts actively participate in the regulation of skin physiology processes such as tissue development and differentiation, as well as remodeling and repair during the wound healing process (Stunova and Vistejnova 2018).

Dermal fibroblasts play an important role under the stress conditions of healing wound environments in both the inflammatory and proliferative phases of the wound healing process. They make up key players in terms of maintaining skin homeostasis and orchestrating physiological tissue repair. During the early stages of the wound repair process, dermal fibroblasts become highly proliferative, migrate to the wound site from the regional connective tissue, and produce various ECM components including glycosaminoglycans (GAG) and collagen so as to form provisional granulation tissue. This granulation tissue further provides a scaffold for the migration of resident cells from local tissue and blood circulation toward the wound (Tracy et al. 2016). The regulation of fibroblast proliferation and migration occurs through the local release of various cytokines, e.g., IL6 and IL8, which contribute toward enhancing inflammation in the wound (Zgheib et al. 2014). Granulation tissue

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degradation occurs in the later stages of the wound healing process via the action of matrix metalloproteinases (MMPs) produced by fibroblasts and other cells. The balance between MMPs and metalloproteinase tissue inhibitors (TIMPs) is essential for the remodeling of the ECM into the scar tissue, thus resulting in normal wound resolution (Caley et al. 2015) either via the degradation of the ECM or indirectly via their ability to affect cell behavior (Martins et al. 2013). Some of the fibroblasts differentiate into myofibroblasts during the wound healing process following stimulation exerted by transforming growth factor-beta (TGF-β). Myofibroblasts with stress fibers containing α-smooth muscle actin (α-SMA) contract and close the wound. In addition, TGF-β promotes the production of collagen by dermal fibroblasts (Desmouliere et al. 1993). The final phase of healing consists of the remodeling of the granulation tissue into more mature and stronger scar tissue. The signaling changes and the proliferative phase are suppressed in disrupted healing; the fibroblasts produce a higher level of pro-inflammatory cytokines and exhibit decreased migratory and mitotic activity, insufficient ECM production, and imbalance between the secretion of MMPs and TIMPs (Greaves et al. 2013).

Several in vitro models exist that simulate the wound environment with respect to dermal fibroblasts, the most common of which involve fibroblasts isolated directly from wounds (Hehenberger et al. 1998; Wall et al. 2008; Schwarz et al., 2013). Dermal fibroblasts derived from chronic wounds exhibit decreased adhesion, proliferation, and ability to withstand oxidative stress compared to those derived from uninjured skin, thus resulting in the early onset of senescence. Moreover, they express lower levels of chemokine genes (CXCL-1, -2, -3, -5, -6, 12) and are unable to correctly express a stromal address code which leads to the requirement for the guidance of infiltrating leukocytes to the site of injury (Hehenberger et al. 1998). The ratio of differentiated myofibroblasts was higher in the chronic wound group compared to dermal fibroblasts derived from properly healing wounds. The examination of several cytokines led to the conclusion that the production of IL6 was enhanced in the chronic wound group while the production of IL8 was enhanced in the properly healing wound group (Schwarz et al. 2013). Burn wounds exhibited a significantly higher production of IL8 than did the healed wounds and intact skin; no difference was determined in terms of the production of IL8 between the two latter groups (Iocono et al. 2000). On the other hand, although IL8 typically comprises a major cytokine expressed by many cells following LPS stimulation, certain fibroblast subtypes (e.g., lung fibroblasts) did not produce IL8 following LPS stimulation (Rolfe et al. 1991; Xing et al. 1993).

A further wound environment in vitro concept consists of the culturing of dermal fibroblasts with inflammatory stimuli, e.g., bacterial lipopolysaccharide (LPS). The treatment of fibroblasts with LPS was found to cause a decrease in viability and the increased production of the MCP-1, MIP-2, CINC, and RANTES chemokines (Xia et al. 1997; Basso et al. 2015), which initiated the recruitment of hematopoietic cells which, in turn, led to the further activation of the fibroblasts via the secretion of TNFα (Smith et al. 1997). Moreover, fibroblasts stimulated with LPS exhibited a 300-fold increase in IL8 mRNA compared to non-affected fibroblasts (Abbott 2003). LPS has also been studied in an ex vivo human skin organ culture and was found to enhance the production of IL6 and IL8 in a dose-dependent manner (Gvirtz et al. 2020). LPS has also been used in in vivo studies; 10 µg/ml of K. pneumoniae–derived LPS delayed wound closure, prolonged the inflammatory response, reduced collagen deposition, induced early cell death in granulation tissue, and inhibited wound edge proliferation in mice (Crompton et al. 2016). A further in vivo study showed that the production of collagen and pro-inflammatory cytokines (e.g., IL6, IL1β) and the wound breaking force were higher in mice treated with LPS (Kostarnoy et al. 2013).

A further important factor present in delayed wounds comprises a lack of nutrition supply due to improper vascularization followed by peripheral arterial disease, which is manifested by tissue ischemia and which, in chronic mode, can lead to delayed wound healing (Li et al. 2017). In the in vitro setup, fetal bovine serum represents the main source of nutrition for cells, and the decreasing of the concentration thereof is used regularly as a stress factor (Liang et al. 2007).

Nevertheless, no complex description of the behavior of dermal fibroblasts in the wound environment has yet been performed. Importantly, since several stress factors present in the wound environment have been characterized, it is possible to speculate which factor or factors exert an effect and the extent to which they are able to influence dermal fibroblasts.

The study involved the selection of two wound stress factors—low nutrition and pro-inflammatory stimuli represented by a decreased concentration of fetal bovine serum in the culture media and the addition of bacterial LPS to the cell culture media, respectively, and the study of the typical behavior of dermal fibroblasts in terms of wound healing under both conditions. Typical parameters for the description of the behavior of dermal fibroblasts include metabolic activity and the production of pro-inflammatory cytokines (IL6 and IL8) and matrix metalloproteinases (MMPs) as inflammatory phase behavioral patterns, and the proliferation, migration, contraction ability, and production of collagen type I and α-SMA as characteristics of the proliferative phase.

Material and methods

Cell isolation and culture Normal human dermal fibroblasts (NHDF) were isolated from skin residues via the digestion-migration method following plastic surgery interventions. The
isolation of NHDF from skin residues was carried out following approval by the local ethics committee of the University Hospital in Pilsen, E. Benesova 13, 305 99 Pilsen, Czech Republic, decision of November 5th, 2015. Guidelines were followed as set out in the Declaration of Helsinki. Written informed consent was provided prior to intervention by the parents of the children who participated in the study. The NHDF were isolated from 8 donors (Table 1). Immediately following skin biopsy, the samples were immersed in physiological solution and transported to the cell culture lab for immediate isolation. The samples were washed in Hank’s balanced salt solution (HBSS) (Merck KGaA, Darmstadt, Germany) containing penicillin (100 U/ml)/streptomycin (0.1 mg/ml) (Biochrom, Cambridge, UK) and gentamicin (50 µg/ml) (Biochrom). The samples were cut into 3 mm² pieces and digested overnight at 37 °C in Petri dishes (Techno Plastic Products, Trasadingen, Switzerland) in HBSS containing collagenase type I (100 U/ml, Thermo Fisher Scientific, Waltham, MA). On the following day, the suspension containing the digested tissue was shaken intensively employing a vortex for 30 s and filtered through a 100 µm nylon cell strainer (Falcon™, Thermo Fisher Scientific), penicillin (100 U/ml)/streptomycin (0.1 mg/ml) (Biochrom), 0.5% of L-glutamine (Biosera, Nuaille, France), and 1.0% of non-essential amino acids (Biosera). The NHDF were cultured at 37 °C, 5% CO₂ up to 80% confluence and then passaged. Only those NHDF from the 3rd to 5th passages were used in the experiments. The media were changed every 2nd–3rd day of cell culturing.

### In vitro simulation of wound conditions

Two types of wound conditions were simulated. Low nutrition conditions were simulated. Low nutrition conditions were simulated by means of a culture medium containing only 2% of FBS, and low nutrition + LPS conditions were simulated by means of a culture medium containing 2% of FBS and 0.1 µg/ml of lipopolysaccharide (LPS) that originated in pathogenic E. coli strain type O111:B4 (Merck KGaA). The NHDF culture medium mentioned in “Cell isolation and culture” provided for the control conditions.

#### Metabolic activity

The metabolic activity was estimated using alamarBlue assay that converted blue resazurin to pink resorufin. The NHDF were seeded on 96-well plates (Techno Plastic Products) at a density of 6000 cells/cm² (for the metabolic activity and cell proliferation) in a culture medium and cultured overnight at 37 °C and 5% CO₂. On the following day, the NHDF were treated as described above (“In vitro simulation of wound conditions”). The media were collected and frozen at −80 °C at time points 0, 1, 2, 3, and 5 d following culture treatment and subjected to the performance of alamarBlue assay; 100 µl of alamarBlue solution (Thermo Fisher Scientific) 10 × diluted in the same media as used for the culture treatment was added to each well and the NHDF were incubated for 2 h at 37 °C, 5% CO₂. Subsequently, 100 µl of the media was transferred to a black 96-well test plate (Thermo Fisher Scientific) and the fluorescence was measured at 530 nm (ex) and 590 nm (em) in a microplate reader (Synergy HT, Biotek, Winoski, VT). The change in the metabolic activity over time was expressed as the ratio of the metabolic activity at days 1, 2, 3, and 5 to that at day 0 for each treatment. The results were expressed as the mean ± standard deviation from 8 independent NHDF donors.

#### Cell proliferation

Cell proliferation was estimated via a total nuclei count. Following the performance of alamarBlue assay, the NHDF were washed in phosphate buffer saline (PBS) and the nuclei were stained using Hoechst #33342 (Thermo Fisher Scientific) diluted at 1:2000 with the culture media for 5 min at 37 °C, 5% CO₂. The NHDF were then washed in PBS and images were captured of the whole of the well areas (0.335 cm²) using an Olympus IX-83 (Olympus, Tokyo, Japan). Nine sequential pictures were taken covering the whole of the well areas using an Olympus UPlanFL N 4x/0.13 objective followed by automatic whole well area picture decomposition employing VisiView software (Visitron Systems, Puchheim, Germany). The number of nuclei in each well was determined via counting in Fiji software (National Institute of Health, Bethesda, MD). The change in the proliferation rate over time was expressed as the ratio of the cell count at days 1, 2, 3, and 5 to that at day 0 for each treatment. The results were expressed as the mean of the cell number ± standard deviation from 8 independent NHDF donors.

#### Scratch wound assay

The NHDF were seeded on 6-well test plates (Techno Plastic Products) at a density of 30,000 cells/cm² in the culture medium and cultured overnight at

| Donor No. | Gender | Age | Demographics | Site of excision | Passage of cells in experiment |
|-----------|--------|-----|---------------|------------------|-------------------------------|
| 1         | F      | 10  | Caucasian     | Ear              | 2                             |
| 2         | M      | 12  | Caucasian     | Ear              | 5                             |
| 3         | F      | 10  | Caucasian     | Ear              | 2                             |
| 4         | F      | 10  | Caucasian     | Ear              | 2                             |
| 5         | M      | 9   | Caucasian     | Ear              | 5                             |
| 6         | F      | 10  | Caucasian     | Ear              | 3                             |
| 7         | F      | 9   | Caucasian     | Ear              | 4                             |
| 8         | F      | 6   | Caucasian     | Ear              | 3                             |
37 °C and 5% CO₂. On the following day, the cell monolayers were scratched by means of a 10-μl pipette tip so as to create four crosses (wounded areas) per well thus mimicking the “wound bed.” The detached cells were washed carefully in PBS and treated with 0.1 µg/ml of LPS in a serum-free medium (low nutrition + LPS) in order to eliminate the cell proliferation effect, and cultured for 5 d. The serum-free culture medium (low nutrition) was applied as the control treatment. Immediately following treatment, images of the four wounded positions in each well were captured using an Olympus UPlanFL N 4×/0.13 objective and Olympus IX-83 inverted microscope. The positions were saved using cellSens Dimension 1.12 microscope software (Olympus). Images of the migrating cells were taken at time points 0, 1, 2, 3, and 5 d following treatment. The final images were analyzed using TScratch software (CSELab, Zurich, Switzerland) via the quantification of the wounded areas (i.e., the areas without cells) as a percentage of the whole picture area. The change in the migration rate of the cells was expressed as the ratio of the wounded area at days 1, 2, 3, and 5 to the wounded area at day 0. The results were expressed as the mean ± standard deviation from 3 independent NHDF donors.

**IL6, IL8, and collagen type I quantification** The culture media were collected at each time point, i.e., 0, 1, 2, 3, and 5 d, and stored at −80 °C. The concentrations of IL6, IL8, and collagen type I were assessed by means of ELISA using the Human IL-6 ELISA Ready-SET-Go! Kit (Thermo Fisher Scientific), the Human IL-8 ELISA Ready-SET-Go! Kit (Thermo Fisher Scientific), and the Human Pro-Collagen I alpha 1 ELISA Kit (Abcam, Cambridge, UK), respectively. The assays were performed according to the manufacturer’s instructions. The change in the time for the production of IL6, IL8, and collagen type I was expressed as the ratio of picograms per cell (IL6 and IL8) or picograms per 1000 cells (collagen type I) at days 1, 2, 3, and 5 to picograms per cell (IL6 and IL8) or picograms per 1000 cells (collagen type I) at day 1. The results were expressed as the mean ± standard deviation from 6–8 independent NHDF donors.

**MMP2 and MMP9 quantification** The culture media were collected at time points 1 and 5 d and stored at −80 °C. The pure cell culture media containing either 10% or 2% of FBS were analyzed so as to determine the basal MMP activities of the culture media. The MMP2 and MMP9 were analyzed via gelatin zymography using an SDS-PAGE system (7.5% separating gel, 4% stacking gel) containing 0.05% of gelatin and using a PageRulerPlus Prestained Protein Ladder, 10 to 250 kDa (Thermo Fisher Scientific) as a marker of MW. Following electrophoresis, the gels were washed in 2.5% Triton X-100 for 30 min while being shaken so as to remove the SDS and to renature the MMPs in the gels. The gels were then incubated overnight in a gelatinase activation buffer (100 mM Tris–HCl, pH 7.4; 5 µM CaCl₂; 1 μM ZnCl₂), stained with Coomassie Brilliant Blue G-250 for 30 min and de-stained using de-staining solution. The gels were scanned using the ChemiDoc MP Imaging System (BioRad Laboratories, Hercules, CA). The gels (Fig. 4A) were then cropped from one original scan using free Inkscape software (https://inkscape.org/). The MMP2 and MMP9 activities were determined via the software quantification of the degraded bands in Fiji software. The MMP activity was determined in two stages. Firstly, the basal MMP activity values of the pure culture media were subtracted from those of the culture media of the analyzed samples; secondly, the MMP activity values were normalized to the total cell number. Subtracting the higher values of MMPs in pure culture media from those of the culture media of the analyzed samples resulted in negative MMPs activity values, which is consistent with the evaluation approach. The results were expressed as the mean ± standard deviation from 4 independent NHDF donors.

**α-SMA and collagen type I visualization and α-SMA quantification** The NHDF were seeded on a µ-Slide 4-well (Ibidi, Graftelfing, Germany) at a density of 6000 cells/cm² (visualization) and on a 96-well plate (quantification) in the culture medium and cultured overnight at 37 °C and 5% CO₂. On the following day, the NHDF were treated as described above (“In vitro simulation of wound conditions”). Five days following treatment, the NHDF were fixed with fresh 4% paraformaldehyde. The cells were then permeabilized with cold 0.1% Triton X-100 solution for 15 min. After rinsing with PBS, the non-specific bonds were blocked with 5% BSA in PBS for 30 min at room temperature. Subsequently, the samples were incubated overnight at 4 °C with the following primary antibodies diluted in PBS: anti-alpha-smooth muscle actin antibody (ab7817, Abcam) diluted to a final concentration of 1 µg/ml and collagen I alpha I antibody (MAB 6220, R&D Systems, Minneapolis, MN) diluted to a final concentration of 125 µg/ml. Following rinsing with PBS, a secondary goat anti-mouse IgG H&L antibody conjugated with Alexa Fluor 488 (ab150113, Abcam) was applied to the samples (diluted in PBS to a final concentration of 1 µg/ml) for 1 h at room temperature in the dark. The nuclei were stained with Hoechst #33342 diluted at 1:2000 in the final 10 min. The samples were then rinsed with PBS and scanned using an Olympus IX-83. An Olympus UPlanFL N 20×/0.50 objective was used for the visualization of the α-SMA. The visualization of the collagen type I was conducted using an Olympus LUCPlanFL N 40×/0.60 objective. With respect to the quantification of the α-SMA, nine sequential images covering the whole well area were captured by means of an Olympus UPlanFL N 4×/0.13 objective followed by automatic whole well area picture decomposition using VisiView software. The number of nuclei and the area...
of the α-SMA were determined from the same well by means of Fiji software. The results were expressed as the mean of the α-SMA area at day 5 (mm²) normalized to 1000 cells at day 5 ± standard deviation from 3 independent NHDF donors.

**NHDF contraction ability** The NHDF were seeded in hydrogels produced from rat tail collagen type I. The lyophilized rat tail collagen was dissolved in 0.1% acetic acid to a concentration of 5 mg/ml followed by the preparation of a neutralized collagen suspension containing NHDF (final collagen concentration 3 mg/ml, NHDF density 30,000 cells/ml). Two milliliters of the collagen suspension with NHDF was added to the wells of 24-well plates and left at 37 °C and 5% CO₂ to polymerize. After 1 h, once the hydrogels were completely stiff, 1 ml of the culture medium was added to the hydrogels. On the following day, the NHDF were treated as described above (“In vitro simulation of wound conditions”). An equal volume of fresh culture medium was replaced three times per week. The diameter of the hydrogels was measured using a caliper at time points 1, 6, and 13 d. The results were expressed as the mean ± standard deviation from 6 independent NHDF donors.

**Statistical analysis** All the data was expressed as the mean ± standard deviation from 3 to 8 independent NHDF donors depending on the type of analysis conducted. The Shapiro–Wilk test was used to ascertain data normality and Leven’s test was employed for the assessment of the equality of the variances for a variable calculated for two or more groups. The statistical significance of the differences between the control, low nutrition, and low nutrition + LPS groups was determined via one-way ANOVA (p < 0.05) followed by the Fisher’s LSD test for the quantification of the α-SMA and MMP2 and by the Kruskal–Wallis test for the metabolic activity, proliferation, contraction, and quantification of the IL6, IL8, collagen type I, and MMP9. The Mann–Whitney U test (p < 0.05) was used for the scratch wound assay for migration since only two groups were compared. The statistical significance of the differences between the time points within each group was determined using the Mann–Whitney U test (p < 0.05). All the statistical analyses were performed in Statistica v12 software (Tibco Software, Palo Alto, CA).

**Results**

**Metabolic activity is not affected by low nutrition and low nutrition + LPS** The effect of low nutrition and low nutrition + LPS conditions on the NHDF metabolic activity was estimated by means of the alamarBlue assay. The NHDF were cultured for 5 d under control, low nutrition, and low nutrition + LPS conditions, and the metabolic activity was estimated after 0, 1, 2, 3, and 5 d of treatment (Fig. 1A). Under all three conditions, the cultured NHDF exhibited increasing tendencies in Fig. 1A in terms of metabolic activity during the whole of the cultivation period (Mann–Whitney U test; p < 0.05). The comparison of the metabolic activity of the NHDF cultured under the various conditions revealed no significant difference between the groups, with the exception of the occurrence of a significantly higher NHDF metabolic activity under the low nutrition (4.08 ± 0.76) conditions than that of the control (2.60 ± 0.59) at day 3 (Kruskal–Wallis test; p < 0.05).

**The dermal fibroblasts did not proliferate under either low nutrition or low nutrition + LPS conditions** The effect of low nutrition and low nutrition + LPS conditions on NHDF metabolic activity, proliferation, contraction, and quantification of the IL6, IL8, collagen type I, and MMP9. The Mann–Whitney U test (p < 0.05) was used for the scratch wound assay for migration since only two groups were compared. The statistical significance of the differences between the time points within each group was determined using the Mann–Whitney U test (p < 0.05). All the statistical analyses were performed in Statistica v12 software (Tibco Software, Palo Alto, CA).

**Figure 1.** The course of metabolic activity differs from the course of proliferation of dermal fibroblasts. (A) Metabolic activity and (B) proliferation of NHDF in the stress conditions. The change was expressed as a ratio of the signal/cell number at days 1–5 to the signal/cell number at day 0 (mean ± standard deviation, n = 8). Control = culture media with 10% of FBS; low nutrition = culture media with 2% of FBS; low nutrition + LPS = culture media with 2% of FBS and 0.1 µg/ml of LPS. *p < 0.05.
proliferation was evaluated by means of the total cell count (Fig. 1B). The NHDF were found not to have proliferated after 5 d under either low nutrition or low nutrition + LPS conditions. The comparison of the proliferation of the NHDF under the various conditions and at particular time points revealed no significant difference between the low nutrition and low nutrition + LPS conditions at any of the time points. However, at days 3 and 5, the proliferation of the NHDF was observed to be significantly lower under both the low nutrition (1.86 ± 0.78 for day 3; 1.67 ± 0.41 for day 5) and the low nutrition + LPS (1.89 ± 0.79 for day 3; 2.17 ± 1.11 for day 5) conditions than under the control conditions (3.30 ± 0.69 for day 3; 4.90 ± 0.90 for day 5) (Kruskal–Wallis test; p < 0.05).

**LPS stimulated the migration of dermal fibroblasts** The effects of low nutrition and low nutrition + LPS conditions on NHDF migration were evaluated by means of scratch wound assay. The NHDF migrated for 5 d and the wounded area was measured 0, 1, 2, 3, and 5 d following wounding (Fig. 2). Interestingly, the low nutrition + LPS conditions, particularly with respect to the addition of LPS, were observed to promote NHDF migration significantly from day 2. After 2 d of migration, the wounded area under low nutrition conditions was determined at 71.5 ± 12.4% and under low nutrition + LPS conditions at 56.7 ± 7.0%. At the subsequent time points, the wounded areas were determined at: 62.7 ± 11.0% (low nutrition) versus 42.2 ± 4.7% (low nutrition + LPS) after 3 d and 56.8 ± 13.3% (low nutrition) versus 36.5 ± 9.7% (low nutrition + LPS) after 5 d (Mann–Whitney U test; p < 0.05). The healthy physiological condition of NHDF throughout scratch wound assay is demonstrated in Fig. 2B. Third control group containing 10% FBS was avoided in this assay to prevent the effect of proliferation on wound area closure as used in other studies (Cai et al. 2010; Li et al. 2018).

![Figure 2](image_url). The migration of dermal fibroblasts measured by means of scratch wound assay is potentiated by low nutrition + LPS. (A) The wounded area (in %) normalized to day 0 was calculated as the ratio of the open area at days 1–5 to the open area at day 0 (mean ± standard deviation, n = 8). Low nutrition = culture media with 2% of FBS; low nutrition + LPS = culture media with 2% of FBS and 0.1 µg/ml of LPS. (B) Representative pictures of wounded area in culture of dermal fibroblasts showing healthy physiological condition of the cells. *p < 0.05.
Low nutrition + LPS promoted the pro-inflammatory behavior of the dermal fibroblasts. The pro-inflammatory response of the NHDF under low nutrition and low nutrition + LPS conditions was estimated via the quantification of IL6 (Fig. 3A) and IL8 (Fig. 3B) in the cell culture media and normalized to the cell number at each day and, subsequently, to the value at day 1. The production of IL6 by the NHDF was significantly higher under the low nutrition + LPS conditions than under the low nutrition and control conditions over time from day 2 (Kruskal–Wallis test; \( p < 0.05 \)). The production values were determined at 1.35 ± 0.50 (low nutrition + LPS), 0.85 ± 0.18 (low nutrition), and 0.78 ± 0.20 (control) at day 2; 2.16 ± 1.04 (low nutrition + LPS), 0.83 ± 0.49 (low nutrition), and 0.78 ± 0.55 (control) at day 3; and 5.47 ± 2.07 (low nutrition + LPS), 1.07 ± 0.60 (low nutrition), and 1.16 ± 1.02 (control) at day 5. The production of IL8 by the NHDF exhibited a greater increasing tendency over time when cultured under low nutrition + LPS conditions than it did under the low nutrition and control conditions. However, the concentration of IL8 did not differ significantly between the groups at any of the time points (Kruskal–Wallis test; \( p > 0.05 \)).

Both low nutrition and low nutrition + LPS stimulated MMP2 activity and did not affect the MMP9 activity in the dermal fibroblasts. The effects of low nutrition and low nutrition + LPS conditions on the NHDF secretion of MMP2 and MMP9 were evaluated via gelatin zymography at the 1- and 5-d time points. The signals from the MMP2 and MMP9 activities in the pure cell culture media without cells were subtracted from the signals of the tested samples and normalized to the cell number (Fig. 4A–C). In both, the low nutrition and low nutrition + LPS conditions, the activity of MMP2 per cell at day 1 was significantly promoted (Fig. 4B), with MMP2 per cell counts of 1.25 ± 0.60 (low nutrition + LPS) and 0.58 ± 0.26 (low nutrition) versus −0.68 ± 0.37 (control). The MMP9 increased significantly under the low nutrition conditions at day 1, evincing an MMP9 per cell count of 0.38 ± 0.17 (low nutrition) versus −0.37 ± 0.33 (control). The negative values of the control conditions were the result of the higher activity in the control cell culture media without cells (Fig. 4A). We decided to use negative values instead of simple zero values. Similarly, at day 5 (Fig. 4C), the activity of MMP2 per cell was increased significantly under both the low nutrition and low nutrition + LPS conditions, with MMP2 per cell counts of 1.48 ± 0.50 (low nutrition + LPS) and 1.54 ± 0.82 (low nutrition) versus 0.35 ± 0.17 (control). The level of MMP9 per cell was not found to have changed at day 5 under either the low nutrition or low nutrition + LPS conditions; however, the level of MMP9 per cell under the control conditions was observed to attain the values of the low nutrition and low nutrition + LPS (one-way ANOVA; \( p < 0.05 \) followed by Fisher’s LSD for the MMP2; Kruskal–Wallis test; \( p < 0.05 \) for the MMP9).

Both the low nutrition and low nutrition + LPS conditions reduced the NHDF contraction ability. The impacts of the low nutrition and low nutrition + LPS conditions on the contraction ability of the NHDF were evaluated via the diameter measurement of collagen hydrogels seeded with the NHDF at the 3-, 6- and 13-d time points (Fig. 5 and Suppl. Fig. S1). After 1 d and 6 d of culturing under the control, low nutrition, and low nutrition + LPS conditions, the contraction ability of the NHDF was found not to have changed. After 13 d of culturing, the highest NHDF contraction ability was observed in the control conditions, with scaffold diameters of 10.27 ± 1.3 mm (control) versus 11.60 ± 0.99 mm (low nutrition) and 11.89 ± 1.33 mm (low nutrition + LPS) (Kruskal–Wallis test; \( p < 0.05 \)).

**Figure 3.** The production of IL6 and IL8 by dermal fibroblasts estimated by means of ELISA is potentiated by low nutrition + LPS. The change in the production of A IL6 and B IL8 is expressed as the ratio of pg produced by a single cell at days 1–5 to those produced by a single cell at day 1 (mean ± standard deviation, \( n = 6–8 \)). Control = culture media with 10% of FBS; low nutrition = culture media with 2% of FBS; low nutrition + LPS = culture media with 2% of FBS and 0.1 µg/ml of LPS. *\( p < 0.05 \).
Figure 4. MMP activity of dermal fibroblasts assessed by means of zymography is potentiated by low nutrition and low nutrition + LPS. (A) Gels of particular culture conditions at days 1 and 5 including baseline MMP level in culture media (culture media without cells). (B, C) The graph depicts MMP activity as a fraction of the area of the sample (baseline MMP levels subtracted) normalized to the cell number (mean ± standard deviation, n = 4). Control = culture media with 10% of FBS; low nutrition = culture media with 2% of FBS; low nutrition + LPS = culture media with 2% of FBS and 0.1 µg/ml of LPS. *p < 0.05.
Both the low nutrition and low nutrition + LPS conditions decreased the area of the α-SMA positive NHDF, with the more profound effect of the low nutrition + LPS. The area of α-SMA positive NHDF per the total cell number was visualized and quantified after 5 d of culturing under the control, low nutrition, and low nutrition + LPS conditions (Fig. 6A–D). The area of α-SMA decreased under both the low nutrition and low nutrition + LPS conditions after 5 d of culturing compared to the control conditions. Moreover, the low nutrition + LPS conditions acted to decrease the area of α-SMA to a greater extent than did the low nutrition conditions, with areas of α-SMA 0.17 ± 0.04 mm²/1000 cells (control), 0.14 ± 0.02 mm²/1000 cells (low nutrition), and 0.07 ± 0.04 mm²/1000 cells (low nutrition + LPS) (Fig. 6A) (one-way ANOVA; *p < 0.05 followed by the Fisher’s LSD test). The differences between the control, low nutrition, and low nutrition + LPS conditions are apparent from the selected representative microscopy images shown in Fig. 6B–D and Suppl. Fig. S2.

Neither the low nutrition or low nutrition + LPS conditions exerted an effect on the synthesis and production of collagen type I by the NHDF. The synthesis of intracellular collagen type I by the NHDF was visualized using fluorescent microscopy after 5 d of cultivation under the control, low nutrition, and low nutrition + LPS conditions. The production of collagen type I by the NHDF was evaluated by means of ELISA from the culture media. The synthesis of intracellular collagen type I was found to be comparable under all the tested conditions (Fig. 7B–D). The production of collagen type I increased during culturing in the low nutrition + LPS, low nutrition, and control media (Fig. 7A); however, no difference was detected between the groups at any of the time points (Kruskal–Wallis test; *p > 0.05).

Discussion

The dermal fibroblasts cultured under the low nutrition conditions exhibited a slightly higher metabolic activity level than those cultured under the control conditions; however, the increase was significant at day 3 only. A quiescence phenotype and pathways that generated NADPH were induced when the dermal fibroblasts were cultured under low nutrition conditions represented by a medium with 0.1% of FBS (Lemons et al. 2010). alamarBlue containing resazurin, the substrate used in this study for the estimation of the metabolic activity, is able to be reduced by NADPH (Rampersad 2012). Therefore, we hypothesized that low nutrition conditions used in our study induce a quiescence phenotype and that the metabolic activity of the dermal fibroblasts under low nutrition and low nutrition + LPS conditions is, paradoxically, increased due to the generation of increased levels of NADPH produced by the activated metabolic pathways. The results of the metabolic activity response obtained in other studies were inconsistent. 0.2 µg/ml of LPS was observed not to affect the metabolic activity of the dermal fibroblasts (Eleftheriadis et al. 2011). 10 µg/ml of LPS (E. coli) in a medium with 10% of FBS acted to decrease the metabolic activity of gingival fibroblasts (Basso et al. 2015), and 25 µg/ml of LPS (derived from E. coli O111:B4) in a medium containing 10% of FBS was seen to increase the metabolic activity of gingival fibroblasts (Xi et al. 2016). The inconsistency in the data of our and other studies was presumably the result of the differing sources of the fibroblasts and differences in the experimental setups.

The dermal fibroblasts did not proliferate under either the low nutrition or the low nutrition + LPS conditions. Similarly, the serum-free medium exhibited a sharp decrease in
With respect to the addition of LPS, 1 µg/ml of LPS in a medium containing 10% of calf serum led to an increase in the proliferation of mouse lung fibroblasts (He et al. 2012), while, conversely, 10 µg/ml of LPS in a medium containing 10% of FBS acted to decrease the proliferation of gingival fibroblasts (Basso et al. 2015). We assume that the dermal fibroblasts in our study did not proliferate due to the low nutrition conditions rather than the presence of LPS, which is supported by the results obtained by Ejiri et al. (2015). Importantly, the comparison of the metabolic activity and proliferation presented in our paper provides evidence that while the two methods are sometimes erroneously interchanged (Cai et al. 2010; Zhang et al. 2011; Xi et al. 2016), the metabolic activity does not equate to proliferation.

Scratch wound assay was found to provide an appropriate method for the assessment of cell migration into the wounded area (Liang et al. 2007). We demonstrated that dermal fibroblasts cultured in the presence of LPS migrate to a greater extent than do dermal fibroblasts cultured without LPS. The enhanced migration rate of mouse adventitial fibroblasts was observed in a culture medium with 10 µg/ml of LPS (Cai et al. 2010). Similarly, treatment with 0.4 µg/ml of LPS increased the migration of mouse embryo cell line fibroblasts through the positive feedback between β-catenin and COX-2 in concentration in a time-dependent manner (Li et al. 2018).

The production of IL6 was significantly enhanced when the dermal fibroblasts were cultured under low nutrition + LPS conditions independent of the cell count. Similarly, other researchers have shown that the secretion of IL6 increased when gingival fibroblasts were cultivated with 0.1 µg/ml of LPS from *E. coli* in a medium containing 10% of fetal calf serum (Jin et al. 2012). Similarly, 10 µg/ml of LPS from *P. aeruginosa* significantly enhanced the production of IL6 by nasal polyp-derived fibroblasts (Cho et al. 2014). Therefore, we propose that the presence of LPS exerts a primary effect on the production of IL6. In addition, a complex of IL6 with the IL6 receptor suppresses IL1β, TNFα, and PDGF-AA-induced dermal fibroblast proliferation (Mihara et al. 1996). These findings correlate well with the non-proliferating fibroblasts in our experiment. An increased level of IL6 following LPS treatment was also observed in vivo (Kostarnoy et al. 2013). Our results suggest that dermal fibroblasts are able to produce a higher level of IL8 under low nutrition + LPS conditions, especially after 5 d of culturing. The production of IL8 by dermal fibroblasts increased significantly following treatment with 0.2 µg/ml of LPS (Eleftheriadis et al. 2011). Similarly, 10 µg/ml of LPS from *P. aeruginosa* significantly enhanced the production of
IL8 by nasal polyp-derived fibroblasts (Cho et al. 2014). The origin of the fibroblasts and the origin and concentration of the bacterial LPS appear to be of significant importance. For instance, 10 µg/ml of LPS from E. coli enhanced the production of IL8 in human nasal but not lung fibroblasts (Xing et al. 1993). Similarly, LPS from E. coli and S. typhimurium led to an increase in the levels of IL6 and IL8 in an ex vivo human skin organ culture, while LPS that originated in S. enteritidis failed to do so (Gvirtz et al. 2020). Our IL8 assay data exhibited a high degree of variability, thus rendering the differences between the experimental groups insignificant. Interleukin values were observed in our study for 5 d of cell culturing while in the studies mentioned above the researchers observed interleukin production up to just 3 d of cell culturing. Therefore, we attribute the discrepancies between our results and those of other research teams to the differing time points of the analysis. Moreover, we used the primary dermal fibroblasts isolated from different human donors, which make this study unique; however, it is affected by higher data variability.

The presence of MMPs in wounded tissue is essential with respect to cell migration, tissue remodeling, and the regulation of the level of cytokines (Howard et al. 2012; Martins et al. 2013). The control conditions applied in this study contained more FBS (10%) than did the low nutrition and low nutrition + LPS conditions (2%). Our results revealed that the FBS contained MMPs regardless of those produced by the cells (Fig. 4A; culture media without cells). Nevertheless, the results also indicated higher levels of MMP2 under the low nutrition and low nutrition + LPS conditions than that of the control conditions over time and independent of the cell count. We propose that the increases in the MMP2 levels in both the stress media were due primarily to low nutrition rather than the presence of LPS. The level of MMP9 remained unchanged under both stress conditions. After 48 h of the exposure of bovine dermal fibroblasts to LPS (5 µg/ml) from E. coli, increased levels of MMP2 and MMP9 were observed (Akkoc et al. 2016). Our findings and those of Akkoc et al. (2016) indicate that LPS is capable of enhancing the production of MMP2 and MMP9 by dermal fibroblasts in concentrations of higher than 0.1 µg/ml. The release of MMPs is dependent not only upon the origin of the LPS but also on that of the fibroblasts (Lindner et al. 2012). An increased level of MMP9 in nasal polyp-derived fibroblasts was determined following exposure to 10 µg/ml of LPS (P. aeruginosa) for 12 h (Cho et al. 2014). We conclude that, with respect to our experimental setup, the dermal fibroblast secretion of MMP2 was induced primarily by low nutrition, and that the LPS concentration of 0.1 µg/ml was too low to enable the release of the active form of MMP9.

Our study revealed that the contraction ability of dermal fibroblasts decreased under both the low nutrition and low nutrition + LPS conditions, most likely due to the decreased number of cells under the low nutrition conditions. These results are
consistent with those obtained from a previous in vivo study; 10 µg of LPS (originated in *K. pneumoniae*) subcutaneously injected prior to wounding delayed the wound closure process (Crompton et al. 2016). However, other researchers have observed the opposite effect. Gels containing 0.2, 2, and 10 mg/g of LPS from *S. typhi* (mg LPS/g of gel) were applied to the incision-wounded skin of mice. The wound closure process was enhanced in the presence of LPS and was dose-dependent (Kostarnoy et al. 2013). A similar effect was also observed in vitro. 2 and 5 µg/ml of LPS (origin not specified) in a culture medium with 1% of FBS was found to enhance the contraction of intestine fibroblast–mediated collagen in a dose-dependent manner (Burke et al. 2010). Whether the differences in the types of fibroblasts and/or origin and concentrations of LPS were responsible for the discrepancies in the results remains open to debate.

**Figure 8.** Summary of the effects of two wound stress factors low nutrition and low nutrition + LPS on the typical behavior or dermal fibroblasts in wound healing. Dermal fibroblasts exhibited similar responses in the most of analyzed characteristics in both conditions. Their different behavior was observed for IL6 production which was higher in low nutrition + LPS compared to low nutrition and for α-SMA expression which decreased under both conditions compared to control conditions and even more decreased in low nutrition + LPS.
α-SMA constitutes one of the typical characteristics of myofibroblasts, a contractile type of fibroblast that appears later in the proliferative phase of the wound healing process (Darby et al. 1990; Tomasek et al. 2002; Hinz et al. 2007). We observed a decrease in the area of α-SMA positive cells normalized to 1000 cells under the low nutrition conditions and a more pronounced decrease under the low nutrition + LPS conditions, which was accompanied by a decrease in the contraction ability, thus indicating decreased myofibroblast differentiation. The impact of LPS on the expression of α-SMA by dermal fibroblasts has not previously been reported in the literature. However, a decreased α-SMA protein level has been observed in human cardiac fibroblasts following LPS treatment (1 µg/ml) (Bolívar et al. 2017).

The production of collagen type I by dermal fibroblasts was confirmed in this study via the application of two independent methods. The production of collagen type I and hyaluronic acid in dermal fibroblasts is dependent on growth factor and cytokine changes. The research team determined that neither IL6 nor IL8 exerted changes with concern to the production of either of the molecules compared to the control (Kim et al. 2014). These findings correspond to our results, i.e., the production of collagen by dermal fibroblasts is not affected by pro-inflammatory conditions modeled in the form of increased levels of LPS or IL6/IL8 in the culture media.

Conclusion

This study describes a number of new observations on the behavior of dermal fibroblasts via the analysis of the typical functions thereof under wound stress conditions. The results led to the conclusion (summarized in Fig. 8) that although both low nutrition and low nutrition + LPS conditions do not affect the naturally increasing metabolic activity of cells, this phenomenon is not accompanied by increasing proliferation, as evinced by the absence of a change in the cell number under either of the conditions. Furthermore, low nutrition + LPS served to potentiate the migration and pro-inflammatory phenotype of the cells as evidenced by enhanced in vitro wound closure and the increased production of IL6, respectively. The potential of dermal fibroblasts to contract collagen hydrogel declined under the low nutrition conditions as the result of the low cell number. Moreover, the expression of α-SMA decreased under the low nutrition conditions as well as (to a greater extent) in the low nutrition + LPS conditions. The remodeling ability of the cells was influenced by both wound conditions, as documented by the enhanced MMP2 and unchanged MMP9 activities. Finally, the production of collagen type I was not affected by either of the conditions. We believe that, taken overall, our results provide a comprehensive functional insight into the response of dermal fibroblasts to in vitro modeled wound stress conditions.

The unique benefit of this study concerns the provision of evidence that increased metabolic activity does not necessarily reflect cellular proliferation under particular stress conditions; rather it reflects the energy required to trigger a cellular response to alarming or changing conditions in general. Furthermore, the study shows that even a single stress factor is able to retard the healing process, since the low nutrition conditions alone acted to reduce the cell proliferation and the contractile capacity and to enhance the production of MMPs by the dermal fibroblasts. The nutrition supply thus appears to be the one key factor with respect to the support of dermal fibroblasts in the healing process. Moreover, the pro-inflammatory behavior of dermal fibroblasts in the healing process should also be taken into account since they actively produce pro-inflammatory cytokines in the pro-inflammatory conditions of wounds. Last but not least, when discussed with literature the origin of dermal fibroblasts and the origin and the concentration of LPS are overlooked. These variables bring different results followed by different conclusions made from them. In conclusion, we can state that the differing experimental setups simulating wound conditions in vitro led to inconsistent results making the generalization of dermal fibroblasts functions upon these conditions challenging.

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Author contribution Anna Zavadakova and Lucie Vistejnova designed the experiments and prepared the figures. Anna Zavadakova and Pavla Tonarova performed the experiments and collected the data. Data analysis was performed by all authors. The first draft of the manuscript was written by Anna Zavadakova and all authors commented on previous versions of the manuscript. All authors have read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare no competing interests. The funders had no role in the design of the study; in the collection, analysis or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.
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