Biochimica et Biophysica Acta 1842 (2014) 32–43

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Neurotensin-loaded collagen dressings reduce inflammation and improve wound healing in diabetic mice

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ARTICLE INFO

Article history:
Received 12 July 2013
Received in revised form 25 September 2013
Accepted 15 October 2013
Available online 23 October 2013

Keywords:
Collagen
Wound dressing
Diabetic foot ulcer
Neurotensin
Wound healing

ABSTRACT

Impaired wound healing is an important clinical problem in *diabetes mellitus* and results in failure to completely heal diabetic foot ulcers (DFUs), which may lead to lower extremity amputations. In the present study, collagen based dressings were prepared to be applied as support for the delivery of neurotensin (NT), a neuropeptide that acts as an inflammatory modulator in wound healing. The performance of NT alone and NT–loaded collagen matrices to treat wounds in streptozotocin (STZ) diabetic induced mice was evaluated. Results showed that the prepared dressings were not-cytotoxic up to 72 h after contact with macrophages (Raw 264.7) and human keratinocyte (HaCaT) cell lines. Moreover, those cells were shown to adhere to the collagen matrices without noticeable change in their morphology. NT–loaded collagen dressings induced faster healing (17% wound area reduction) in the early phases of wound healing in diabetic wounded mice. In addition, they also significantly reduced inflammatory cytokine expression namely, TNF-α ($p < 0.01$) and IL-1β ($p < 0.01$) and decreased the inflammatory infiltrate at day 3 post-wounding (inflammatory phase). After complete healing, metalloproteinase 9 (MMP-9) is reduced in diabetic skin ($p < 0.05$) which significantly increased fibroblast migration and collagen (collagen type I, alpha 2 (COL1A2) and collagen type III, alpha 1 (COL3A1)) expression and deposition. These results suggest that collagen-based dressings can be an effective support for NT release into diabetic wound enhancing the healing process. Nevertheless, a more prominent scar is observed in diabetic wounds treated with collagen when compared to the treatment with NT alone.

1. Introduction

Diabetes mellitus is an important health problem that affects millions of people over the world and its prevalence is expected to rise up to 439 million patients by 2030 [1]. One severe and chronic complication of diabetes is the diabetic foot ulcer (DFU) that results from peripheral neuropathy and impaired wound healing (characterized by chronic inflammation, impaired angiogenesis and decreased collagen production). DFUs lead to frequent hospitalizations and in extreme cases, to amputations that result in elevated hospital costs and poor quality of life for patients [2,3].

Recently it has been demonstrated that peripheral nerves and cutaneous neurobiology contribute to a correct wound healing process [4]. In DFU, the loss of peripheral sensory and autonomic nerves reduces the production, and consequently the levels, of neuropeptides that are important for proper wound healing [3]. Neurotensin (NT) is a bioactive neuropeptide widely distributed in the brain and in several peripheral tissues [5,6] that acts on immune cells (leukocytes, mast cells, dendritic cells and macrophages) and leads to cytokine release and chemotaxis necessary for a correct immunomodulation response. In addition, NT affects microvascular tone, vessel permeability, vasodilation/vasoconstriction and new vessel formation which helps to improve angiogenesis during wound healing processes [3,7,8].

Some neuropeptides namely, substance P and neuropeptide Y have been topically applied to improve diabetic wound healing [9,10]. However, the major problem of topical administration of peptides is their short half-life and loss of bioactivity in the peptidase-rich wound environment [11]. A strategy to overcome this problem is the use of wound dressings made of biocompatible materials for the sustained

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AUC, area under the curve; COL1A1, collagen type I, alpha 1; COL1A2, collagen type I, alpha 2; COL3A1, collagen type III, alpha 1; DFU, diabetic foot ulcer; EGF, endothelial growth factor; ECM, extracellular matrix; FBS, fetal bovine serum; IL-1β, interleukin-1β; IL-6, interleukin-6; KC, interleukin-8; MMP-9, metalloproteinase 9; NT, neurotensin; NO, nitric oxide; PBS, phosphate buffer solution; PDGF, platelet-derived growth factor; PMN, polymorphonuclear leukocytes; SEM, scanning electron microscopy; STZ, streptozotocin; TGF β1, transforming growth factor β1; TGF β3, transforming growth factor β3; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor

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http://dx.doi.org/10.1016/j.bbadis.2013.10.009

delivery of neuropeptides. Besides protecting peptides from rapid biodegradation, wound dressings should also replicate skin characteristics in order to promote the proliferation and migration of fibroblasts and keratinocytes, as well as to enhance collagen synthesis, leading to proper healing with low scar formation [12].

After injury, the application of wound dressings based on natural polymers has been proposed to simulate the original cellular environment and extracellular matrix (ECM) [13]. Collagen is the most abundant protein of ECM being naturally present in human tissues such as skin, bones, cartilage, tendon and ligaments [14,15]. This biopolymer interacts with cells and regulates cell anchorage, migration, proliferation and survival [12]. Furthermore, collagen-based dressings have shown to promote increase in fibroblast production and stimulate a faster wound healing [16].

In addition, collagen is biocompatible, non-toxic, with low antigenicity [17] and able to absorb large quantities of wound exudate while simultaneously preserving a moist environment [18]. Due to its characteristics, it is usually considered as an ideal biomaterial for tissue engineering and wound dressing applications [19]. Its efficiency in the treatment of DFU has also been evaluated. Recent studies comproved the efficacy of collagen dressings to decrease infection by bacteria and to favoring granulation tissue formation stimulating a faster wound healing in DFU patients [16,20–22]. Moreover collagen based dressings have already been used for the delivery of several bioactive agents. Different approaches tested so far include the incorporation of glucose oxidase in a collagen matrix in order to enhance the sustained delivery of reactive oxygen species (ROS), natural compounds (such as polyphenols), growth factors (such as bFGF), antibiotics (such as doxycycline and levofloxacin) and ionic silver as an antimicrobial agent [20,23,24].

The aim of this study was to develop and apply wound dressings, prepared from collagen extracted from mouse tails, for the delivery of NT into diabetic and control wounds. The *in vivo* progression of skin wound healing in both diabetic and control mice was also evaluated. Moreover, the effect of NT on the production of the inflammatory and collagen when applied in skin wounds alone (in solution) or loaded into collagen-based dressings was analyzed using a mouse model of wound healing.

2. Material and methods

2.1. Materials

Ketamine (Clorketam 1000) was obtained from Vétoquinol (Portugal) and xylazine (Rompun) from Bayer HealthCare (Germany). NT was purchased from Bachem (Switzerland). The antibodies against TNF-α and MMP-9 were purchased from Cell Signaling Technology (USA) and the antibody against actin was purchased from the Millipore Corporation (USA).

2.2. Preparation of collagen dressing

Collagen isolation and preparation were carried out following the procedures from Espinosa et al. [43]. Briefly, collagen dressings were manufactured from type I collagen isolated from mouse-tail tendons. Mouse tails were washed, and disinfected (2% sodium hypochlorite) and the tendons were dissected and cut into small pieces. After, they were suspended in 0.5 M acetic acid (4 °C during 24 h) and the suspension was centrifuged. The supernatant collagen was dissolved in 0.1 M acetic acid and poured in tissue culture dishes, frozen at −20 °C and freeze-dried to yield collagen dressings. The dressings were then rehydrated and cross-linked with glutaraldehyde (0.02%, 4 °C during 24 h). Several washes with water were performed to remove glutaraldehyde residues that did not react during the crosslinking. Collagen dressings were cut in circular pieces with 6 mm of diameter and then sterilized with ethylene oxide.

2.3. Cell culture

Mouse leukaemic monocyte macrophages (Raw 264.7) and human keratinocyte (HaCaT) cells were cultured in DMEM medium, pH 7.4, supplemented with 10% heat inactivated fetal bovine serum (FBS), 3.02 g/l sodium bicarbonate, 30 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37 °C in a humidified incubator containing 5% CO_2. Sub-culturing was performed according to ATCC recommendations. Raw 264.7 and HaCaT cell lines were purchased from ATCC (number TIB-71) and CLS (number 300493), respectively.

2.4. Scanning electron microscopy (SEM)

Collagen samples were saturated with the DMEM medium before being placed to 96-well plates. Raw 264.7 (3×10^4 cells/well) and HaCaT (1.5×10^4 cells/well) cells were seeded individually onto the surface of the collagen samples with 200 μl of DMEM. After 24, 48 and 72 h of incubation, the cells on the materials were prepared for SEM. At each time point, the samples with adherent cells were washed with 0.1 M phosphate buffer and fixed with 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer for 4 h, at 4 °C. After, they were washed with 0.1 M phosphate buffer and dehydrated by different percentages of alcohol (50%, 75%, 95% and 100%). Finally, samples were coated with gold (approximately 300 Å) in an argon atmosphere and SEM micrographs were obtained at 15 kV (JEOL, model JSM-5310, Japan).

2.5. In vitro evaluation of collagen dressing's biocompatibility

Raw 264.7 (3×10^4 cells/well) and HaCaT (1.5×10^4 cells/well) cells were plated individually in 96-well plates with 200 μl of DMEM above previously sterilized collagen dressings. After 24, 48 and 72 h of incubation, the medium was removed and 200 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/ml) was added to each well. The plates were further incubated at 37 °C for 4 h, in a humidified incubator containing 5% CO_2. After this period, 200 μl of acidic isopropanol (0.04 N HCl in isopropanol) was added. Formazan produced was quantified using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm. Each sample was analyzed in duplicate.

2.6. NO production — Griess method

Raw 264.7 (3×10^4 cells/well) cells were plated in 96-well plates with 200 μl of DMEM, above previously sterilized collagen dressings. After 24, 48 and 72 h of incubation, 170 μl of medium supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-naphthelenediamine dihydrochloride in 2.5% phosphoric acid). After 30 min of incubation in the dark, the absorbance was measured at 550 nm in a microplate reader (SLT, Austria). Nitrite concentration was calculated from a previously obtained nitrite standard curve.

2.7. In vivo wound closure

Male C57BL/6 mice (Charles River Corporation Inc., Barcelona, Spain) weighing 25–30 g were used in this work. The animals were maintained at normal room temperature (22–24 °C) on a 12 h light/dark cycle, with free access to commercial pellet diet and water. After the wounding procedure, the animals were kept in individual cages. All experiments were conducted according to the National and European Community Council directives on animal care.

Diabetes was induced by intraperitoneal injections of STZ (50 mg/kg body weight), in citrate buffer pH 4.5, during five consecutive days. Four days after diabetes induction, blood glucose levels were measured by the Accu-Chek Aviva glucometer (Roche Diagnostics, Germany). The animals with blood glucose levels higher than 300 mg/dl were considered diabetic. Mice were anesthetized by intraperitoneal injection of

xylazine (13 mg/kg) and ketamine (66.7 mg/kg). The dorsal hair of control and diabetic mice was shaved and two 6 mm diameter full-thickness wounds were created with a biopsy punch.

For each animal, two wounds were performed: one in a top position (closer to the neck) and other in a bottom position (closer to the tail). Each animal was randomly distributed in groups and identified by a number (1, 2, 3, etc. in each group). Animals with odd numbers received treatment in the top wound while those with even number received treatment in the bottom wound (to minimize area dependent healing profiles).

C57BL/6 mice were divided into six groups of treatment for control (non-diabetic) and other six similar groups for diabetic mice — three groups for day 3 (d3) (I, II, III) and three similar groups for day 12 (d12) (IV, V, VI): groups I and IV were treated with collagen dressings alone, groups II and V with topical application of 50 μg/ml NT and groups III and VI with 50 μg/ml NT–loaded collagen dressings. For each animal one of the wounds worked as control (PBS application only) and the other received treatment. For each group, an average of 5 to 8 animals were included, either for control or diabetic mice. The dried type 1 collagen foams were applied over the wounds and wetted with 5 μl of PBS or NT solution (50 μg/ml) to originate hydrogels. By visual inspection it was possible to observe that the dressings persist into the wound approximately until days 6–7. The progress of wound healing was evaluated periodically by acetate tracing till day 12. Topical application of PBS or NT (alone or loaded into the prepared collagen dressing) was performed daily. At day 3 (d3) or after complete healing (fd), C57BL/6 mice were sacrificed and around 2 mm of tissue and skin surrounding the wound were harvested. Complete healing day varies between days 13–16 for control mice and days 14–16 for diabetic mice. These time points were chosen to evaluate the inflammatory (day 3) and the proliferating/remodeling (fd) phases of wound healing.

2.8. Real time RT-PCR

Total RNA was isolated from skin samples with the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, USA). First strand cDNA was synthesized using High Capacity cDNA Reverse Transcription. Then, real-time RT-PCR was performed in a BioRad My Cycler iQ5. Primer sequences are given upon request. Gene expression changes were analyzed using iQ5 Optical system software v2. The results were normalized using a housekeeping gene, TATA box binding protein (TBP), which was previously validated in our lab. Quantitative RT-PCR results were analyzed through delta CT calculations.

2.9. Western blotting

Skin tissue lysate was homogenized in RIPA buffer (50 mM Tris HCl pH8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, protease inhibitor cocktail, phosphatase inhibitor cocktail and 1 mM DTT). Protein concentration was determined using the BSA method and the skin lysates were denatured at 95 °C, for 5 min, in sample buffer. 40 μg of total protein were resolved on 12% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T), for 1 h, at room temperature. After blocking, membranes were incubated with primary antibodies against TNF-α (1:500), and MMP-9 (1:500), overnight at 4 °C. After incubation, membranes were washed and incubated for 1 h at room temperature, with anti-rabbit antibody (1:5000), or anti-mouse antibody (1:5000). The membranes were exposed to the ECF reagent followed by scanning on the VersaDoc (Bio-Rad Laboratories, Portugal). For normalization, the membranes were reprobed with an anti-actin antibody (1:10000). The generated signals were analyzed using the Image-Quant TL software.

2.10. Hydroxyproline content

This analysis was performed using a Hydroxyproline Assay Kit (Sigma Aldrich). Briefly, 10 mg of skin tissue were homogenized in 100 μl of water and hydrolyzed with HCl 12 M at 120 °C for 3 h. 25 μl of the supernatant were transferred to 96-well plate and evaporated in the incubator at 60 °C till total dryness. After, 100 μl of the Chloramine T/Oxidation Buffer and 100 μl of the Diluted DMAB Reagent were added to each sample and incubated for 90 min at 60 °C. Quantification was performed using an ELISA automatic microplate reader (SLT, Austria) at 560 nm. Hydroxyproline content was calculated from a previously obtained hydroxyproline standard curve.

2.11. Histopathological analysis

For histological preparations, the skin was fixed in 10% neutral buffered formalin and then embedded in paraffin. Skin tissues were sectioned in 3 μm thickness slices for histopathological examination by hematoxylin/eosin (H&E) and for collagen formation by Masson's Trichrome staining. The stained sections were observed with a microscope Nikon H600L with Digital Camera DXM 1200F (Nikon, Germany). Analysis of stained skin sections was performed by an experienced pathologist.

2.12. Statistical analysis

Results are expressed as mean ± SEM (Structural equation modeling). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison tests or through the unpaired and paired *t* tests by GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) and p values lower than 0.05 were considered statistically significant.

3. Results

3.1. In vitro biocompatibility of collagen dressings

SEM analysis show that both Raw 264.7 and HaCaT cell lines adhere to the collagen dressing surfaces without apparent change in their morphology (Fig. 1A).

There was also no significant difference in the viability of macrophages, Raw 264.7, after exposure to collagen dressings during 24, 48 and 72 h, and when compared to control, as shown in Fig. 1B. However, the viability of HaCaT is significantly increased after contact with the collagen dressings for 24 and 48 h (Fig. 1C). NO is produced by macrophages in response to inflammatory stimuli. The production of nitrites, the final stable breakdown product of NO, measured after exposure of the cells to the collagen dressings (Fig. 1D) was not significantly affected when compared to control.

3.2. In vivo wound healing experiments

Fig. 2 shows the effect of the different topical treatments studied: NT alone, collagen dressings alone and NT–loaded collagen dressings both in control (A) and diabetic (B) mice. PBS was applied in non-treated wounds. For control mice, no major differences were verified among the treatments (Fig. 2A). However, in diabetic mice, significant differences were observed after day 3 post-wounding, where NT alone reduced diabetic wound sizes by 11% ($p < 0.05$) and NT–loaded collagen by 17% ($p < 0.001$), compared to the PBS treated wounds (Fig. 2B). Collagen treated wounds achieved the significance only at day 5, by 18% compared to control wounds.

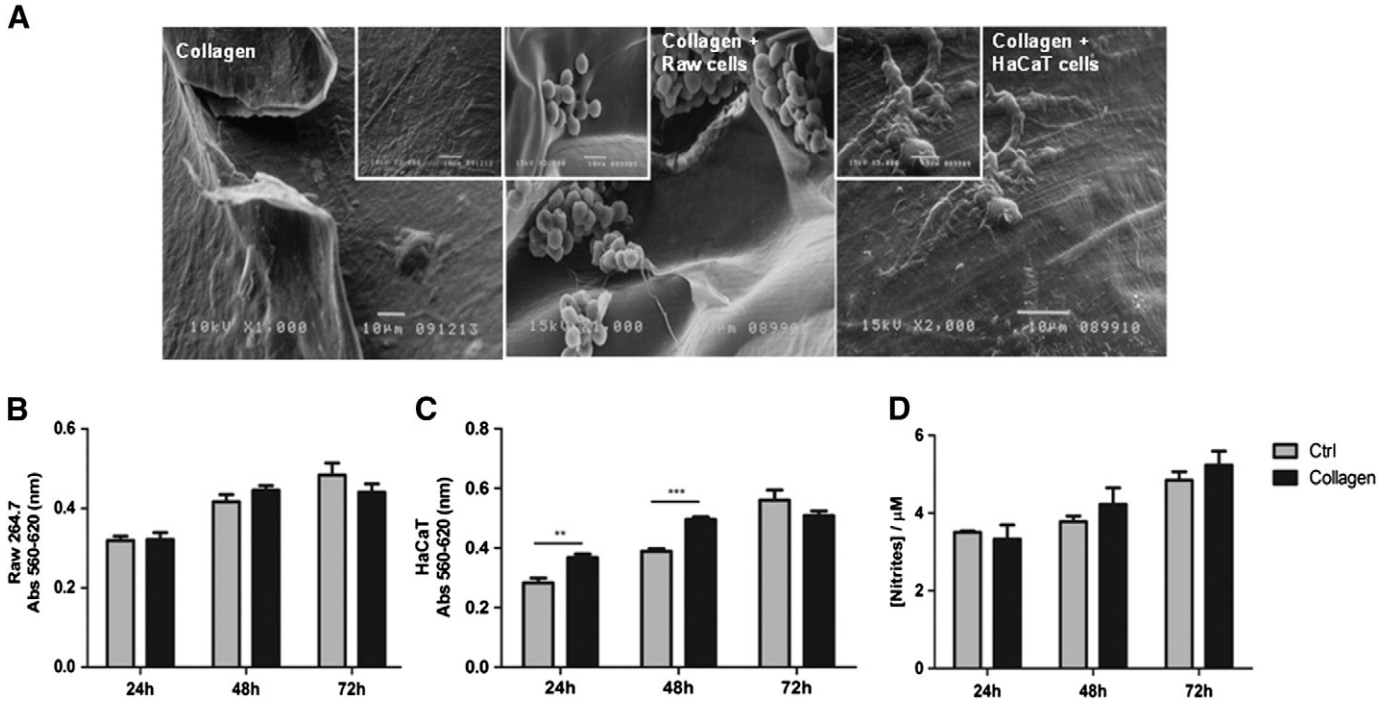

Fig. 1. SEM micrographs for non-loaded collagen foam structures and/or in contact with Raw 264.7 and HaCaT cells obtained by freeze-drying (A). Inner images represent magnifications. Cell viability of Raw (B) and HaCaT (C) cells in the presence of collagen foams, during 24, 48 and 72 h. NO production in Raw cells (D). Results are presented as mean ± SEM of three to six independent experiments.

3.3. Cytokine expression at the wound site

In order to address the pattern of cytokine expression in untreated (d0) or treated wounds at day 3 post-wounding and after complete wound healing (fd), gene expression of inflammatory cytokines (TNF-α and IL-1β), MMP-9, TGFβ1, TGFβ3 and several types of collagen genes (COL1A1, COL1A2, COL3A1) was measured and the results are presented in Fig. 3A–P. Gene expression for IL-6, KC and growth factors (EGF, VEGF and PDGF) was also performed, however few differences were observed and consequently they are presented in Supplementary Fig. S1.

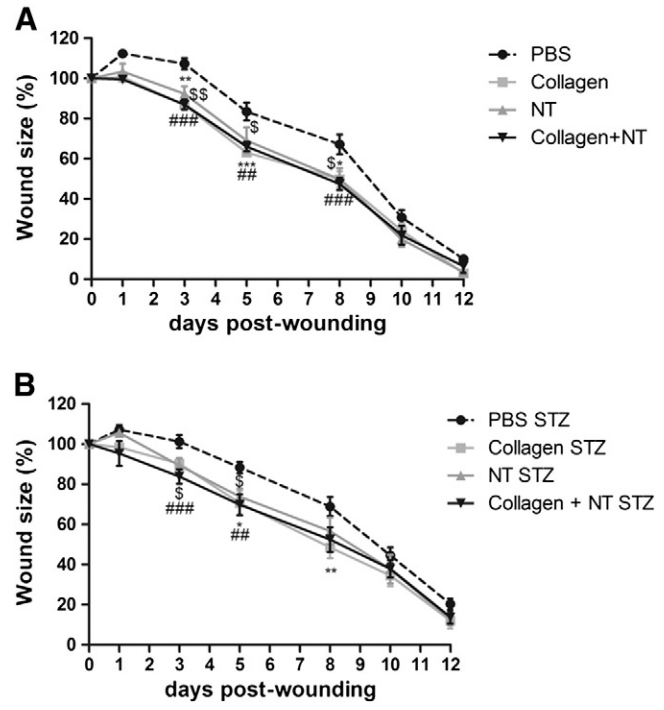

Fig. 2. Wound size for collagen, NT and NT–loaded collagen foam treatments in control (A) or diabetic (B) mice. The wound size was determined at days 0, 1, 3, 5, 8, 10 and 12 post-wounding. Results are presented as mean ± SEM of five to twenty four independent experiments. *$p < 0.05$ collagen compared to PBS, **$p < 0.01$ collagen compared to PBS, *** $p < 0.001$ collagen compared to PBS, ## $p < 0.01$ collagen + NT compared to PBS, ### $p < 0.001$ collagen + NT compared to PBS, $ $p < 0.05$ NT compared to PBS, $$ $p < 0.01$ NT compared to PBS.

In unwounded skin (day 0, baseline), and when comparing with control mice, all the mRNA measured for the inflammatory cytokines and MMP-9 were significantly increased in the skin of diabetic animals (Fig. 3A–F). On the other hand, the expression of TGFβ3 and all types of collagen genes analyzed was significantly reduced (Fig. 3K–P).

At day 3 post-wounding, NT alone stimulated the expression of TNF-α ($p < 0.01$), TGFβ1 ($p < 0.05$) and all types of collagen ($p < 0.01$) in diabetic mice while, in control, IL-1β expression and TGFβ1 expression are decreased ($p < 0.01$) (Fig. 3A, G, K, M, O). For instance, NT–loaded collagen reduced the expression of inflammatory cytokines TNF-α ($p < 0.01$), and IL-1β ($p < 0.01$) (Fig. 3A and C). COL1A2 was significantly increased ($p < 0.01$) in diabetics while in controls all types of collagen are reduced ($p < 0.01$) (Fig. 3K, M and O). Collagen alone did not affect inflammatory cytokine expression either in diabetic or control skin, while in diabetics the MMP-9 expression is reduced ($p < 0.001$) and TGFβ1 ($p < 0.05$) and TGFβ3 ($p < 0.01$) are significantly stimulated. In control mice, expression of VEGF is significantly stimulated ($p < 0.05$) and COL1A1, COL2A1 and COL3A1 are reduced ($p < 0.05$; $p < 0.01$; $p < 0.001$, respectively) (Fig. 3G, I, K, M and O).

After complete healing (fd), NT alone and NT–loaded collagen reduced significantly the expression of TNF-α ($p < 0.001$) and MMP-9 ($p < 0.01$) in control skin. However both treatments increased significantly the expression of TNF-α ($p < 0.05$) and reduced the expression of MMP-9 ($p < 0.001$) in diabetic skin (Fig. 3B and F). Moreover, all treatments stimulated significantly the expression of TGFβ1, COL1A1, COL1A2 and COL3A1 in control skin. However, NT alone reduced the expression of COL1A1 ($p < 0.05$) in diabetic skin while collagen alone or combined with NT significantly increased COL1A2 and COL3A1 expression (Fig. 3H, L, N and P).

3.4. Protein expression at the wound site

Western blots were performed to evaluate the protein expression in the skin at the wound site (Fig. 4). At day 0, MMP-9 is significantly increased ($p<0.05$) in non-treated diabetic skin when compared to control. In contrast, at day 3, NT–loaded collagen significantly reduced MMP-9 ($p<0.05$) protein levels and increased significantly the protein expression of TNF-α ($p < 0.05$) in diabetic skin. Moreover, collagen

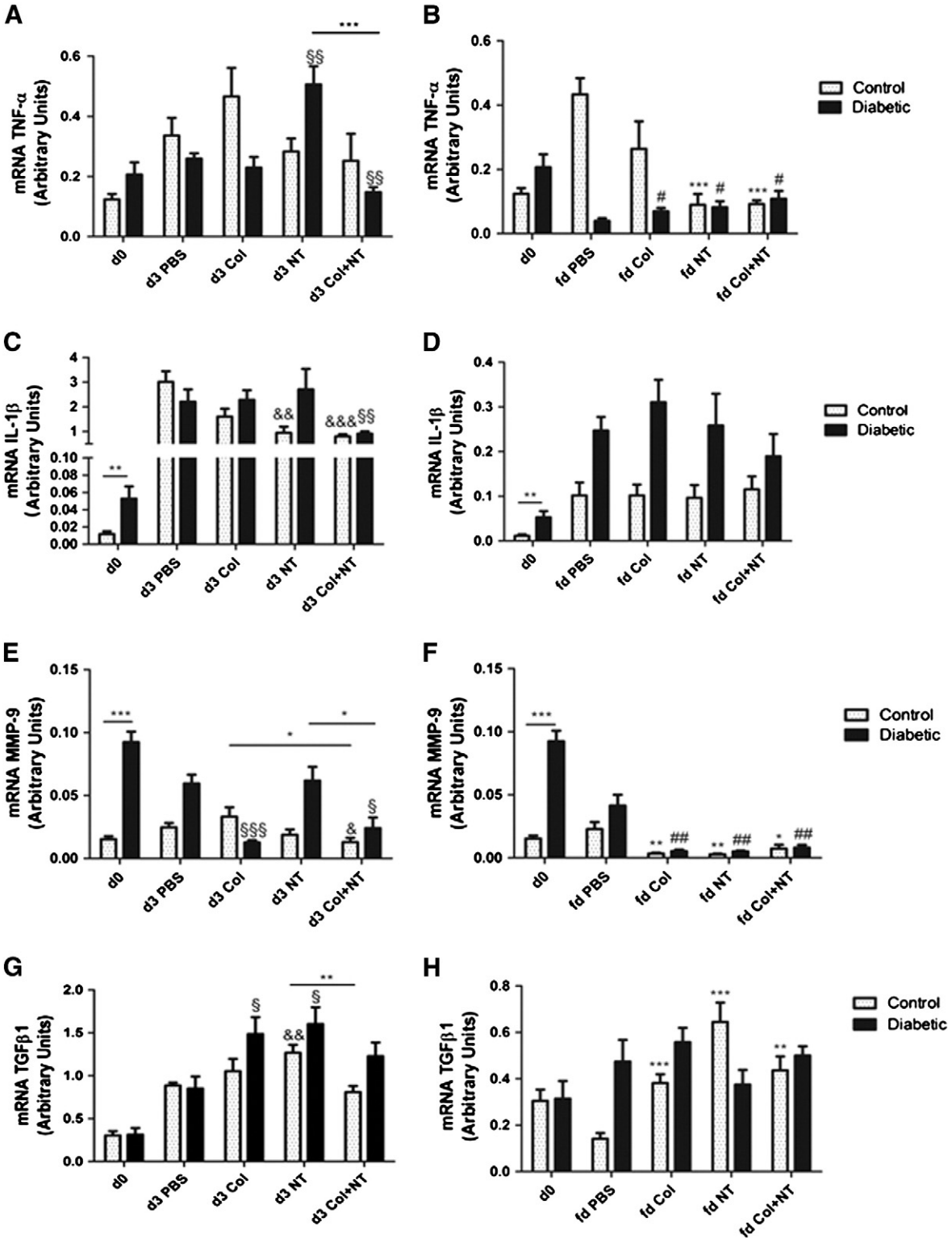

Fig. 3. The gene expression profile of TNF-α, IL-1β, MMP-9, TGFβ1, TGFβ3, COL1A1, COL1A2 and COL3A1 in skin biopsies before and after treatments, at either day 3 (A, C, E, G, I, K, M and O) or total wound healing day (fd) (B, D, F, H, J, L, N and P) post wounding. Results are presented as mean ± SEM of five to eighteen independent experiments. & $p<0.05$ compared with PBS d3, && $p<0.01$ compared with PBS d3, &&& $p<0.001$ compared with PBS d3, *$p<0.05$ compared with PBS fd, **$p<0.01$ compared with PBS fd, ***$p<0.001$ compared with PBS fd, § $p<0.05$ compared with diabetic PBS d3, §§ $p<0.01$ compared with diabetic PBS d3, # $p<0.05$ compared with diabetic PBS fd, ##$p<0.01$ compared with diabetic PBS fd, ###$p<0.001$ compared with diabetic PBS fd.

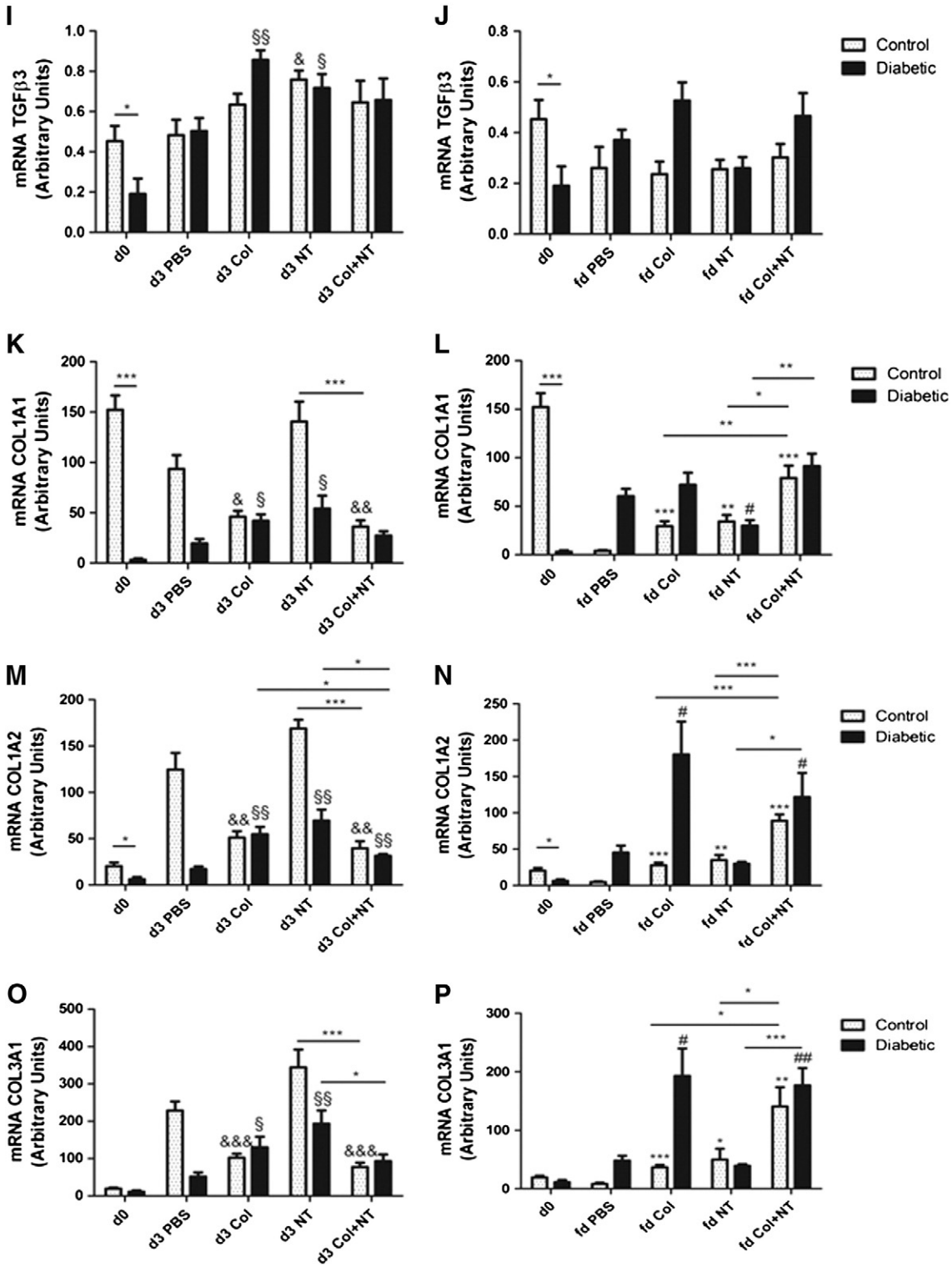

Fig. 3 (*continued*).

alone was also able to decrease the expression of TNF-α. After complete healing (fd), NT–loaded collagen dressings significantly reduced MMP-9 protein expression comparing with PBS in diabetic skin. In addition, an overall increase in MMP-9 expression was observed at the final day as compared to day 3 post-wounding. No major differences were observed in VEGF protein expression with all treatments and time points. In addition, TNF-α protein expression was not detected by western blot analysis after complete healing (fd).

3.5. Hydroxyproline content at the wound site

Hydroxyproline levels were measured to evaluate collagen deposition, in both unwounded and wounded, treated and non-treated wounded skin (Fig. 5). In unwounded skin, hydroxyproline levels were significantly decreased ($p<0.05$) in diabetic mice comparing with control skin. At day 3 post-wounding, no differences were observed with the different treatments. However treatments with collagen (with and

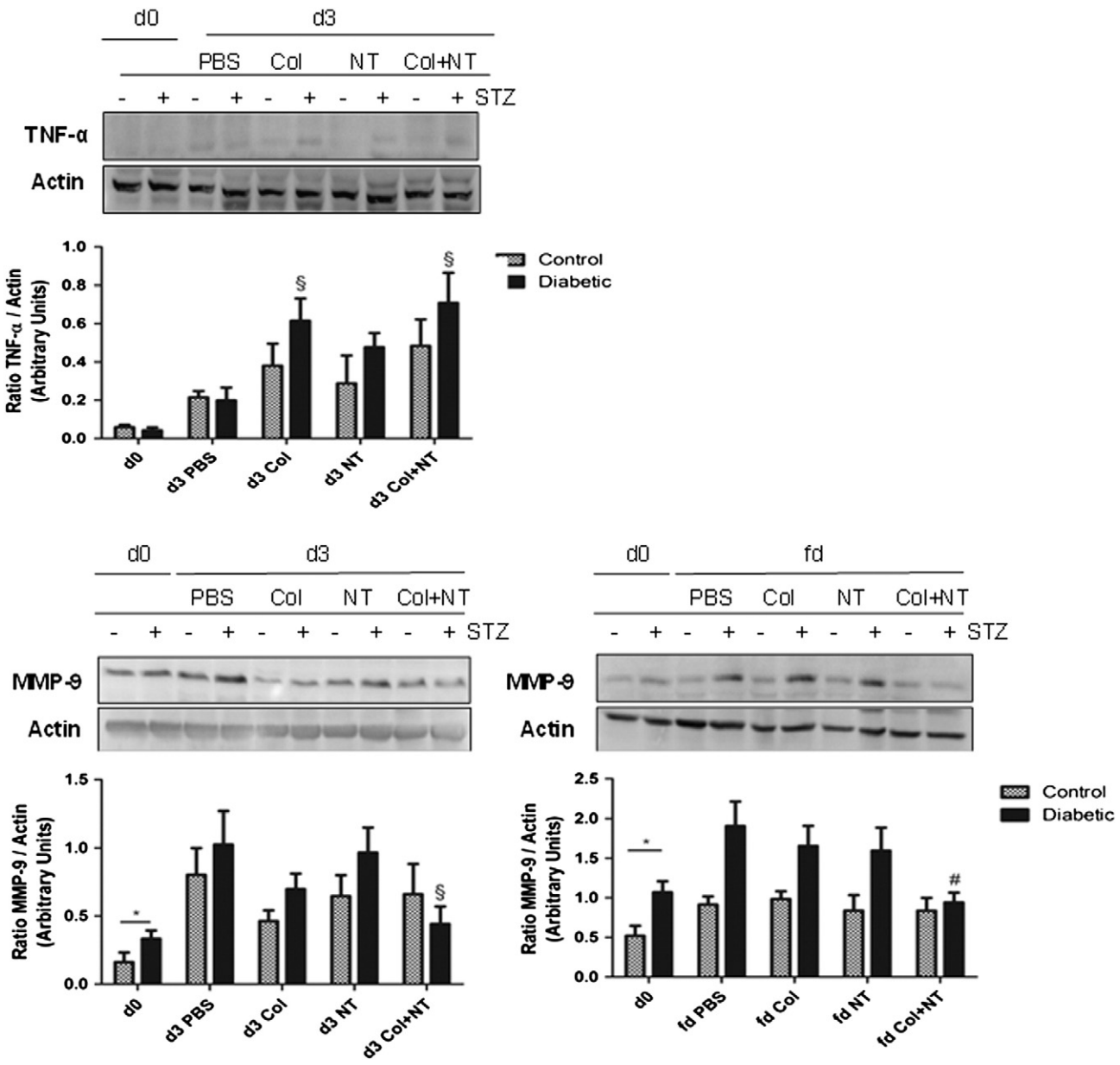

Fig. 4. Protein expression of TNF-α and MMP-9 in unwounded skin (day 0) or after treatments, at either day 3 or total wound healing day (fd). Results are presented as mean ± SEM of three to five independent experiments. § $p < 0.05$ compared with PBS d3, * $p < 0.05$ compared with diabetic PBS d3, #$p < 0.05$ compared with diabetic PBS fd.

without NT) stimulated significantly an increase in the hydroxyproline content in control skin after complete healing (fd). The same effect was observed for NT–loaded collagen in diabetic skin.

3.6. Histopathological analysis of the wound

We performed the histopathological analysis (H&E and Masson's Trichrome staining) of unwounded and wounded control and diabetic skin treated with collagen or NT or NT–loaded collagen (Fig. 6A and B). In unwounded diabetic skin, the thickness of the epidermis (that includes stratus lucidum, epithelial layers and basal layer) is increased compared to control skin. At day 3 post-wounding, all the treatments stimulated an increase in the epidermis thickness. The most significant epidermal thickness was found in the non-loaded and NT–loaded collagen treatments in diabetic skin (Table 1).

A specific re-epithelialization profile was observed: in control mice, re-epithelialization occurred from bottom to top with basal cells in the epidermis covering the scar; in diabetic mice, the re-epithelialization occurred over the granulation inflammatory tissue while it was suffering repair, without correlation with the treatments and in both groups (Table 1).

At day 3 post-wounding, none of the treatments affected the number of polymorphonuclear leukocytes (PMN) in control skin, however in diabetic skin, these inflammatory cells were less recruited to the wound site treated with collagen alone, NT alone or NT–loaded collagen compared with the PBS (Table 3). No plasma cells were observed either in control or in diabetic skin. However, less fibrin and a higher number of fibroblasts were observed at the wound site after treatments in diabetic skin. In addition, more loose collagen was observed in NT and NT–loaded collagen treated skin either in control or diabetic wounds.

After complete healing (fd), epidermal thickness was similar either in control or diabetic skin (Table 2). In addition, inflammatory cells (PMN and plasma cells) were not present at the wound site either in control or diabetic skin treated or non-treated, with exception of lymphocytes that persisted in diabetic treated skin (Table 4). No fibrin was observed either in control or diabetic skin. There was increased

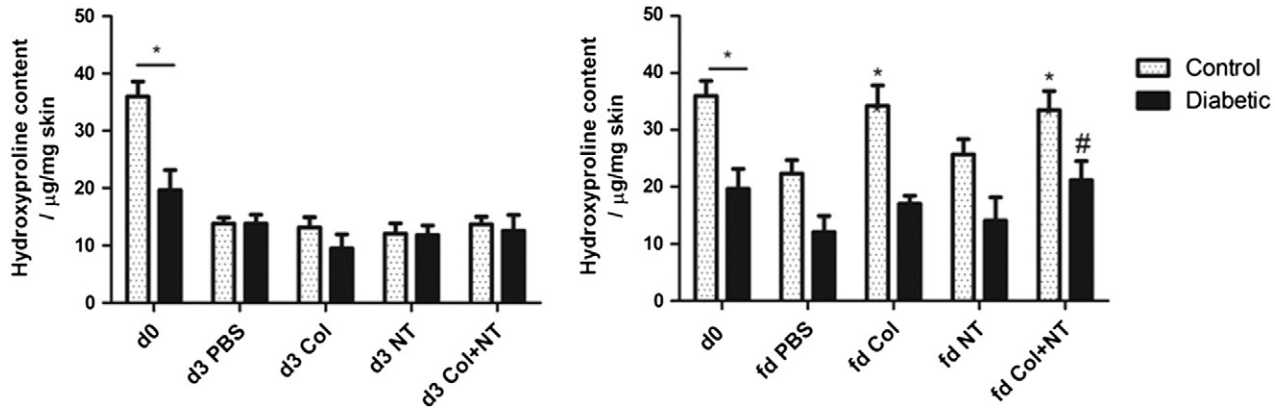

Fig. 5. Hydroxyproline content levels in unwounded skin (d0) or after treatments, at either day 3 or total wound healing day (fd). Results are presented as mean ± SEM of four to six independent experiments. *$p < 0.05$ compared with PBS fd.

migration of fibroblasts in wounded tissue after NT–loaded collagen treatments followed by collagen and NT treatments in diabetic skin. In addition, more loose collagen is present in NT and NT–loaded collagen treated diabetic skin compared with PBS, and the scar is more pronounced in collagen than in NT treated skin.

A summary of cytokine expression and corresponding cell type production, in wounded control and diabetic skin, at either day 3 post-wounding or total wound healing day, is represented on Table 5.

4. Discussion

Treatment, in the particular case of DFUs, should actively promote wound healing by correcting the expression of biological factors involved in the healing process, namely neuropeptides. Nabzdyk and co-authors [25] verified that in DFU, neuropeptides such as substance P and neuropeptide Y control cytokine release from leukocytes and affect endothelial cell function. In the present study, we evaluated the effect of the topical application of NT and NT–loaded collagen in control and diabetic wound healing, using a full skin thickness wound mouse model. Non-loaded collagen and PBS alone were also studied for comparison. Collagen was used as support for NT as an alternative to facilitate the application of the neuropeptide into the wound site and also to evaluate its capacity to sustain its delivery and to avoid biodegradation. This biopolymer has been frequently used in as wound dressing material to accelerate healing [26,27]. Among other advantages it has a prominent role to maintain the biological and structural integrity of the extracellular matrix (ECM) [28].

Results for the *in vitro* biocompatibility of collagen with cells present in skin, namely macrophage Raw 264.7 and keratinocyte HaCaT cell lines showed that the materials prepared in this work were non-toxic against these cell lines (up to 72 h) when compared with control cells (without collagen). The viability of HaCaT cells was even significantly increased after 24 and 48 h in contact with collagen. SEM analyses showed that both macrophage Raw 264.7 and keratinocyte HaCaT cells adhere perfectly to collagen without apparent change in their morphology. Similar results were observed by Yahyouche and co-authors, [29] which reported that Raw 264.7 macrophages attached to collagen scaffolds, proliferated and aggregated into it (similarly to our SEM images). Moreover, in another formulation (nanofibers) collagen showed to have good adherence after contact with keratinocyte cell line NHEK [30].

Wound closure results showed that the topical application of NT reduced significantly the wound area in either control and diabetic mouse skin compared with PBS treated wounds. These results are in agreement with previous data reported in the literature showing that different neuropeptides, namely substance P, induce diabetic wound healing [9,31,32]. Moreover, NT–loaded collagen dressings were slightly more effective in reducing wound area, especially in diabetic mice already at day 3 post-wounding. These results suggest a synergistic behavior between the bioactivity of NT alone and collagen dressing properties. All the treatments lead to total healing, however larger scars were developed over diabetic wounds that were treated with collagen dressings.

An overexpression of inflammatory cytokines (IL-1β) and matrix metalloproteinases (MMP-9) was observed in unwounded diabetic skin which is in agreement with previously reported data [33]. On the contrary, the expression of TGFβ3 and all of the collagen genes analyzed decreased in diabetic skin when compared to control. This may suggest a decrease in the capacity of diabetic mouse skin to produce essential components of the skin matrix that would guarantee correct healing [34]. For instance, a decrease in the migration of fibroblasts can lead to a decrease in COL1A1, COL1A2 and COL3A1 levels and justify the lower amount of collagen deposited as observed from the hydroxyproline assay results. Similar results were observed by Black and co-authors [35] which reported a decrease by 40% in the deposition of collagen in type 1 diabetes and its influence in the wound healing process.

The inflammatory phase is an important step in diabetic wound healing that frequently becomes stalled promoting a pro-inflammatory status that originates chronic non-healing diabetic ulcers. TNF-α and IL-1β are inflammatory cytokines involved in the recruitment of cells, such as neutrophils and macrophages to the wound site, to stimulate the immune response. In the skin, TNF-α produced by inflammatory cells and fibroblasts stimulates adhesion molecules and chemokines leading to the attachment of inflammatory cells to vessels, rolling, migration, and eventually chemotaxis into the skin [33]. In addition, IL-1β, produced by macrophages, fibroblasts, keratinocytes and epithelial cells is also an important player in the early phase of inflammation and in the wound healing process [36]. In the present work, the inflammatory phase, which was monitored at day 3, was characterized by a decrease in the expression of IL-1β in control mice after treatment with NT and NT–loaded collagen, suggesting a decrease in the inflammatory status, which promotes healing. However, in diabetic mice, NT stimulated an increase of the TNF-α gene expression while NT–loaded collagen led to a decrease in the protein expression of this inflammatory marker. In addition, all of the treatments that were studied decreased the recruitment of inflammatory cells to the wound site when compared to control. This can suggest that the high expression of TNF-α at day 3, in diabetic mice, is not only produced by the inflammatory cells present in the wound site but also by other cells, such as fibroblasts and other skin cells. These results can justify the contraction of the wound, stimulation of granulation tissue formation and a faster re-epithelialization of the wound site when NT–loaded collagen is applied. Consequently this treatment has a potential positive effect in the early phases of wound repair. Similar results were previously obtained in our group using NT–loaded chitosan based derivatives as wound dressings [37].

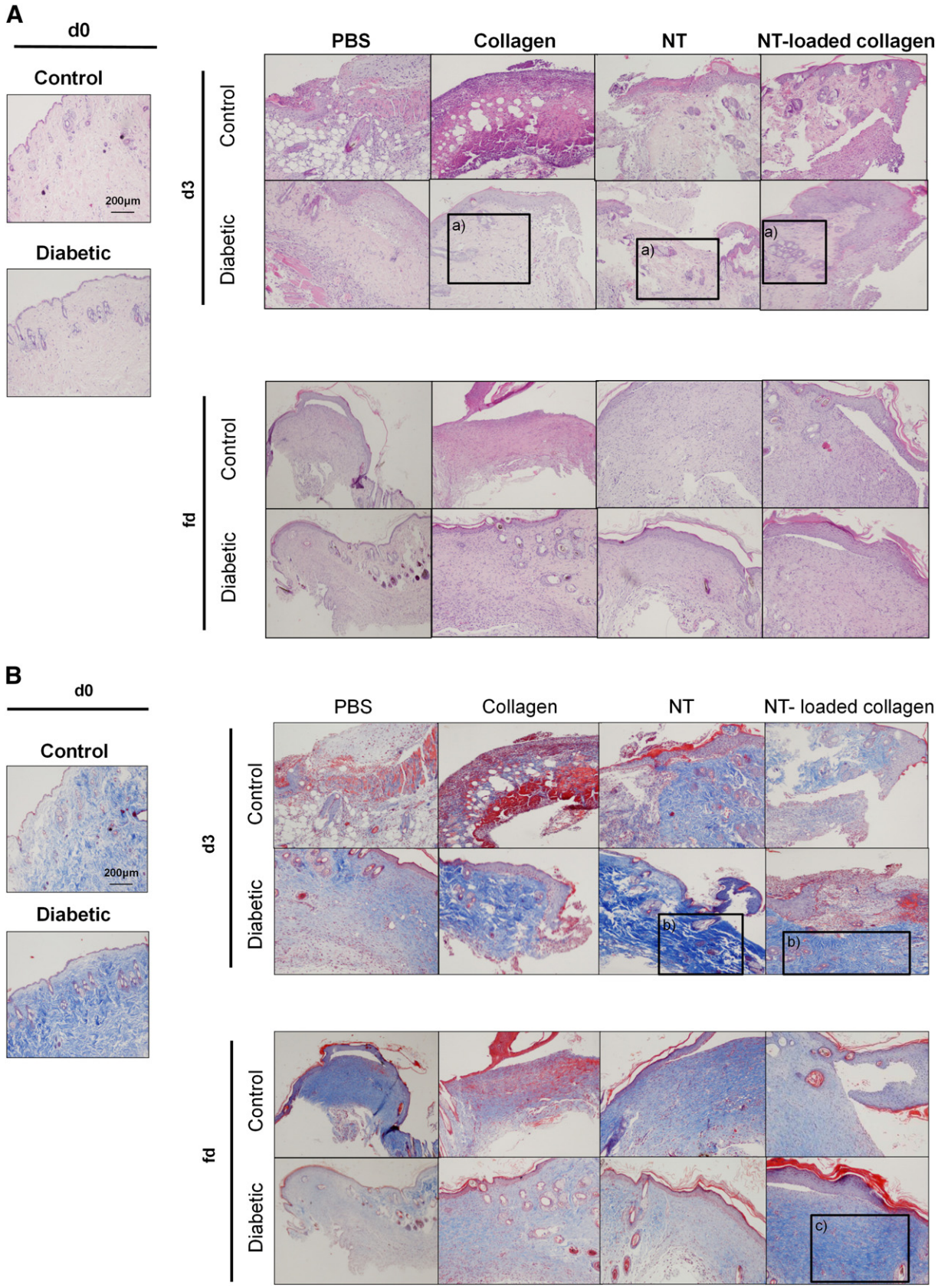

Fig. 6. Histopathological analysis of hematoxylin and eosin (H&E) (A) and Masson's Trichrome (B) stainings for control and diabetic mouse skin, untreated or treated with collagen, NT and NT–loaded collagen foams (magnification 100×). Wounds are in continuum with preserved skin. Representative images of three skin staining were analyzed. a) Less infiltrated PMN and lymphocytes in the granulation tissue after collagen, NT or NT–loaded collagen application in diabetic mice, at day 3 post-wounding; b) increased collagen deposition in NT and NT–loaded collagen treated wounds in diabetic mice, at day 3 post-wounding. c) Increased collagen deposition in NT–loaded collagen treated wounds in diabetic mice at total wound healing day (fd).

Table 1
Histological analysis of unwounded skin and in the presence of NT, collagen and NT–loaded collagen dressing treated wounds at day 3, by H&E staining. − absence or no alterations, + presence <10%, ++ presence 10%–50%, +++ presence > 50%, na — not applicable.

	Skin (d0)		Day 3							
			PBS		Collagen		NT		NT–loaded collagen	
	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic
Epidermis thickness										
– Stratus lucidum	−	+	−	+	++	+++	−	++	++	+++
– Epithelial layers	−	+	−	+	++	+++	−	++	++	+++
– Basal layer	−	+	−	+	+	++	−	++	+	++
Wound area (mm^2)	27.67 ± 3.1	26.66 ± 2.7	29.6 ± 0.19	26.98 ± 0.41	23.99 ± 0.37	24.05 ± 0.32	25.54 ± 0.31	23.83 ± 0.24	24.05 ± 0.22	22.34 ± 0.18
Re-epithelialization										
– From bottom	na	na	+	−	+	−	+	−	+	−
– Top cover	na	na	−	+	−	+	−	+	−	+

Table 2
Histological analysis of skin in the presence of NT, collagen and NT–loaded collagen dressings treated control and diabetic wounds after complete healing (fd), by H&E staining. + presence <10%, ++ presence 10%–50%, +++ presence >50%.

	Complete wound healing (fd)							
	PBS		Collagen		NT		NT–loaded collagen	
	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic
Epidermis thickness								
– Stratus lucidum	+	++	+++	+++	++	+++	+++	+++
– Epithelial layers	+	++	+++	+++	++	+++	+++	+++
– Basal layer	+	++	++	+++	++	+++	++	+++

Moreover, in diabetic mice, the reduction of MMP-9 gene expression observed for the NT–loaded collagen treated wounds can contribute to the resolution of the persistent inflammation. MMP-9 is produced by several different types of cells in the skin, including fibroblasts, keratinocytes, macrophages and endothelial cells [38]. However, no differences were verified in MMP-9 protein expression after NT–loaded collagen treatment.

Furthermore, TGFβ1 and TGFβ3 are significantly increased after collagen and NT treatments alone, however no significant differences were observed when NT–loaded collagen was also used as treatments in diabetic mice. This result reinforces the fact that cells (besides inflammatory cells) may contribute to the resolution of inflammation. The TGF-β family of proteins attracts macrophages into the wound area and stimulates them to produce additional cytokines, to enhance fibroblast and smooth muscle chemotaxis and to modulate collagen expression and consecutively scar formation [39].

Type I collagen is the most expressed collagen in skin, followed by type III and type IV, which contribute for the stability of the epidermis and are responsible for its tensile strength. In this work, it was verified that all the treatments significantly increased the expression of COL1A1, COL1A2 and COL3A1 in the skin of diabetic mice. On the contrary, treatments with collagen (alone or loaded with NT) in the skin of control mice led to a decrease in the expression of these genes. Recent studies show that a decrease in the expression of type III collagen, in early granulation tissue, promotes myofibroblast differentiation and an increase in scar deposition in cutaneous wounds [40]. Although these results were not reproduced by the measured hydroxyproline levels, results from histopathological analysis showed an increase in the amount of fibroblasts and loose collagen matrix in the treated diabetic skin already at day 3.

After complete healing (fd), the inflammatory process in control skin has already been mostly concluded, as expected. The decrease in the MMP-9 protein levels, in diabetic skin, contributes to the proteolytic degradation of the ECM and consequently to repair and remodel cutaneous wounds. During the re-epithelialization and remodeling phases, the initial ECM formed is being replaced by collagenous matrix [41]. Fibroblasts promote re-epithelialization by the production of ECM proteins such as collagen [42]. Fibroblast accumulation at the wound site was

Table 3
Skin inflammatory and granulation tissue histological analysis in the presence of NT, collagen and NT–loaded collagen dressings treated control and diabetic wounds at day 3, by H&E and Masson's Trichrome staining. Symbols represent: − absence or no alterations, + presence <10%, ++ presence 10%–50%, +++ presence >50%; < not relevant, > predominant.

	Day 3							
	PBS		collagen		NT		NT–loaded collagen	
	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic
Inflammation status								
– PMN	++	+++	++	+	++	+	++	+
– Lymphocytes	+	++	+	+	+	+	++	++
– Plasma cells	−	−	−	−	−	−	−	−
– Fibrin	<	>	>	<	>	<	>	<
Repair								
– Fibroblasts	<	>	<	>	<	>	<	>
Collagen matrix								
– Loose	−	−	−	+	+	+	++	++
– Scar	−	−	−	−	−	+	−	−

Table 4
Skin inflammatory and granulation tissue histological analysis in the presence of NT, collagen and NT–loaded collagen dressings treated control and diabetic wounds after complete healing (fd), by H&E and Masson's Trichrome staining. Symbols represent: − absence or no alterations, + presence <10%, ++ presence 10%–50%, +++ presence >50%.

	Complete wound healing (fd)							
	PBS		Collagen		NT		NT–loaded collagen	
	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic
Inflammation status								
– PMN	−	+	−	−	−	−	−	+
– Lymphocytes	+	+	+	++	+	+++	+	++
– Plasma cells	−	−	−	−	−	−	−	−
– Fibrin	−	−	−	−	−	−	−	−
Repair								
– Fibroblasts	++	+	+	++	+	++	+	+++
Collagen matrix								
– Loose	+	+	+	++	++	+++	++	+++
– Scar	+	+	+	++	+	+	+	++

increased in diabetic skin after treatment with collagen (with and without NT), enhancing COL1A2 and COL3A1 expression and collagen deposition and consecutively the formation of a more organized loose collagen matrix, as observed by both H&E and Masson's Trichrome staining. However, a more prominent scar is evident when these treatments with collagen are applied in diabetic skin when compared with the treatment with NT alone (without the collagen support). This may indicate that NT stimulates wound closure with a better esthetic appearance. A similar matrix/dressing effect was previously observed in our group after application of a chitosan derivative (5-methylpirrolidinone) for the treatment of diabetic wounds [37].

5. Conclusions

The results obtained in this work show that NT–loaded collagen dressings are effective as wound-healing accelerators in diabetic mice, at day 3 post-wounding (inflammatory phase), reducing the inflammatory infiltrate in the early phase of healing and the proteolytic degradation of ECM by MMP-9. Moreover, NT–loaded collagen stimulated fibroblast accumulation in tissue granulation, collagen expression and deposition at the wound site, which lead to the production of a more organized collagen matrix. On the contrary, the treatment with NT alone presented a lower inflammatory potential however, it stimulated the expression of all of the collagen types studied in this work. Special attention was given to the effect of each treatment at different time points (0, 3, 10 days post-wounding and after complete healing) which correspond to different stages of the wound healing process. Table 5 summarizes the cytokine expression in wounded non-diabetic and diabetic skin, at day 3 and after complete healing. All together these results indicate that NT can enhance diabetic wound healing and that its activity can be even enhanced when it is applied on collagen based dressings. These effects were particularly evident during the inflammatory phase. Despite of the promising results, the NT–collagen treatment still led to the formation of a more pronounced scar after complete healing. Therefore further studies will have to be done to overcome this issue and to develop a dressing material that can originate improved esthetic results. The promising results obtained in this work need also to be complemented with human studies to further investigate the potential application of NT–loaded collagen wound dressings for diabetic foot ulcer treatment.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.10.009.

Conflict of interest

The authors do not have any conflicts of interest.

Table 5
Summary of protein expression in wounded control and diabetic skin, at day 3 post-wounding and after complete healing (fd).

Day	Cytokine/growth factor	Control mice	Diabetic mice	Cell type that produce this protein
3	TNF-α	=	↑ NT, ↓Col + NT	Macrophages, fibroblasts
	IL-1β	↓ NT, Col + NT	=	Macrophages, epithelial cells
	MMP-9	↓ Col + NT	↓ Col, Col + NT	Fibroblasts, keratinocytes, macrophages, endothelial cells
	TGFβ1	↑ NT	↑ Col, NT	Macrophages, PMN, fibroblasts, keratinocytes, epithelial cells
	TGFβ3	↑ NT	↑ Col, NT	Macrophages, PMN, fibroblasts, keratinocytes, epithelial cells
	COL1A1	↓ Col, Col + NT	↑ Col, NT	Fibroblasts
	COL1A2	↓ Col, Col + NT	↑ Col, NT, Col + NT	Fibroblasts
	COL3A1	↓ Col, Col + NT	↑ Col, NT	Fibroblasts
fd	TNF-α	↓ NT, Col + NT	↑ Col, NT, Col + NT	Macrophages, fibroblasts
	IL-1β	=	=	Macrophages, epithelial cells
	MMP-9	↓ Col, NT, Col + NT	↓ Col, NT, Col + NT	Fibroblasts, keratinocytes, macrophages, endothelial cells
	TGFβ1	↑ Col, NT, Col + NT	=	Macrophages, PMN, fibroblasts, keratinocytes, epithelial cells
	TGFβ3	=	=	Macrophages, PMN, fibroblasts, keratinocytes, epithelial cells
	COL1A1	↑ Col, NT, Col + NT	↓ NT	Fibroblasts
	COL1A2	↑ Col, NT, Col + NT	↑Col, Col + NT	Fibroblasts
	COL3A1	↑ Col, NT, Col + NT	↑Col, Col + NT	Fibroblasts

= means no differences; ↓ means significant decrease; ↑ means significant increase.

Acknowledgements

This work was financially supported by COMPETE, FEDER and Fundação para a Ciência e Tecnologia (FCT-MEC) under contract PTDC/SAU-MII/098567/2008, PTDC/SAU FAR/121109/2010, PEst-C/EQB/UI0102/2011 and PEst-C/SAU/LA0001/2013-2014, in addition to the RIMADEL — Rede Iberoamericana de Nuevos Materiales para el Diseño de Sistemas Avanzados de Liberación de Fármacos en Enfermidades de Alto Impacto Socioeconómico, EFSD/JDRF/Novo Nordisk European Programme in Type 1 Diabetes Research and Sociedade Portuguesa de Diabetologia (SPD).

Liane I. F. Moura, Ana M. A. Dias and Ermelindo Leal acknowledge FCT-MEC for their fellowships SFRH/BD/60837/2009, SFRH/BPD/40409/2007 and SFRH/BPD/46341/2008, respectively.

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