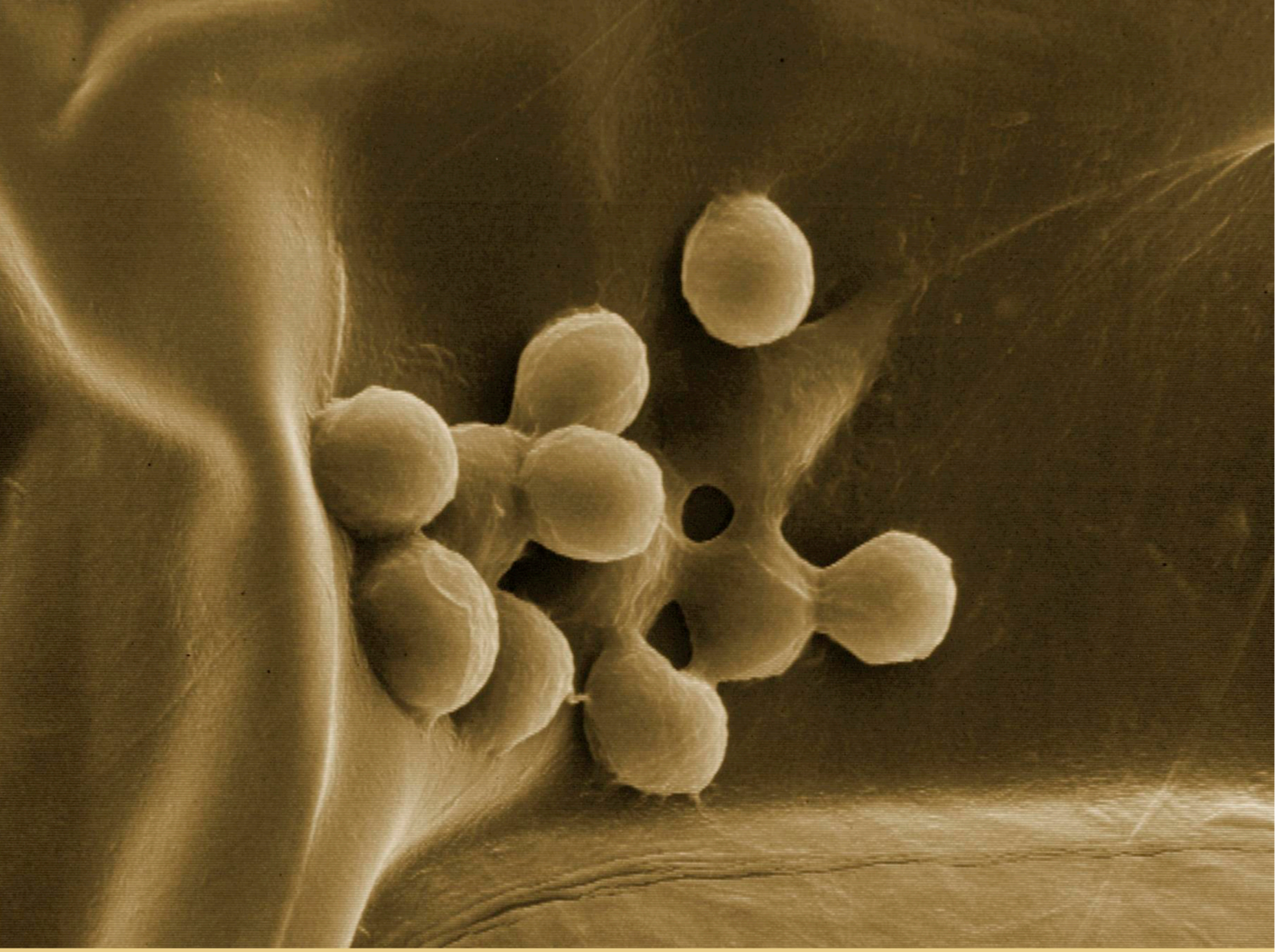

Liane Isabel Ferreira Moura

TESE DE DOUTORAMENTO

DEVELOPMENT OF NOVEL THERAPEUTIC APPROACHES FOR WOUND HEALING IN DIABETES

2013

UNIVERSIDADE DE COIMBRA

Liane Isabel Ferreira Moura

Development of novel therapeutic approaches for wound healing in diabetes

A Thesis submitted for the degree of

Doctor of Philosophy in Chemical Engineering (pré-Bolonha) – specialization in Biotechnology

Coimbra 2013

Financial support by:

Agradecimentos / Acknowledgments

Um trabalho deste âmbito não poderia ser realizado sem a ajuda fulcral de algumas pessoas às quais pretendo manifestar a minha profunda gratidão.

À minha orientadora, Dra Eugénia Carvalho, o meu obrigado por ter confiado e dado a oportunidade de realizar este trabalho de doutoramento, com todas as condições necessárias à sua realização.

Ao meu co-orientador, Professor Dr. Hermínio C. de Sousa por toda a ajuda e conhecimento científico e não científico que me transmitiu ao longo destes 4 anos. Muito obrigada por todas as sugestões e comentários na redacção desta tese.

À Dra Ana Dias, o meu agradecimento especial pela simpatia, dedicação e imensa disponibilidade com que sempre acompanhou o meu trabalho.

À Professora Dra Maria Teresa Cruz por toda a disponibilidade, simpatia e orientação relativa ao trabalho *in vitro* presente nesta tese.

À Dra Lina Carvalho e todo o serviço de Anatomia Patológica da FMUC o meu obrigada por me terem recebido sempre com simpatia e pela ajuda na análise histopatológica realizada.

Ao Ermelindo e à Ana Tellechea por terem sido os primeiros a acompanharem-me neste desafio e, mesmo longe, sempre tiverem uma palavra de apreço e motivação.

Aos meus amigos por terem estado sempre presentes com palavras de apoio e motivação e por todos os momentos bem passados que tornaram mais fácil ultrapassar todas as dificuldades deste percurso.

Ao todos os meus amigos/colegas de laboratório, no CNC e no DEQ, que tornaram os longos dias de trabalho, momentos de boa-disposição e companheirismo o meu muito obrigada.

À FCT-MEC, comparticipada pelo Fundo Social Europeu e por fundos nacionais do Ministério da Educação e Ciência pela Bolsa de Doutoramento SFRH / BD / 60837 / 2009.

Ao CNC e ao DEQ um agradecimento especial pela disponibilização dos serviços e laboratórios necessários para a execução das experiências.

E por fim, aos meus pais Luís e Isabel, a minha eterna gratidão pelo esforço, pelo incentivo e por terem sempre apoiado todas as minhas decisões; ao meu irmão Romão, por estar sempre presente ao longo destes anos e ao meu sobrinho Pedro, por me fazer acreditar num futuro bem melhor.

Abstract

Diabetes mellitus is one of the chronic diseases that affect more people worldwide. Patients with diabetes are susceptible to develop chronic, non-healing diabetic foot ulcers (DFU) which cause pain, suffering, poor quality of life and, in extreme cases, lower extremities amputations. This fact together with the high prevalence of DFU among the diabetic population (around 15%) increases the necessity to find new and more efficient approaches for DFU treatment.

Some neuropeptides, such as Substance P (SP) and Neuropeptide Y (NPY), are known to have an important role in the inflammatory, proliferative and reparative processes after tissue injury. However, little is known about the effect of other neuropeptide, neurotensin (NT), in these processes. Recent studies indicated that some of the above referred neuropeptides may act as inflammatory modulators and that may improve the diabetic wound healing process through topical application into wounds. However, one of the major problems associated to the topical administration of peptides, in general and neuropeptides in particular, are their short half-lives and the corresponding loss of bioactivity in the peptidase-rich wound environment. An alternative strategy to overcome these issues can be the use of biocompatible wound dressings for the sustained delivery of these neuropeptides. Some biopolymers such as collagen and chitosan derivatives may be employed for these purposes mostly due to their well known favorable properties and biological behavior, namely their ability to load/release bioactive substances, and their biocompatibility, biodegradability and non-toxicity.

In this thesis, the *in vitro* effects of NT in the migration, proliferation and regulation of cytokine expression of skin cells, namely in macrophages and keratinocytes, under hyperglycemic and/or inflammatory conditions were studied. Moreover, the analysis of the expression of NT and of its receptors was also performed under the above described conditions. In addition, the development and characterization of three chitosan derivatives (*N*-carboxymethyl chitosan (CMC), 5-methyl pyrrolidinone chitosan (MPC) and *N*-succinyl chitosan (SC)) and of type I mice collagen-based dressings as supports for the topical delivery of NT into diabetic wounds were performed. The evaluation of the progression of wound healing and of modulation of inflammatory, angiogenic and

re-epithelializating factors were performed (*in vivo*) using MPC and collagen-based dressings (with or without the release of NT) in a full-thickness wound healing model in diabetic mice.

From *in vitro* results, it was concluded that NT impairs macrophage migration under hyperglycemic conditions as well as it decreases their pro-inflammatory cytokines (IL-1β and IL-12) expression under hyperglycemic and inflammatory conditions. In addition, it was also found that hyperglycemia modulates NT and NT receptor expression in both tested conditions.

On the other hand and for human keratinocytes, the presence of NT strongly stimulated NT and NTR2 expression. However, results also showed that NT did not affect cell proliferation and migration, as well as the expression of some inflammatory cytokines (IL-1β and IL-8) and growth factors (EGF, VEGF and PDGF) under hyperglycemic conditions. These results thus suggest that NT did not exert a direct effect on keratinocytes function, but it seems to present a paracrine effect on other skin cells such as fibroblasts, macrophages and dendritic cells.

From *in vivo* tests, it was found that NT alone induced faster healing in either control (22%) or diabetic (29%) wounds at day 3 (if compared to non-treated wounds). MPC alone and NT-loaded MPC dressings presented different wound healing profiles either in control or in diabetic mice, at day 1 post-wounding, leading to significant reductions in wound sizes (48% and 43%, respectively, in control, and 35% and 50%, respectively in diabetic animals). RT-PCR analysis showed that NT-loaded MPC dressings reduced inflammatory cytokines expression (TNF-α) and decreased the inflammatory infiltrate at day 3. At day 10, the MMP-9 expression was also reduced in diabetic mouse skin, and led to increased fibroblast migration and to a higher collagen (COL1A1, COL1A2 and COL3A1) expression and deposition in wound sites.

Results obtained when using NT-loaded collagen dressings showed that, in diabetic mice, a faster healing was achieved (17% wound area reduction). In addition, this strategy significantly reduced the inflammatory cytokine expression (TNF-α and IL-1β) as well as the inflammatory infiltrate, at day 3 post-wounding. After complete healing (fd), the MMP-9 expression was also reduced in diabetic mouse skin. Once again, this probably led to fibroblast migration and to higher collagen (COL1A2 and COL3A1) expression and deposition.

Finally and in conclusion, all the obtained results (*in vitro* and in *vivo*) indicate that NT may enhance diabetic wound healing and its activity can be further improved when it is loaded into MPC or collagen based dressings.

The results presented in this thesis show that NT is a promising neuropeptide that can be used for the treatment of diabetic wounds, either alone or, preferably, combined with biocompatible and biodegradable wound dressings. Therefore, these results can/should be further developed in order to obtain new and more efficient bioactive wound dressings for treatment of DFU and even of other types of wounds.

Resumo

Diabetes mellitus é uma das doenças crónicas que afecta mais pessoas em todo o mundo. Os pacientes com diabetes são susceptíveis ao desenvolvimento de úlceras crónicas e não cicatrizantes do pé diabético (DFU), que causam dor, sofrimento fraca qualidade de vida e, em casos extremos, amputações das extremidades inferiores. Estes factos juntamente com a alta prevalência das DFU entre a população diabética (cerca de 15%) aumenta a necessidade de encontrar novas e mais eficientes abordagens para o tratamento das DFU.

Alguns neuropeptídeos, nomeadamente a Substância P (SP) e o Neuropeptídeo Y (NPY), são conhecidos por desempenharem um papel importante no processo inflamatório, proliferativo e reparativo após a lesão dos tecidos. Contudo, pouco é conhecido acerca do efeito do neuropeptídeo, neurotensina (NT) nestes processos. Estudos recentes indicam que alguns dos neuropeptídeos referidos atrás podem actuar como modeladores inflamatórios e que podem melhorar o processo de cicatrização nos diabéticos através da aplicação tópica destes nas feridas. Contudo, um dos maiores problemas associados à administração tópica de peptídeos, em geral e neuropeptídeos em particular, são os seus tempos de meia vida e correspondente perda de bioactividade em feridas ricas em peptidases. Uma estratégia alternativa para superar estas questões passa pelo uso de *dressings* (pensos curativos ou apósitos) biocompatíveis para a libertação continuada destes neuropeptídeos. Alguns biopolímeros como o colagénio e os derivados do quitosano podem ser utilizados neste âmbito devido às suas propriedades favoráveis conhecidas e ao seu comportamento biológico, nomeadamente à sua capacidade de carregar/libertar substâncias bioactivas, e à sua biocompatibilidade, biodegradabilidade e não toxicidade.

Nesta tese, os efeitos *in vitro* da NT na migração, proliferação e regulação da expressão das citocinas nas células da pele, nomeadamente em macrófagos e em queratinócitos, em condições hiperglicémicas e/ou inflamatórias foram estudados. Além disso, a análise da expressão de NT e seus receptores foi também realizada nas condições acima descritas. Também se desenvolveram e caracterizaram *dressings* baseados em três derivados do quitosano (*N*-carboximetil quitosano (CMC), 5-metilpirrolidinona quitosano (MPC) e *N*-succinil quitosano (SC)) e baseados em

colagénio tipo 1 de ratinho como suporte para a libertação tópica de NT em feridas diabéticas. A avaliação da progressão da cicatrização das feridas e a modulação de factores inflamatórios, angiogénicos e de re-epitelialização foi realizada *in vivo* usando *dressings* baseados em MPC e colagénio (com ou sem a libertação de NT) num modelo de cicatrização de espessura total em ratinhos diabéticos.

Dos resultados *in vitro*, podemos concluir que a NT prejudica a migração de macrófagos em condições inflamatórias assim como diminui a expressão de citocinas pro-inflamatórias (IL-1β e IL-12) em condições hiperglicémicas e inflamatórias. Além disso, verificou-se que a hiperglicemia modula a expressão da NT e seus receptores em ambas as condições testadas.

Por outro lado e nos queratinócitos humanos, a presença de NT estimulou fortemente a expressão de NT e NTR2. Contudo, os resultados também mostraram que a NT não afectou a proliferação e migração celular assim como a expressão de alguns factores inflamatórios (IL-1β e IL-8) e factores de crescimento (EGF, VEGF e PDGF) em condições hiperglicémicas. Estes resultados sugerem que a NT não exerce um efeito directo na função dos queratinócitos, mas parece apresentar um efeito parácrino em outras células da pele como os fibroblastos, os macrófagos e as células dendríticas.

Dos resultados *in vivo*, verificou-se que a aplicação de NT sozinha induziu uma cicatrização mais rápida das feridas tanto nos controlos (22%) assim como nos diabéticos (29%) ao dia 3 após ferimento (quando comparado com feridas não tratadas). Os *dressings* de MPC sozinho ou MPC carregado com NT apresentaram diferentes perfis de cicatrização das feridas tanto nos controlos como nos ratinhos diabéticos, no dia 1 após o ferimento, conduzindo a reduções significativas do tamanho das feridas (48% e 43%, respectivamente nos controlos, e 35% e 50%, respectivamente nos animais diabéticos). A análise por RT-PCR mostrou que os *dressings* de MPC carregados com NT reduziram a expressão de citocinas inflamatórias (TNF-α) assim como reduziram o infiltrado inflamatório ao dia 3. No dia 10, a expressão de MMP-9 foi também reduzida na pele dos ratinhos diabéticos, o que levou ao aumento da migração dos fibroblastos e à maior expressão e deposição de colagénio (COL1A1, COL1A2 e COL3A1) nas feridas.

Os resultados obtidos quando se usaram *dressings* de colagénio carregados com NT mostraram que, foi conseguida uma cicatrização mais rápida em ratinhos diabéticos (redução de 17% da área da ferida). Além disso, esta estratégia reduziu significativamente a expressão de citocinas inflamatórias (TNF-α and IL-1β) assim como o infiltrado inflamatório, no dia 3 após o ferimento. Após completa cicatrização (fd), a expressão de MMP-9 encontra-se também reduzida na pele dos ratinhos diabéticos com este tratamento. Uma vez mais, este resultado conduziu à migração dos fibroblastos e ao aumento da expressão e deposição de colagénio (COL1A2 e COL3A1).

Finalmente e em conclusão, todos os resultados obtidos (*in vitro* e *in vivo*) indicam que a NT pode estimular a cicatrização de feridas diabéticas e a sua actividade pode ser melhorada quando é carregada em *dressings à* base de MPC ou colagénio.

Os resultados apresentados nesta tese mostram que a NT é um neuropeptídeo promissor que pode ser usado no tratamento de feridas diabéticas, quer sozinha ou preferivelmente combinada com *dressings* biocompativeis e biodegradáveis. Portanto, estes resultados podem/devem ser posteriormente desenvolvidos de modo a obter novos e mais eficientes *dressings* para o tratamento da DFU e até mesmo para outros tipos de feridas.

List of publications

The thesis is based on the following papers:

Moura LI, Dias AM, Carvalho E, de Sousa HC (2013) Recent advances on the development of wound dressings for diabetic foot ulcer treatment – A review. *Acta Biomaterialia* 9: 7093–7114.

Moura LI, Silva L, Leal EC, Tellechea A, Cruz MT, Carvalho E (2013) Neurotensin modulates the migratory and inflammatory response of macrophages under hyperglycemic conditions. (Accepted in *BioMed Research International- in press*).

Moura LI, Cruz MT, Carvalho E; The effect of neurotensin in human keratinocytes – implication on impaired wound healing in diabetes. (Submitted to *Experimental Biology and Medicine*).

Moura LI, Dias AMA, Leal EC, Carvalho L, de Sousa HC, Carvalho E; Chitosan-based dressings loaded with neurotensin as an efficient strategy to improve early diabetic wound healing (Submitted to *Acta Biomaterialia*).

Moura LI, Dias AMA, Suesca E, Casadiegos S, Leal EC, Duque MRF, Carvalho L, de Sousa HC, Carvalho E; Neurotensin-loaded collagen dressings reduce inflammation and improve wound healing in diabetic mice (Submitted to *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*).

List of contents

Chapter 5 – *In vivo* study

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

List of figures

List of tables

List of acronyms

bFGF	Basic fibroblast growth factor
BMSCS	Bone marrow stem cells
CBC	*N*-carboxybuthyl chitosan
CGRP	Calcitonin gene-related peptide
CMC	*N*-carboxymethylchitosan
CNS	Central nervous system
COL1A1	Collagen type I, alpha 1
COL1A2	Collagen type I, alpha 2
COL3A1	Collagen type III, alpha 1
CSF-1	Colony-stimulating factor 1
DFU	Diabetic foot ulcer
DMEM	Dulbecco's Modified Eagle Medium
DTNB	Ditio-bis(nitrobenzoic acid)
DTT	Ditiotreitol
ECM	Extracellular Matrix
EDTA	Ethylenediamine tetraacetic acid
EGF	Endothelial growth factor
EPC	Endothelial progenitor cells
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FSDC	Fetal skin dendritic cell
GAG	Glycosaminoglycans
GSH	Glutathione
HLA	Human Leukocyte Antigen
HPRT-1	Hypoxanthine-guanine phosphoribosyltransferase
IDDM	Insulin-dependent diabetes mellitus
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
KC	Interleukin-8 related protein in rodents

LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MIP-2	Macrophage inflammatory protein – 2
MMP-9	Metalloproteinase 9
MPC	5-methyl pyrrolidinone chitosan
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEP	Neutral endopeptidade
NIDDM	Non-insulin-dependent diabetes mellitus
NK	Tackykinin receptors
NKA	Neurokinin A
NO	Nitric oxide
NPY	Neuropeptide Y
NT	Neurotensin
NTR	Neurotensin receptor
PBS	Phosphate buffer solution
PDGF	Platelet-derived growth factor
PMN	Polymorphonuclear leukocytes
PNS	Peripheral nervous system
PVDF	Polyvinylidene difluoride
REK	Rat epidermal keratinocytes
ROS	Reactive oxygen species
SC	*N*-succinyl chitosan
SDF-1α	Stromal cell-derived factor 1 alpha
SEM	Scanning electron microscopy
SP	Substance P
STZ	Streptozotocin
TBP	Tata binding protein
TGF β1	Transforming growth factor β1
TGF β3	Transforming growth factor β3
TNF-α	Tumor Necrosis Factor -α
VEGF	Vascular endothelial growth factor

Scope, motivations, goals and thesis structure/organization

Diabetes mellitus is one of the most prevalent chronic diseases worldwide which prevalence is expected to rise up to 439 million by 2030. It is characterized by an impaired blood glucose homeostasis and can cause poor circulation in the extremities, particularly in patients with neuropathy, compromising the immune system and increasing the incidence of infections. This disease leads to severe complications such as diabetic foot ulcer (DFU). This chronic, non-healing disease affects around 15% of all diabetic population and it leads to a poor quality of life of patients, to high hospital costs and, in extreme cases, to lower extremities amputations. Diabetic peripheral neuropathy, peripheral vascular disease, impaired angiogenesis and chronic inflammation are some multifactors that initiate the impaired diabetic wound repair.

Proper inflammatory (recruitment of leucocytes and macrophages) and re-epithelialization (migration and proliferation of fibroblasts and keratinocytes) phases are essential for the restoration of wounded tissues. In addition, the peripheral nervous system can improve wound healing in diabetes through stimulation of above referred processes by the release of some neuropeptides such as SP and NPY. Neuropeptides can activate specific receptors on target cells in the skin, namely keratinocytes, mast cells, macrophages, fibroblasts and endothelial cells. However, it is important to evaluate the involvement of other neuropeptides in wound healing such as neurotensin (NT) whose effect in the skin has been poorly studied. NT is a promising candidate to improve diabetic wound healing, since it is expressed in the skin and it was found to modulate inflammation and neovascularization in dendritic cells, important events in the progression of wound healing. However, the function of NT in other cells involved in wound healing is poorly understood.

In addition, to enhance the DFU healing process, wounds should be dressed with appropriate biomaterials to protect and avoid contaminations or to provide the sustained and effective release of a given bioactive substance. Moreover, the topical administration of neuropeptides has a major problem related with its short half-lives. A strategy for the release of these substances is through their loading into proper dressings. Collagen and chitosan derivatives were used as biopolymers for

NT delivery/protection systems due to their favorable properties, such as biocompatibility, biodegradability and non-toxicity.

The aims of this thesis are:

Aim 1: To analyze the effects of NT in skin cells through *in vitro* studies using mouse macrophages and human keratinocytes cell lines:

a) to understand how NT modulates LPS-stimulated macrophage migratory, inflammatory and angiogenic responses through its receptors expression, either under normal/hyperglycemic conditions;
b) to study how NT modulates keratinocyte functions (migration, proliferation and cytokine expression) under hyperglycemic conditions, through its receptors expression.

Aim 2: To analyze the effects of topical wound dressings for NT delivery, into the wound site in control and diabetic mice - an *in vivo* study:

a) to develop, prepare and characterize efficient wound dressings, using chitosan derivatives (CMC, MPC and SC) and type I collagen as biopolymers.
b) to evaluate the effects of topically applied NT alone, chitosan or collagen-based dressings alone, or loaded with NT into skin wounds of control and diabetic mice and evaluate the progression of wound healing and modulation of inflammatory, angiogenic and re-epithelializating factors.

The Thesis is divided into six chapters (1-6). Chapter 1 is an introduction to the main topics covered during the study. Briefly, it is presented an overview of *diabetes mellitus* and its complications in the skin, namely diabetic foot ulcers, and its impaired wound healing is described. The importance of the skin as a neuroimmune organ and the impact of neuropeptides, such as neurotensin, in tissue repair have been focused. Different wound dressings and polymers used for DFU treatment are reviewed. Chapters 2 and 3 focus on *in vitro* studies performed in skin cells,

namely macrophages and keratinocytes, respectively, to analyze the effect of NT in hyperglycemic and/or inflammatory conditions. Chapter 4 and 5 describe the development and characterization of chitosan derivatives and collagen-based dressings and their topical application in diabetic wound healing. Chapter 6 includes the major conclusions that may be addressed from the developed work as well as future work directions.

Chapter 1

State of the art

Introduction

This Chapter comprises the work published in *Acta Biomaterialia (2013) Recent advances on the development of wound dressings for diabetic foot ulcer treatment – A review. 9: 7093-114* by **Moura LI**, Dias AM, Carvalho E, de Sousa HC.

1.1 Diabetes mellitus and its complications

Diabetes mellitus is one of the most prevalent chronic diseases worldwide. In 2010, it was estimated that 285 million adults had diabetes in the world and its prevalence is expected to rise up to 439 million by 2030 (Shaw *et al.*, 2010b, Whiting *et al.*, 2011). In North America and Europe, the number of adults with diabetes is expected to increase by 42.4% and 20%, respectively with a major burst in Africa where the number of adults with diabetes is expected to increase by 98.1% from 2010 to 2030 (Shaw *et al.*, 2010a, Whiting *et al.*, 2011) (Figure 1.1).

The main factors responsible for the increase in the number of patients with diabetes are the growth and aging of the population and changes in the life style (Chittleborough *et al.*, 2007, Shaw *et al.*, 2010a).

Diabetes mellitus is a metabolic disorder of multiple aetiology characterized by chronic hyperglycemia and by disturbances in carbohydrate, lipid and protein metabolism which are caused by alterations in insulin secretion, insulin sensitivity or both of these processes in insulin target tissues (Alberti and Zimmet, 1998, Ahmed and Glodstein, 2006). The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. Characteristic symptoms of *diabetes mellitus*

are thirst, polyuria, blurring of vision and weight loss, in extreme cases it can lead to lethargy, coma and in the absence of effective treatment, dead (Alberti & Zimmet, 1998).

Etiologically, diabetes can be classified into type 1, type 2 and gestational diabetes. In the last years, various forms of diabetes with genetic abnormalities have been identified namely genetic defects in the β-cell function and insulin action, as well as endocrinopathies, that are treated as a separate category (2006).

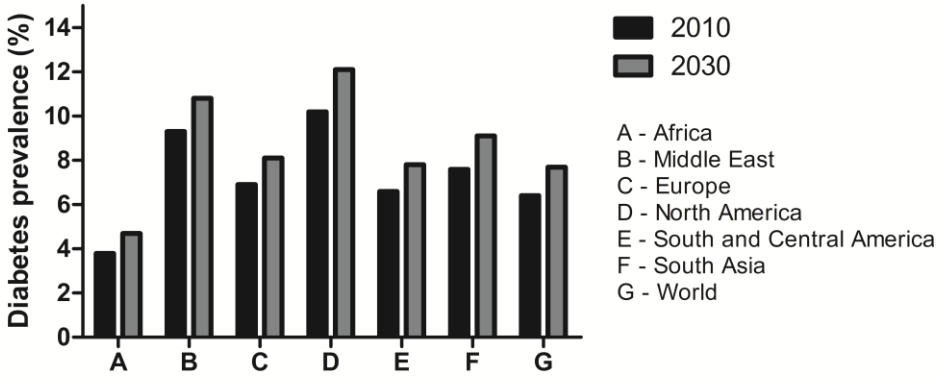

Figure 1.1: Diabetes prevalence in the world, in 2010 and 2030.

Type 1, insulin-dependent *diabetes mellitus* (IDDM), or juvenile-onset diabetes, is characterized by an autoimmune destruction of pancreatic β-cell, leading to absolute insulin deficiency and, consequently, to the total dependence on exogenous insulin to sustain life (Ahmed and Glodstein, 2006, Daneman, 2006). This type of diabetes develops in association with certain hereditary factors, such as Human Leukocyte Antigen (HLA) alleles, as well as with environmental factors, such as viral infections (Seino *et al.*, 2010).

The incidence of type 1diabetes is around 5-10% of the diabetic population, being type 1 diabetes usually higher under the age of 15 regardless that only 20-50% of the patients are diagnosed before this age. In addition, the Caucasian population tends to present higher risk for type 1 diabetes when compared to all other ethnic groups (Vehik and Dabelea, 2011).

Type 2 *diabetes mellitus*, also known as non-insulin-dependent *diabetes mellitus* (NIDDM) or as adult-onset diabetes, is characterized by insulin resistance which may be combined with relatively reduced insulin secretion levels. Type 2 diabetes affects approximately 90-95% of all diabetic

patients and its main risk factors are high plasma glucose concentrations in the fasting state and after an oral glucose load, overweight and a sedentary lifestyle (Wang *et al.*, 2008). However, this type of diabetes can be delayed or prevented by proper nutrition and by regular physical exercise (Knowler *et al.*, 2002, Kahn, 2008). It is associated with a strong genetic predisposition (more than type 1 diabetes), however its genetics are complex and have not been clearly defined (2006).

Finally, gestational diabetes or impaired glucose intolerance, which is firstly diagnosed during pregnancy, is defined as the carbohydrate intolerance resulting in hyperglycemia of variable severity during gestation (Alberti and Zimmet, 1998, McCance, 2011). Gestational diabetes affects approximately 14% of pregnancies and it is also an important risk factor for type 2 diabetes in women in the future (Kim *et al.*, 2002, Ali and Dornhorst, 2011).

Generally, the injurious effects of hyperglycemia are separated into macrovascular (coronary artery disease, peripheral vascular disease and stroke) as well as microvascular complications (diabetic nephropathy, neuropathy and retinopathy) (Fowler, 2008) (Table 1.1).

Diabetes mellitus can lead to a high frequency of atherosclerosis, caused by chronic inflammation and injury of the arterial wall in the peripheral or coronary vascular system that increases the risk of stroke and/or heart attack (Vinik and Vinik, 2003). People with *diabetes mellitus* are 2 to 4 times more susceptible to die from heart disease than people without diabetes. Moreover, diabetes is the principal cause of new blindness cases in adults as a consequence of severe visual disability due to retinopathy and macular edema (Fowler, 2008).

Table 1.1: Macrovascular and microvascular diabetes complications.

Diabetes complications	
Macrovascular	Microvascular
- Stroke - Coronary heart disease (angina, myocardial infarction) - Peripheral vascular disease	- Retinopathy - Nephropathy - Neuropathy → diabetic foot ulcer

The prevalence of retinopathy is greater in type 1 diabetes while macular edema is more important in type 2 diabetes. Diabetic retinopathy is closely related to the duration of diabetes (Davis, 1992). Diabetic nephropathy is the leading cause of chronic kidney disease in United States of America and Western societies. The functional alterations in kidney include an early increase in the glomerular filtration rate with intraglomerular hypertension and subsequent proteinuria, systemic hypertension and eventual loss of renal function (Ritz, 1999, Ayodele *et al.*, 2004). Due to severity of disease, patients with diabetic nephropathy make up the fastest-growing group of renal dialysis and transplant recipients. Another microvascular complication of diabetes, diabetic peripheral neuropathy, leads to a loss of sensitivity and it is a major contributor to non-traumatic lower extremity amputations. Diabetic neuropathy affects almost 50% of all the diabetic population, it is one of the most common and troublesome complication affecting diabetic patients (Vinik *et al.*, 1992). Chronic diabetic neuropathy, defined as temporary or permanent nerve tissue damage, is characterized by a progressive loss of peripheral nerve fibers that is caused by a decreased blood flow, high glycemic levels and diminished neuropeptide production (Dyck *et al.*, 1993, Pradhan *et al.*, 2009b, Basic-Kes *et al.*, 2011). The duration and intensity of the exposure to hyperglycemia strongly influences the severity of neuropathy (Dyck *et al.*, 1993). Diabetic neuropathy can be classified as peripheral, autonomic, proximal or focal, depending on the affected body part (Aring *et al.*, 2005, Vinik *et al.*, 2006). It occurs in both type 1 and type 2 diabetes and it is more frequent in older people. However, many diabetic patients may never develop neuropathy while others may develop this condition rather early (Boulton *et al.*, 2004, Vinik *et al.*, 2006). On average and after the diabetes diagnosis, the neuropathy symptoms begin to appear within 10-20 years and approximately 50% of diabetic patients will develop nerve damages to some extent (Rathur and Bloulton, 2005).

1.2 Skin and types of wounds

Skin is the outermost covering and the largest organ of the human body, in terms of weight and area. This organ has an important active role in protect our internal organs and tissues from

external contaminants such as toxins and potential dangerous microorganisms (Kanitakis, 2002, Yildirimer *et al.*, 2012). In addition, skin prevents body dehydration through the regulation of body temperature and provides support to blood vessels and nerves (Bottcher-Haberzeth *et al.*, 2010, Pereira *et al.*, 2013). Moreover, skin has also an aesthetic relevance being related with healthier and more attractive (Sachs and Voorhees, 2011).

The skin has a complex three-layer structure stratified as epidermis, dermis and hypodermis or subcutis (Figure 1.2) which, under physiological conditions is self-renewable, a new layer of skin is developed every 2–3 weeks (Yildirimer *et al.*, 2012, Pereira *et al.*, 2013). Epidermis is the superficial, outermost layer that varies in thickness from 0.05 mm on the eyelids to 0.8mm on the soles of the feet and palms of the hand. It serves as a physical and chemical barrier between the interior body and exterior environment. It is mainly constituted by stratified squamous epithelium keratinocytes which synthesize the protein keratin contributing to the rigidity and permeability of the epidermis. These cells move progressively from the epidermal basement membrane towards the skin surface forming various well defined layers (Yildirimer *et al.*, 2012). The epidermis is also constituted by melanocytes (responsible for skin coloration), Langerhans cells (responsible for immune response) and Merkel cells that are associated with cutaneous nerves (responsible for light touch sensation) (Slominski and Wortsman, 2000). The epidermis has no blood vessels and is nourished by simple diffusion of nutrients from the underlying connective tissue (Zhong *et al.*, 2010).

The dermis is the middle layer, it varies in thickness, ranging from 0.6 mm on the eye lids to 3 mm on the back, palms and soles. It contains a vascularized extracellular matrix (ECM) rich in fibroblasts that produce type I and III collagen, reticulum fibers, elastin and glycosaminoglycans (GAGs) (Zhong *et al.*, 2010, Yildirimer *et al.*, 2012). They are responsible for the strength, toughness, elasticity, viscosity and hydration of the skin. Fibroblasts, the major cell type present in the dermis, are able to produce remodeling enzymes (proteases and collagenases) that play and important function in wound healing process (Shaw and Martin, 2009). In addition, immune-competent such as mast cells and macrophages, endothelial cells and smooth muscle cells are also found in the dermis (Pereira *et al.*, 2013).

The hypodermis or subcuties is located below the dermis and it is mainly constituted by adipose tissue and collagen. It contains larger blood vessels and nerves that are also found in the dermis. This skin layer acts as an insulator and conserves body heat (Yildirimer *et al.*, 2012, Pereira *et al.*, 2013). Many reports do not include this layer as part of the skin referring to it only as subcutaneous tissue.

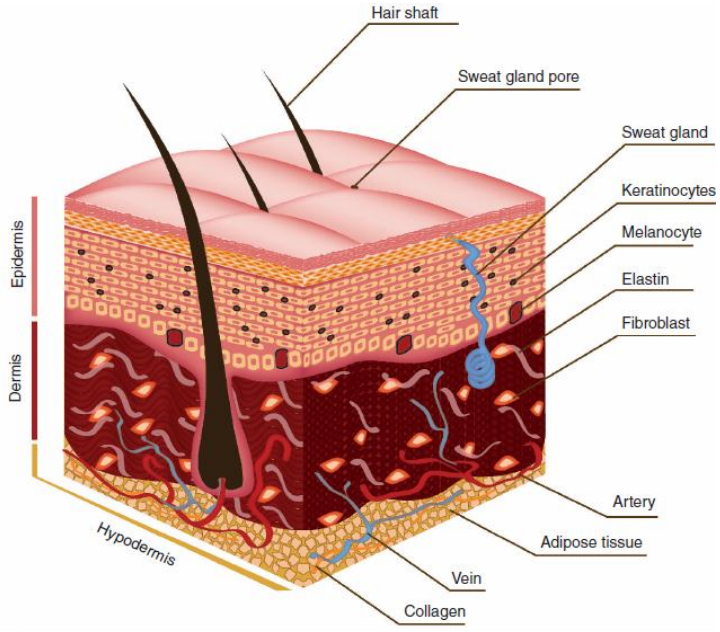

Figure 1.2: Anatomy of skin. Image obtained from Pereira *et al.*,2013

The loss of skin integrity because of injury (acute wounds) or illness (chronic wounds – venous, pressure, diabetic and leg ulcers) may result initially in the substantial physiologic imbalance and, in extreme cases, in significant disability or even death (Clark *et al.*, 2007, Yildirimer *et al.*, 2012). Wounds can be divided into epidermal, superficial partial-thickness, deep partial-thickness and full thickness according to the depth of the injury (Papini, 2004, Shevchenko *et al.*, 2010). Epidermal injuries are caused by sunburns, light scalds or grazing and do not require specific treatment since only the epidermis is affected and it rapidly regenerates without scarring (Shevchenko *et al.*, 2010). Superficial partial-thickness wounds, such as thermal trauma, affect the epidermis and the superficial part of the dermis causing severe pain. These wounds heal by epithelialization from the margins of the wound where keratinocytes proliferate and migrate to cover the wounded area

(Papini, 2004). On the other hand, deep partial- thickness wounds involve huge dermal damage with longer healing time and pronounced scar. In full- thickness wounds there is total destruction of epithelial-regenerative tissues causing huge scars. This type of wounds cause functional and cosmetic defects and may require skin grafting to stimulate wound healing (Papini, 2004, Shevchenko *et al.*, 2010).

1.3.Diabetic foot ulcers (DFU) and impaired wound healing

Diabetic foot ulcers (DFU) are chronic, non-healing neuropathic ulcers that occur in around 15% of all the diabetic population (Brem and Tomic-Canic, 2007) and are normally responsible for huge hospital costs besides affecting the patient's life quality (Falanga, 2005, Brem and Tomic-Canic, 2007, Pradhan *et al.*, 2009a). Once a DFU has developed there is an increased risk of wound progression that may ultimately lead to amputation (more than 85% of foot amputations in patients are caused by DFU) (Snyder and Waldman, 2009).

Diabetic neuropathy and peripheral vascular disease are usually the major factors involved in DFU. These two factors may act alone, together, or in combination with other conditions such as microvascular disease, biomechanical abnormalities, limited joint mobility and increased susceptibility to infection (Rathur and Bloulton, 2005, Snyder and Waldman, 2009). Some studies refer that the difficulties associated with DFU healing are mostly due to the excessive and persistent activity of metalloproteinases (MMP) and/or due to low levels of MMPs inhibitors (Lobmann *et al.*, 2006, Liu *et al.*, 2009). In addition, ischemia and vascular disease usually reduce the healing capacity due to the reduced oxygen and nutrients supply to the wound area (Guo and Dipietro, 2010). There are also impaired granulocytic, chemotactic and macrophage functions, as well as prolonged inflammation and deregulation of the neovascularization phase (Acosta *et al.*, 2008, Blakytny and Jude, 2009). These issues are mainly due to the impaired growth and angiogenic factors expression, namely VEGF and PDGF (Bloomgarden, 2008). Finally, there may be also nitric oxide abnormalities, collagen accumulation (Brem and Tomic-Canic, 2007),

abnormal migration and proliferation of fibroblasts and of keratinocytes (Bloomgarden, 2008), as well as accumulation of ECM components and their remodeling by MMPs (Muller *et al.*, 2008).

Wound healing is a complex process that involves the simultaneous action of soluble mediators, blood cells, extracellular matrix as well as parenchymal cells. This process can be divided into several phases: homeostasis/coagulation, inflammation, proliferation (granulation tissue formation), reepithelialization and remodeling (Li *et al.*, 2007, Enoch and Leaper, 2008). These phases are not typically associated with a rigorous and well-defined period of time and may overlap (Sidhu *et al.*, 1999, Falanga, 2005, Silva *et al.*, 2010, Delavary *et al.*, 2011). The transition between phases usually depends on the maturation and differentiation of keratinocytes, fibroblasts, mast cells and macrophages which are the most important cells involved in the wound healing process (Singer and Clark, 1999, Monaco and Lawrence, 2003, Rodero and Khosrotehrani, 2010).

The inflammatory phase begins a few minutes to 24h after injury and lasts for about 3 days. It plays a central role in wound healing, through protection of the wound from microbes and participating in the tissue repair processes (Boateng *et al.*, 2008).

After tissue injury, a clot (comprising fibrin, fibronectin, vitronectin, von Willbrand factor, thrombospondin) is formed in order to reestablish homeostasis, and aggregated platelets secrete growth factors and cytokines (such as transforming growth factor beta (TGF-β) and monocyte chemoattractant protein 1 (MCP-1) that recruit neutrophils and monocytes, polymorphonuclear cells to the wound site to minimize bacterial contamination of the wound preventing from possible infection (Enoch and Leaper, 2008). These inflammatory cells induce the expression of colony-stimulating factor 1 (CSF-1), tumor necrosis factor α (TNF-α) and platelet-derived growth factor (PDGF) which are extremely important for the first phase of new tissue formation (Tsirogianni *et al.*, 2006, Wilgus, 2008). This is called the early inflammatory phase. Depending on time, duration of the response and the inflammatory cells involved, inflammation can be divided into the early and the late stages (Enoch and Leaper, 2008). The late inflammatory phase starts 2-3 days after of injury and is characterized by the differentiation of the monocytes into macrophages. Macrophages are crucial for the coordination of later events in response to injury and they function as phagocytic cells. In addition, they are the principal producers of growth factors responsible for the proliferation

and production of the ECM by stimulating fibroblasts, endothelial and smooth muscle cells (DiPietro, 1995). These cells also release proteolytic enzymes such as collagenases for help in the wound debridement (Enoch and Leaper, 2008).

The re-epithelialization process usually begins a few hours after injury. In response to the growth factors, keratinocytes and activated fibroblasts (myofibroblasts) that migrate from the wound edges into the wound site where they proliferate and construct the extracellular matrix that will enhance wound closure (Enoch and Leaper, 2008, Silva *et al.*, 2010). The initial extracellular matrix is gradually replaced by a collagenous matrix with the formation of new blood vessels (angiogenesis) (Singer and Clark, 1999). Angiogenic factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and PDGF induce angiogenesis by stimulating the production of basic fibroblast and vascular endothelial growth factors by macrophages and endothelial cells (Wilgus, 2008, Schreml *et al.*, 2010). Protease expression and activity are also necessary for the angiogenesis process (Singer and Clark, 1999, Schreml *et al.*, 2010). When the wound area is completely filled with new granulation tissue, angiogenesis stops and the apoptosis of many new vessels is then started.

Remodeling is the last phase of the wound healing process and is essential for restoration of the full functionality and a 'normal' appearance of the injured skin (Shaw and Martin, 2009). This phase is characterized by the degradation of the previously formed granulation tissue and by dermis regeneration (Rodero and Khosrotehrani, 2010). It begins 2-3 weeks after injury and it can last for a year or more (Gurtner *et al.*, 2008).

During this stage, the ECM components are remodeled to restore the normal architecture of the dermis, through a delicate balance of collagen synthesis, bundling and degradation. This process is carried out by MMPs that are secreted by fibroblasts, macrophages and endothelial cells. In addition, the new blood vessels within the scar are refined and mature to form a functional network (Gurtner *et al.*, 2008, Shaw and Martin, 2009).

While acute wounds usually progress linearly through the different wound healing phases, the healing process in diabetic patients does not develop through this temporal pathway thus originating chronic non-healing wounds that become stalled in one or more of the above mentioned

healing phases (Falanga, 2005; Tellechea *et al.,* 2009). Figure 1.3 schematizes the phases and growth factors involved in diabetic wound healing processes in comparison with regular wound healing.

1.4 Skin as an neuroimmunoendocrine organ

Many reports describe the importance of the cutaneous peripheral nervous system (PNS) in skin homeostasis and disease. Sensory and autonomic nerves stimulate various physiological (vasoconstriction, vasodilation, body temperature, secretion, growth, differentiation, nerve growth) and pathophysiological processes (inflammation, immune defense, apoptosis, proliferation, wound healing) functions in the skin through neuropeptide action (Lotti *et al.*, 1995, Roosterman *et al.*, 2006). All these processes require a complex communication network between the spinal cord, the central nervous system (CNS) and the immune endocrine system (Roosterman *et al.*, 2006).

Neuropeptides are a heterogeneous group of extracellular messengers, composed of 4 to 40 aminoacids that interact with members of G-protein coupled receptors, and act as neurotransmitters, hormones and paracrine factors. They are involved in the transmission of signals not only between nerve cells but also between nerve and immune cells (Lotti *et al.*, 1995, Schaffer *et al.*, 1998, Roosterman *et al.*, 2006). In addition, skin resident and circulating immune cells express neuropeptides and its receptors identical to those expressed in the central neuroendocrine systems (Slominski and Wortsman, 2000).

After direct stimulation, by physical, chemical, microbiological agents, trauma or inflammation, neuropeptides are released by nociceptive afferent nerve fibers via PNS (principally) or several epidermal and dermal cells, and activate specific receptors on target cells in the skin including immune cells (lymphocytes, macrophages and mast cells), Langerhans cells, endothelial cells, fibroblasts and keratinocytes (Schaffer *et al.*, 1998, Pradhan *et al.*, 2009b) (Figure 1.4). Moreover, neuropeptides may regulate cytokine release and the inflammatory response by inducing its degradation with cell-associated neuropeptide peptidases (NEP) (Silva *et al.*, 2010). The neuropeptides usually involved in neuroimmune regulation and wound healing are Substance P

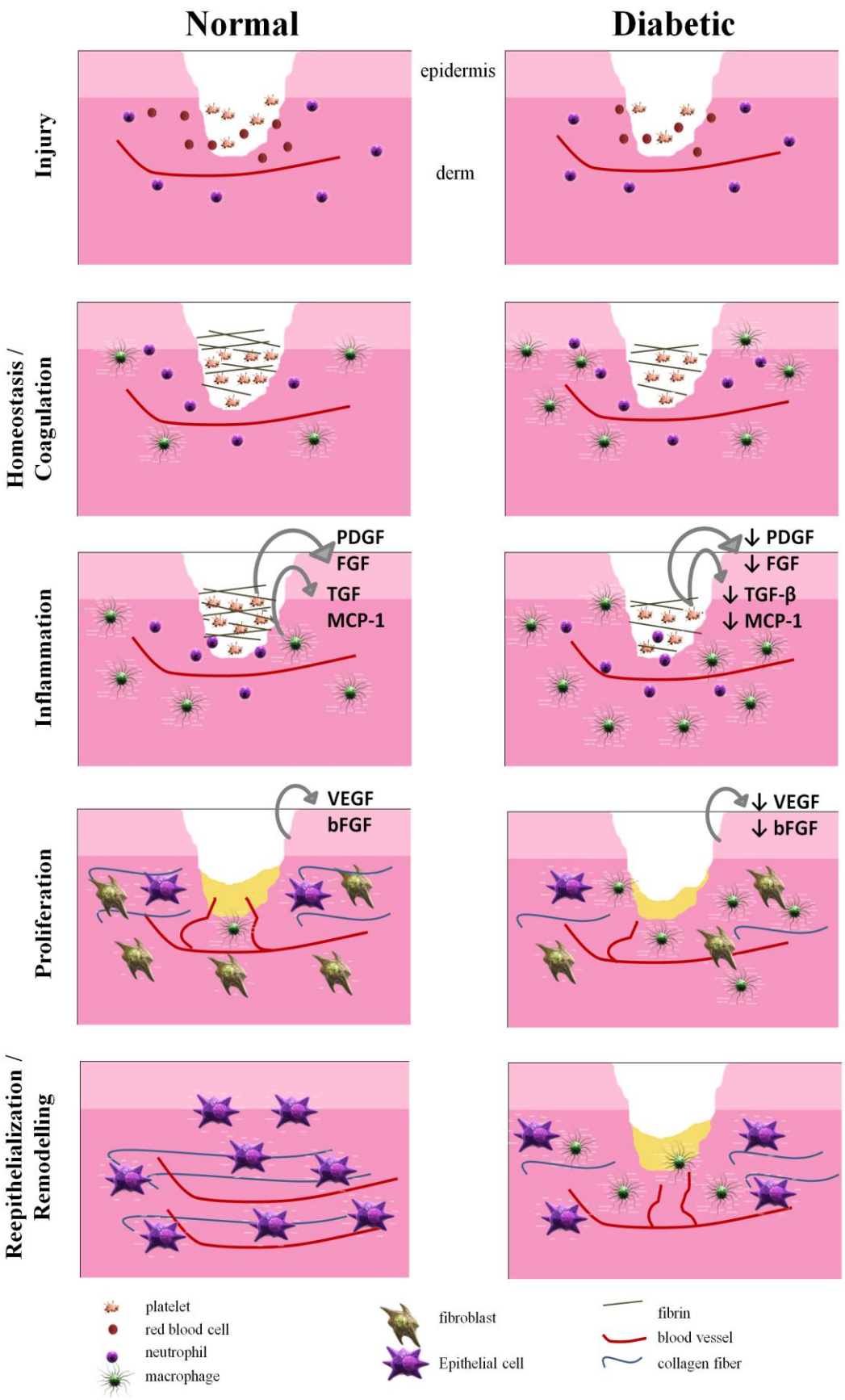

Figure 1.3: Differences in the normal and diabetic wound healing phases.

(SP), calcitonin gene-related peptide (CGRP), neuropeptide Y (NPY), among others (Pradhan *et al.*, 2009b).

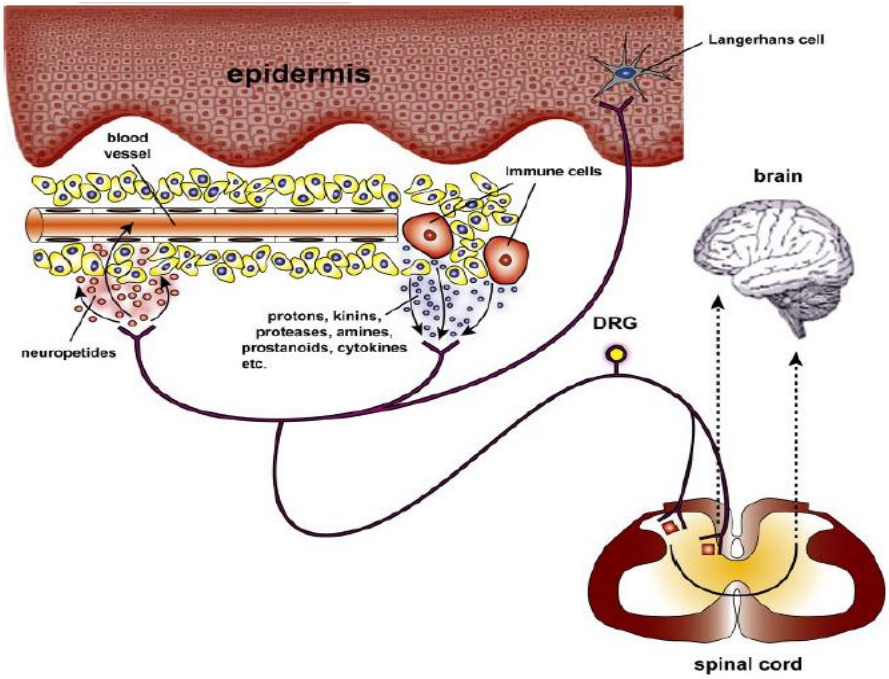

Figure 1.4: Skin as a neuroimmune organ: after an inflammatory stimulus, neuropeptides transmit the response to the spinal cord that activates specific areas in the CNS. Image obtained from Roosterman *et al.*, 2006.

In the next section, a more detailed description of neuropeptides present in skin is presented.

1.5 Neuropeptides

Many neuropeptides namely Substance P (SP), neurotensin, neurokinin A (NKA), somatostatin, calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY) are found in the skin (Lotti *et al.*, 1995, Pradhan *et al.*, 2009b). A neuropeptide that play an essential role in tissue repair and has been well studied is SP. SP is an 11-aminoacid peptide that is member of tackykinin family. In the PNS, SP is located in immunologic areas namely skin, the gastrointestinal and respiratory tracts (Schaffer *et al.*, 1998). It has three tackykinin receptors: NK1, NK2 and NK3, with the NK1 the predominant receptor. G-protein coupled receptor stimulation leads to activation of phospholipase C and thereafter to the release of Ca^{2+} from internal stores (Schaffer *et al.*, 1998, Pradhan *et al.*,

2009b). SP induces vasodilation, increased vascular permeability and protein extravasation after tissue injury (Lotti *et al.*, 1995). In addition, SP induce nitric oxide (NO) production, enhancing wound healing and increases the density of neutrophils, dendritic cells, endothelial cells and macrophage at the wound site (Silva *et al.*, 2010). In addition, SP stimulates proliferation of fibroblasts and endothelial cells, via NK receptors, important process in wound healing (Brain, 1997).

Largely studied in the nervous system, however poorly studied in the skin is the neuropeptide neurotensin (NT). NT was firstly discovered and isolated, from bovine hypothalamus, in 1973, by Carraway and collegues (Carraway and Leeman, 1973). It is a bioactive tridecapeptide with a primary distribution in the CNS (namely hypothalamus and pituitary) and in the gastrointestinal tract (namely on endocrine cells of the jejunum and ileum) (Brun *et al.*, 2005, Pradhan *et al.*, 2009b). NT mediates its functions through the binding to two G-protein coupled receptors: neurotensin receptor 1 (NTR1) (high affinity receptor) and neurotensin receptor 2 (NTR2) (low affinity receptor) with NTR1 the most predominant receptor and to a third receptor, NTR3, an intracellular type I receptor, a non G-protein coupled receptor with a single transmembrane domain similar with the gp95/sortilin protein (Vincent *et al.*, 1999, Pradhan *et al.*, 2009b). These three receptors recognize the same C-terminal 8-13 sequence and display similar functions towards NT (Vincent *et al.*, 1999).

The NTR1 intracellular signaling occurs through phospholipase C and the inositol phosphate pathway with Ca^{2+} mobilization and also by mitogen-activated protein (MAP) kinase phosphorylation. Furthermore, NTR2 internalization activates the ERK1/2 pathway however no Ca^{2+} mobilization is observed while NTR3 activates both MAP and phosphoinositide (PI) 3-kinase-dependent pathways (St-Gelais *et al.*, 2006).

In the periphery, NT is a paracrine and endocrine modulator of the digestive tract and the cardiovascular system. In addition, it acts as a growth factor on diverse normal and cancer cells.

In the nervous system, NT has a pro-inflammatory role inducing vasodilation, vascular permeability, as well as migration and phagocytosis of macrophages (Goldman *et al.*, 1983, De la Fuente *et al.*, 1993). In the gastrointestinal tract, NT is involved in the pathophysiology of acute

colonic inflammation processes showing that NT and NTR1 have a pro-inflammatory role in the rat colon (Castagliuolo *et al.*, 1999). Moreover, NT induces the expression of pro-inflammatory cytokine IL-8 via Ca^{2+} dependent NF-ƙB and Ras-dependent ERK activation in human colonocyte cells (Zhao *et al.*, 2005). In the repair process of mucosal injuries, NT stimulates epithelial restitution through a COX-2 dependent pathway (Brun *et al.*, 2005).

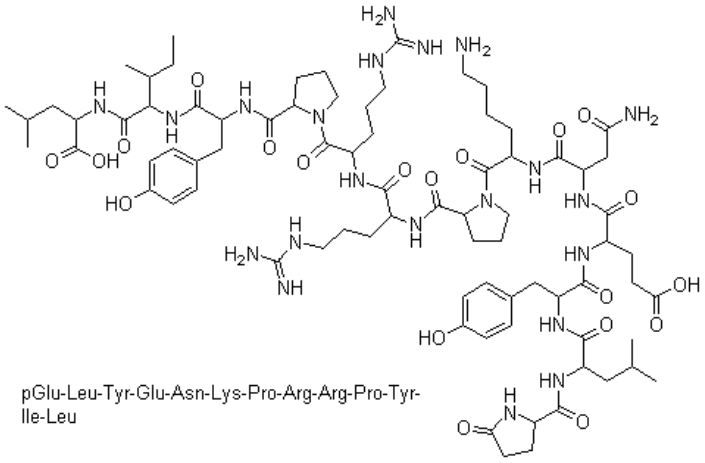

Figure 1.5: Chemical structure of neurotensin (NT). Image from http://www.chemblink.com

1.5.1 Neuropeptides in DFU

Neuropeptides are the link that directly connects neuropathy to wound healing. In diabetic skin there are motor, sensory and autonomic fiber denervation that limits the sensation of pain, pressure and temperature as well as vasodilation of small arteries and weakness of the small intrinsic muscles (Pradhan *et al.*, 2009b, Silva *et al.*, 2010). These symptoms in combination with chronic nerve compression may lead to amputation. Various studies have already demonstrated that there is a decrease in neuropeptide release from nerves in diabetes (Gibran *et al.*, 2002, Pradhan *et al.*, 2011, Nabzdyk *et al.*, 2013). This factor, in part, may explain the impairment of wound healing in diabetic patients.

In diabetic serum, SP levels are significantly reduced compared to control patients. Moreover, in skin biopsies levels of SP are decreased due to elevated levels of the degrading enzyme neutral endopeptidade (NEP) (Pradhan *et al.*, 2009b). No studies have explored the function of NT in diabetic wound healing in the skin.

A better understanding of the pathophysiology and molecular biology of diabetic wounds may help to find improved and more efficient solutions for their treatment. It is currently accepted that DFU therapies should be directed to actively promote wound healing by correcting the expression of those biological factors, namely neuropeptides, which are important in the healing process (O'Loughlin and O'Brien, 2011). Although there are several approaches for the DFU treatment, an efficient medical treatment for DFU still remains a challenge.

1.6 Therapeutic approaches for DFU treatment

During the last decades, several growth factors, such as endothelial growth factor (EGF), as well as PDGF and VEGF, have been in focus as they proved to have the capacity to accelerate chronic wound healing. In addition, stromal cell derived factor-1 alpha (SDF-1α) has been used to stimulate wound healing. Topical application of some peptides or proteins have also shown to be effective in DFU treatment. Gene therapy approaches have also been attempted in recent years, where some specific genes such as SDF-1α, PDGF-BB and HoxB3, have been introduced into wounds through different physical methods or by using biological vectors. Stem cells and progenitor cells have also been used to accelerate DFU healing due to their potential in regenerating dermal and vascular components. Other approaches to promote DFU wound healing have included the topical application of specific drugs, such as nicotin, azelnidipine, simvastatin, naltrexone and ciclopirox olamine or of natural extracts/products from plants such as *Aloe vera*, *Lithospermun erythrorhison*, *Rehmanniae radix*, *Rosmarinus officinalis*, Ampucare , *Astragali radix* and *Annona squamos*. Table 1.2 describes some of the most recent approaches that have been used to stimulate DFU healing. However and to date, their efficacies and/or their application mode were not efficient enough to guarantee adequate DFU healing.

1.6.1 Wound dressings for DFU treatment

Like for acute wounds, it is already well established that to enhance DFU healing processes, wounds should be dressed with adequate biomaterials and in order to protect the long term healing

Table 1.2: Some different and recently proposed approaches to improve DFU treatment (cont).

Bioactive substances		Models used	Results	References
Growth factors	VEGF	db/db diabetic mice	Enhanced neovascularization, mobilization of bone marrow-derived cells into the wound site to accelerate wound healing	Galiano et al., 2004
	PDGF	STZ diabetic rats	Enhanced angiogenesis, cell proliferation and epithelialization. Formed thicker and more highly organized collagen fiber deposition in wounds	Li et al., 2008
	bFGF	Human patients with DFUs	Reduction of 75% of wound area in treated patients. Stimulated granulation and epithelialization of tissues	Uchi et al., 2009
Stem cells	SDF-1α	STZ diabetic mice	Increased EPC mobilization, homing and wound healing	Gallagher et al., 2007
	BMSCS	STZ diabetic rats	Promoted healing and improved the wound breaking strength. In addition, it increased collagen levels and TGF-β, KGF, EGF and VEGF expression	Kwon et al., 2008
	$CD133^{+}$ cells	STZ diabetic rats	Accelerated wound closure and promoted angiogenesis through stimulation of endothelial cell proliferation and migration	Barcelos et al., 2009
	Human adipose-derived stromal cells	db/db diabetic mice	Increased wound closure and stimulated production of extracellular matrix proteins and secreted souble factors	Amos et al., 2010
	Embryonic stem cells	STZ diabetic rats	Reduced significantly wound size and increased expression of EGF and VEGF	Lee et al., 2011
	EPCs	db/db diabetic mice	Promoted wound healing and vascularity and induced expression of VEGF and bFGF	Asai et al., 2012

Table 1.2: Some different and recently proposed approaches to improve DFU treatment (cont).

Bioactive substances		Models used	Results	References
Gene therapy	Adenoviral PDGF-B	db/db diabetic mice	Significantly enhanced wound repair and neovascularization. In addition, adenoviral-PDGF-B stimulated EPC recruitment to the wound site	Keswani et al., 2004
	Lentiviral-containing SDF-1α	STZ diabetic mice obese NOD/Ltj mice	Improved diabetic wound healing with completely epithelialization and increased the granulation tissue.	Badillo et al., 2007
	Adenoviral-Hox B3	db/db diabetic mice	Accelerated wound healing in diabetic mice	Mohebali et al., 2008
Proteins	Substance P	db/db diabetic mice	Enhanced wound repair and increased early inflammatory density in the healing wounds	Gibran et al., 2002, Scott et al., 2008
	Erythropoietin	STZ diabetic rats	Significantly reduced the time of total wound closure, increased micro vascular density, VEGF, and hydroxyproline contents and reduced extent of apoptosis	Hamed et al., 2010
	Insulin	STZ diabetic rats	Enhanced wound healing and stimulated a faster epithelialization.	Lima et al., 2012
Natural products	*Lithospermun erythrorhison* extract	db/db diabetic mice	Decreased vascular permeability, formation of granulation tissue and accelerated wound healing	Fujita et al., 2003
	Rehmanniae radix	STZ diabetic rats	Promoted ulcer healing accelerating the processes of tissue regeneration, angiogenesis and inflammation	Lau et al., 2009
	Aqueous extract of *Rosmarinus officinalis* (Rosemary)	Alloxan diabetic mice	Reduced inflammation and enhancement of wound contraction, re-epithelialization and regeneration of granulation tissue, angiogenesis and collagen deposition in the treated wounds	Abu-Al-Basal, 2010
	Ampucare (polyherbal ingredient)	Alloxan diabetic rats	Significantly reduced the wound size and bacterial count in wound site. Stimulated a well-organized fibrous tissue proliferation, epithelialization and complete scar formation	Dwivedi and Chaudhary, 2012

Table 1.2: Some different and recently proposed approaches to improve DFU treatment.

	Bioactive substances	Models used	Results	References
Other	Nicotine	db/dbdiabetic mice	Accelerated healing and increased wound angiogenesis	Jacobi et al., 2002
	Simvastatin	db/db diabetic mice	Increased VEGF mRNA and protein expression. In addition, simvastatin enhanced NO wound content at day 6 impairing the wound healing process	Bitto et al., 2008
	Azelnidipine	STZ diabetic rats	Accelerated wound healing and improved NO levels in wound fluid. Also, density of collagen fibers, numerical density of fibroblasts and length density of vessels were increased	Bagheri et al., 2011
	Ciclopirox olamine	db/dbdiabetic mice	Enhanced wound clousure, increased angiogenesis and dermal cellularity	Ko et al., 2011
	Naltrexone	STZ diabetic rats	Reduced wound area in 50% compared with control; stimulated DNA synthesis facilitating wound healing process	McLaughlin et al., 2011

from contamination/infection, to prevent wound dissection (providing an ideal moist environment to help wound closure) and, in case of medicated dressings, to provide a sustained and effective release of the applied bioactive substances, as well as to prevent their fast degradation during the healing process (Mulder *et al.*, 2003, Jannesari *et al.*, 2011).

DFU can be medically classified in different ways but all of them define the ulcer in terms of its depth and presence of osteomyelitis or gangrene (Cavanagh *et al.*, 2005, Leung, 2007). As an example, the classification according to the Wagner´s system is based in the following grades: grade 0 (no ulcer with a high risk factor of complication); grade 1 (partial/full thickness ulcer); grade 2 (deep ulcer, penetrating down to ligaments and muscle, but no bone involvement); grade 3 (deep ulcer with cellulites or abscess formation); grade 4 (localized gangrene); and grade 5 (extensive whole foot gangrene) (Oyibo *et al.*, 2001). The classification of DFUs is important as it may facilitate the choice of the adequate dressing depending on the wound type and on its phase (O´Donnell and Lau, 2006).

In recent years, novel wound dressings made of different biocompatible materials, having different shapes/arrangements and improved chemical, physical and biological properties, have been developed in order to ensure the optimal management of DFU. Therefore, the development of novel dressings were looking for better materials to prevent tissue dehydration and cell death, to accelerate angiogenesis, to facilitate the interaction of growth factors with the target cells and to deliver bioactive healing substances in a controlled and efficient manner. This choice depends on several factors that will be discussed in the next sections.

1.7 Types and main characteristics of wound dressings

Natural skin is considered the perfect wound dressing and therefore an ideal wound dressing should try to replicate its properties (Morin and Tomaselli, 2007). Historically, wound dressings were firstly considered to have only a passive and protective role in the healing process. However and in the last decades, wound treatment was revolutionized after the confirmation that moist dressings could help wounds to heal in a faster way (Lloyd *et al.*, 1998, Mulder, 2011). It was proved that the

presence of high amounts of available water, together with the inherent water and oxygen permeability of moist dressings, could create wound environments presenting properties more similar to natural skin (Lloyd *et al.*, 1998, Ishihara *et al.*, 2006). Furthermore, a moist wound environment is also an important factor to induce the proliferation and migration of fibroblasts and keratinocytes as well as to enhance collagen synthesis, leading to reduced scar formation (Harding *et al.*, 2000, Morton and Phillips, 2012).

Besides assuring optimal moisture wound environments, it is currently accepted that a wound dressing should also: i) have the capacity to provide thermal insulation, gaseous exchange, and to help drainage and debris removal thus promoting tissue reconstruction processes; ii) should be biocompatible and not provoke any allergic or immune response reaction; iii) should protect the wound from secondary infections; iv) and should be easily removed without causing trauma (Wittaya-areekul and Prahsarn, 2006, Morin and Tomaselli, 2007).

Due to the distinct characteristics of the different types of wounds and of each of the wound healing stages, there is not a unique dressing that can be efficiently applied in all situations (Boateng *et al.*, 2008). However, it is possible to develop and to optimize different biocompatible wound dressing materials in terms of their chemical and physical properties such as their moisture absorption and permeation capacities and in order to meet most of the wound needs at a particular wound stage (Fonder *et al.*, 2008).

In general terms and according to their main types and characteristics, the most commonly used wound dressings for diabetic wound healing applications can be easily classified as:

i) *Hydrocolloids* - these systems are moist wound dressings and usually comprise a backing material (such as semi-permeable films, foams or non-woven polyester fibers) and a layer with hydrophilic/colloidal particles that may contain biocompatible gels made of proteins (e.g., collagen, gelatin) or of polysaccharides (e.g., cellulose and its derivatives) (Lloyd *et al.*, 1998, Hilton *et al.*, 2004, Fletcher *et al.*, 2011). When in contact with the wound exudates, these dressings will absorb wound fluids thus creating a moist environment (Fletcher *et al.*, 2011, Dumville *et al.*, 2012a). They also

have the capacity to be semi-permeable to water and oxygen (Hilton *et al.*, 2004). However, the application of hydrocolloid dressings in strongly infected wounds have been questioned due to the possible hypoxic and excessive moist environment that could potentiate autolysis of necrotic tissue and therefore increase the risk of infection at the wound site (Jeffcoate *et al.*, 2004, McIntosh, 2007). Hydrocolloids are usually applied in granulating and epithelializing wounds and therefore they may be also used for necrotic wounds in order to promote wound debridement (Dumville *et al.*, 2012a). In average, these materials can be maintained in DFU for more than one week (Hilton *et al.*, 2004). However, there are contradictory studies on whether hydrocolloid-type wound dressings can be used in diabetic foot wounds in the case of superficial wounds, if there are no signs of infection, or if few or moderate wound exudates are present (McIntosh, 2007).

ii) *Hydrogels* – these systems are mostly used to maintain high moist wound environments and are comprised of single or mixed hydrated polymers (i.e., in the form of a gel) presenting at least 20% of their weight in retained water (Fonder *et al.*, 2008, Dumville *et al.*, 2012b). If this water composition is higher than 95% these materials are usually designated as superabsorbents (Jeffcoate *et al.*, 2004). Hydrogels may be covalently or non-covalently cross-linked in order to control their swelling capacities and to maintain their conformational structures (Lloyd *et al.*, 1998) and they may swell (or shrink) reversibly in aqueous environments of specific pH and ionic strength values (Slaughter *et al.*, 2009). Like hydrocolloid dressings, hydrogels are capable to promote the autolytic debridement of necrotic tissues and are usually more efficient to dry wounds with few exudates (Dumville *et al.*, 2012b). Their application in wounds having excess exudates can originate wound maceration and lead to healing problems (Edwards and Stapley, 2010). A great advantage of hydrogel-type wound dressings is that they can usually be applied/ removed without greatly interfering with the wound beds (Hilton *et*

al., 2004, Fonder *et al.*, 2008). In addition, these dressings are flexible, non-antigenic, permeable to water, oxygen and metabolites (Lloyd *et al.*, 1998).

iii) Foams – foam-type dressings were developed as alternatives to hydrocolloid-type dressings for applications in moderate/high draining wounds (Skorkowska-Telichowska *et al.*, 2011). Their capacity to absorb wound fluids is in general dependent on the specific employed polymeric material and on foam thickness (Fonder *et al.*, 2008). These dressings are highly absorbent, cushioning, protective and conformable to body surfaces (Weller and Sussman, 2006). Moreover, they are easy to manipulate and be adapted to the required wound sizes (Hilton *et al.*, 2004, Jeffcoate *et al.*, 2004). Due to its absorbency and protective characteristics, foam-type dressings can be left in wound for up to seven days (Weller and Sussman, 2006). Therefore, foams have been also proposed as potential candidates for DFU treatment (Jeffcoate *et al.*, 2004, Skorkowska-Telichowska *et al.*, 2011).

iv) Films – this type of wound dressings are normally transparent, durable, conformable, easy to manipulate, adhesive, cheap, semi-permeable to oxygen and water vapor, and often impermeable to liquid and to bacterial contamination (Weller and Sussman, 2006, Fonder *et al.*, 2008). A main disadvantage of film-type dressings is the fact that they should only be used in wounds with few exudates, namely as protective dressings in superficial pressure wounds and in applications that usually last 4-5 days before the dressing is replaced (Hilton *et al.*, 2004, Jeffcoate *et al.*, 2004, Fonder *et al.*, 2008). However, they may be used directly in the wound or in association with other types of dressings in order to better fix those in wound bed or to improve their fluid barrier properties (Harding *et al.*, 2000, Fonder *et al.*, 2008). Film-type dressings have been also developed and employed in DFU treatment (Hilton *et al.*, 2004).

The main characteristics of each of these materials are summarized in Figure 1.6.

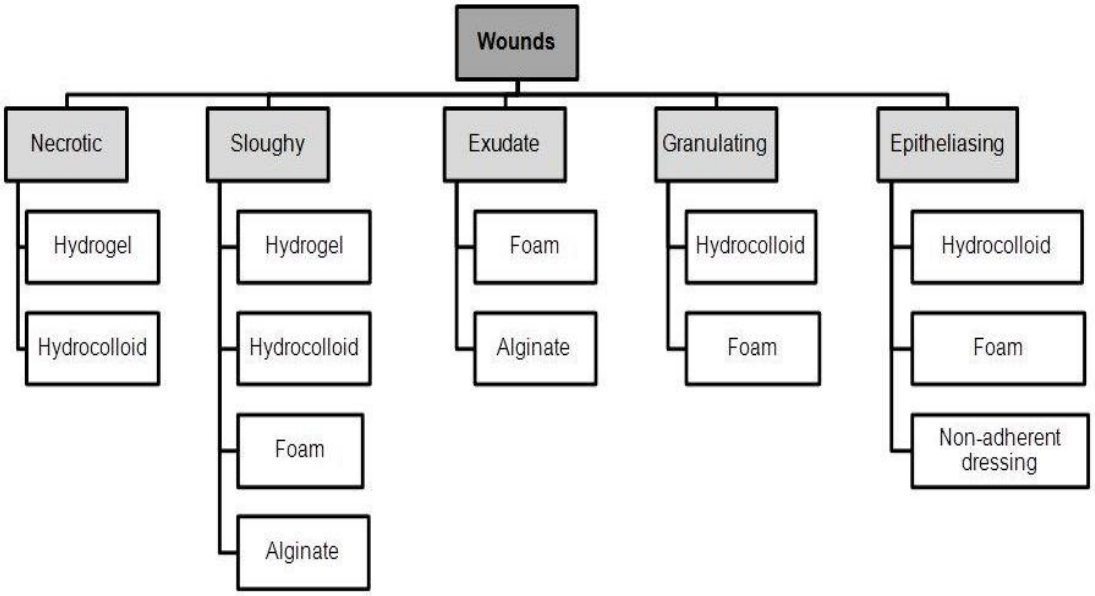

Figure 1.6: Classification of the different dressing types usually used in DFU treatment.

Different synthetic and natural polymer-based biocompatible materials, as well as their mixtures or combinations and different processing methodologies have been proposed and essayed both *in vitro* and *in vivo* for wound dressing (and DFU) applications (Jayakumar *et al.*, 2011, Lee *et al.*, 2012, Meinel *et al.*, 2012). Some of these materials are already commercially available and in clinical use (Nussinovitch and Ben-Zion, 2011, Nisbet, 2012). To supplement and enhance the general wound dressing functions several different strategies have been developed, namely those involving the incorporation of bioactive compounds (like growth factors, peptides, synthetic drugs and/or natural based compounds/extracts) and of stem cells into dressings matrices and in order to prepare medicated dressings (Hong *et al.*, 2008, Altman *et al.*, 2009, Matsumoto and Kuroyanagi, 2010, Suganya *et al.*, 2011).

1.7.1 Polymeric wound dressings for DFU treatment

Wound healing efficiency depends on several factors such as the wound type and stage, injury

extension, patient condition, involved tissues, as well as, on the selected dressing, on the effect of healing enhancers and therapeutic substances (if employed). Wounds can be treated using passive or hydroactive dressings (Zoher *et al.*, 2009). The first are usually used for acute wounds (as they absorb reasonable amounts of exudates and can insure good protection) while the latter are normally used for chronic wounds (as they easily adapt to wounds and are able to maintain a moist environment that can stimulate the healing process) (Weller and Sussman, 2006). In both cases and as already referred, drugs and/or other healing enhancers can be incorporated into the wound dressing polymeric matrices mostly to improve and accelerate healing process.

Different constituent polymeric materials, exhibiting distinct chemical, physical and biological properties may be employed in the preparation of wound dressing systems having different designs, dimensions and shapes, and in order to obtain final products presenting different final functional properties (Boateng *et al.*, 2008, Zahedi *et al.*, 2010). One of the simplest ways to differentiate those polymeric materials is by considering their origin: synthetic- or natural-based polymers and copolymers (Sionkowska, 2011). Modified polymeric materials (those obtained by chemical modification of natural-based polymers) (Muzzarelli and Muzzarelli, 2005) or mixtures/combinations of different polymers and copolymers (Seetharaman *et al.*, 2011) can also be considered.

For DFU applications, there is a wide variety of polymeric materials that has already proved to enhance healing and some of them are by now commercially available (Breen *et al.*, 2008, Yang *et al.*, 2011).

Some of the commonly used natural polymer-based materials to produce dressings for DFU treatment will be presented hereafter.

1.7.2 DFU dressings based on natural polymers

Natural polymers can be classified as those obtained from microbial, animal or vegetal sources that are usually of a protein or polysaccharide nature (Tabata, 2009). Despite these naturally occurring polymers can most closely simulate the original cellular environments and ECMs, and that these

biomaterials are known to undergo naturally-controlled degradation processes, their large heterogeneity and batch-to-batch variations upon their isolation from animal or vegetal tissues are the main limitations for their applications (Malafaya *et al.*, 2007, Sell *et al.*, 2010). Other concerns include the relatively high cost of some of these materials (namely of protein-based materials) and the associated risk of infectious diseases transmission due to the allogenic or xenogenic origins of the original materials (Malafaya *et al.*, 2007). Poor stabilities and mechanical performances also represent drawbacks that may limit their wider application (Huang and Fu, 2010). However, some chemical synthesis and/or processing modifications may be performed in order to overcome some of these disadvantages (Jayakumar *et al.*, 2011). Blending these materials with other polymeric materials (including synthetic polymers) is another viable alternative for these purposes (Tessmar and Gopferich, 2007). Some natural polymers that are being employed in the preparation of wound dressings will be presented and discussed in what follows.

Chitin, chitosan and derivatives - Chitin is one of the most abundant polysaccharides in nature. It can be found in the exoskeleton of arthropods, of crustacean, of some mollusks and in cell wall of fungi (Dai *et al.*, 2011). Common chitin sources (e.g., shell of shrimps and crabs) are very accessible at low cost which makes chitin a commercially attractive biomaterial for various applications (Khoushab and Yamabhai, 2010, Madihally, 2011). Chitin is a linear polysaccharide of *N*-acetyl-D-glucosamine (2-acetylamino-2-deoxy-D-glucose) units linked by β-(1-4) glycosidic bonds (Koide, 1998, Kim *et al.*, 2008, Jayakumar *et al.*, 2010, Dash *et al.*, 2011, Jayakumar *et al.*, 2011). As chitin is not soluble in aqueous solutions, it is usually converted into chitosan by thermochemical deacetylation in the presence of an alkaline solution (Rinaudo, 2006, Dash *et al.*, 2011). Therefore, chitosan is a linear copolymer of D-glucosamine and of *N*-acetyl-D-glucosamine. The term chitosan is also usually employed to describe a series of chitin-derivatives having different deacetylation degrees (defined in terms of the composition of primary amino groups in the polymer backbone and of their average molecular weights) (Rinaudo, 2006). For example, the typical deacetylation degrees of commercial chitosan are usually between 70% and 95% and their most common average molecular weights are between 10 000 and 1 000 000 g/mol.

Despite chitosan chemical, physical and biological properties are directly related to its deacetylation degree and to its molecular weight (Chatelet *et al.*, 2001), chitosan is generally regarded to be biodegradable, biocompatible, non-antigenic, non-toxic, bioadhesive, anti-microbial, bioactive and to have haemostatic effect (Huang and Fu, 2010, Dai *et al.*, 2011, Pérez *et al.*, 2012). It is also easily degraded by chemical hydrolysis as well as by certain human enzymes, namely by lysozyme (Kim *et al.*, 2008, Jayakumar *et al.*, 2010). In addition, chitosan amino and hydroxyl groups can be easily reacted and chemically modified thus allowing a high chemical versatility. For example, chitosan may be modified into *N*-carboxymethyl chitosan (Tan *et al.*, 2011), *N*-carboxybutyl chitosan (Santos *et al.*, 2005, Dias *et al.*, 2010), *N*-succinyl chitosan (Dai *et al.*, 2008), *N*-acyl chitosan (Han *et al.*, 2012), *N*,*O*-(carboxymethyl) chitosan (Chen *et al.*, 2006), *N*-*N*-dicarboxymethyl chitosan (Mattioli-Belmonte *et al.*, 1999), *N*-carboxyethyl chitosan (Weng *et al.*, 2008), *O*-succinyl chitosan (Zhang *et al.*, 2003), *O*-carboxymethyl chitosan (Yin *et al.*, 2007), 5-methylpyrrolidinone (Berscht *et al.*, 1994) and more. Some chitosan derivatives are described below.

Carboxymethylation is a way to improve the water solubility of chitosan. *N*-carboxymethyl chitosan (CMC) is obtained through the reaction of $-NH_2$ group of chitosan with the carbonyl group of aldehyde- glyoxylic acid followed by hydrogenation with $NaBH_4$. The carboxymethyl group is placed on the N-atom with absence of O-substitution (Muzzarelli *et al.*, 1994). CMC has better viscosity, moisture retention, membrane forming, flocculating, chelating and sorption properties comparing with chitosan due the existence of carboxymethyl group in the molecular structure (Mourya *et al.*, 2010).

CMC is non-toxic, modulates cell function namely proliferation and migration of skin fibroblasts promoting *in vitro* wound healing (Chang *et al.*, 2008, Muzzarelli, 2009). In addition, CMC exerts antioxidant, antibacterial and anti-apoptotic activities. CMC has many applications such as in sustained and controlled drug delivery, DNA delivery and permeation enhancer. Commercially it has been used in cosmetic products due its excellent moisture-retention ability (Muzzarelli *et al.*, 1994, Muzzarelli, 2009, Mourya *et al.*, 2010).

N-carboxybutyl chitosan (CBC) was first obtained by Muzzarelli and colleagues (Muzzarelli *et al.*, 1989). It is synthesized from the reaction of $-NH_2$ group of chitosan with the carbonyl group of levulinic acid. But, depending on the chemical conditions the reaction tends to form *N*-carboxybutyl chitosan or 5-methylpyrrolidinone chitosan (MPC), a cyclic derivative in which the amino groups of glucosamine units of the chitosan are partially substituted by methyl pyrrolidinone group in position 5 (Muzzarelli *et al.*, 1993, Mourya *et al.*, 2010). The CBC and MPC present more viscosity compared with corresponding chitosan as well as film-forming ability, moisturizing effect and emulsion stability (Mourya *et al.*, 2010). In addition, MPC combines the properties of chitosan such as biocompatibility and biodegradability with hydrophilic characteristics of pyrrolidinone making it more susceptible to the hydrolysis of lysozyme (Muzzarelli, 1992). MPC has been largely used in dental surgery and accelerating wound and ulcer healings (Berscht *et al.*, 1994, Giunchedi *et al.*, 1998, Rossi *et al.*, 2007) where show to have a potent antimicrobial activity against a broad spectrum of bacteria as well as effective antifungistatic action (Gavini *et al.*, 2008).

Another chitosan derivative is succinyl chitosan (SC) that is obtained by the reaction of chitosan with succinic anhydride. SC has good water solubility at various pH which is favorable in wound dressing applications and cosmetic materials (Tajima *et al.*, 2000, Vanichvattanadecha *et al.*, 2010). Besides biocompatibility, low toxicity and long-term retention in the body, SC has favorable characteristics for drug delivery, namely for anticancer therapy (Kato *et al.*, 2004, Asai *et al.*, 2012).

The employed conditions for the amino group chemical modifications may interfere with the final deacetylation degree and therefore with the cationic nature of obtained materials. Chitosan exhibits a pH-sensitive behavior being a weak poly-base (due to the large number of amino groups). Chitosan easily dissolves at relatively low pH values (while it is insoluble at higher pH values, usually above pH 6.0) and its pH-sensitive swelling mechanism involves the protonation of the amine groups at these low pH conditions (Dai *et al.*, 2011, Censi *et al.*, 2012). Chitosan is also soluble in weak organic acids, interacting with negatively charged molecules, which may facilitate its processing and further integration into particles, membranes, fibers or sponges (Azad *et al.*, 2004, Madihally, 2011). This property has held chitosan and its derivatives (alone or

combined/conjugated with other polymeric materials) to be widely studied as delivery matrices for several pharmaceutical applications (Kumar, 2000, Muzzarelli and Muzzarelli, 2005, Dai *et al.*, 2011, Saranya *et al.*, 2011). At acidic pH, chitosan is positively charged and therefore it is more susceptible to interact with negatively charged molecules such as proteins, anionic polysaccharides and nucleic acids, which are usually present in skin (Lloyd *et al.*, 1998, Bhattarai *et al.*, 2010).

Chitosan is also soluble in weak organic acids, interacting with negatively charged molecules, which may facilitate its processing and further integration into particles, membranes, fibers or sponges. In addition to the fact that chitosan-based materials usually exhibit a positive charge (at typical wound pH values), film-forming capacities, mild gelation characteristics and strong wound tissue adhesive properties, chitosan and its derivatives were also found to enhance blood coagulation and to accelerate wound healing (Kim *et al.*, 2008, Jayakumar *et al.*, 2011). Therefore, these materials clearly present several properties that can potentially permit their use as advantageous and efficient wound dressings. In particular, chitosan films of low deacetylation degree already proved to be efficient for superficial wound dressing applications (Dash *et al.*, 2011). Other works also indicated that these biomaterials enhance the inflammatory functions of polymorphonuclear leukocytes, macrophages and neutrophils promoting a tissue granulation to an appropriate inflammatory response (Takei *et al.*, 2012). Moreover, chitosan may stimulate the proliferation of fibroblasts, angiogenesis, synthesis and a regular deposition of collagen fibers that leads to an improved tissue organization (Kim *et al.*, 2008, Muzzarelli, 2009, Jayakumar *et al.*, 2011).

Chitosan can be also complexed/cross-linked with other charged or non-charged polymers and/or cross-linked agents to change/enhance its physic/chemical/mechanical properties. Through this approach it is possible to optimize and/or to design chitosan based dressings with improved healing characteristics that include enhanced adherent and anti-bacterial capacity, increased exudate absorption capacity, stimulation of angiogenesis and reepithelialization of skin tissue and collagen deposition, sustained delivery of growth factors, etc.

Collagen - Collagen is the most abundant protein of ECMs that are naturally present in human

tissues (e.g. skin, bone, cartilage, tendon and ligaments). It represents 25% of the total protein body content (Lee *et al.*, 2001a, Valenta and Auner, 2004, Slaughter *et al.*, 2009, Sell *et al.*, 2010) providing strength and integrity to tissue matrices (Arul *et al.*, 2007). In addition, collagen can also interact with cells and help essential cell signaling that will regulate cell anchorage, migration, proliferation, differentiation and survival (Chen *et al.*, 2006, Arul *et al.*, 2007, Malafaya *et al.*, 2007).

Twenty-seven types of collagens were already identified being type's I–IV the most common. Type I collagen is the most abundant protein present in mammals and it is the most studied protein for biomedical applications (Malafaya *et al.*, 2007, Mano *et al.*, 2007).

Collagen degrades enzymatically within the body, mostly via collagenases, gelatinases and metalloproteinases (Parenteau-Bareil *et al.*, 2010). In general terms, collagens are rodtype proteins with typical molecular weights around 300 000 g/mol that also present high mechanical strength and good biocompatibility (although they may present some antigenicity) (Malafaya *et al.*, 2007, Cen *et al.*, 2008). Collagen can form stable fibers and its properties, namely mechanical, degradation and water-uptake properties, can be further enhanced by chemical cross-linking (using glutaraldehyde (Lammers *et al.*, 2009), genipin (Antonio *et al.*, 2011), carboiimide (Lin *et al.*, 2009), hexamethylene diisocyanate (Zeugolis *et al.*, 2009)), by physical cross-linking (using freeze-drying) (Kondo *et al.*, 2011) or by binding with other protein/polymers (Lee *et al.*, 2001b, Chen *et al.*, 2010). Low inflammatory and cytotoxic responses and biodegradability are other attractive properties of collagen (Sell *et al.*, 2010).

As a result, and since collagen is one of the major components of human ECMs, it is usually considered as an ideal biomaterial for tissue engineering and for wound dressing applications. Collagen is usually isolated from animal tissues raising some concerns regarding the risks of using collagen derived from animal tissues (Cen *et al.*, 2008, Parenteau-Bareil *et al.*, 2010). However, enzymatic purification techniques (to eliminate those immunogenic telopeptides that induce foreign body response) may be employed (Srinivasan and Sehgal, 2010). Alternatively, the use of recombinant and non-recombinant human collagens can be envisaged but their production still present high costs (Cen *et al.*, 2008). Collagen is also difficult to process and its degradation rate is

not easy to control (Malafaya *et al.*, 2007, Parenteau-Bareil *et al.*, 2010). For example, collagen degradability depends on cell 3D-structure penetration (which causes contraction, inner pressure increase, fluids restrictions and makes collagen less swellable and degradable) and, in addition, collagen is also degradable by other non-specific proteinases (Malafaya *et al.*, 2007). Finally, collagen sterilization may be also an issue as employed sterilization methods may promote chemical and physical modifications in the collagen structure (Parenteau-Bareil *et al.*, 2010).

Due to all the above mentioned characteristics, collagen is frequently used to prepared wound dressings materials in diverse forms that include gels, pads, particles, pastes, powders, sheets or solutions. A large number of commercial collagen based dressings is already available and some are specifically indicated for partial- and full-thickness pressure, venous, vascular and diabetic ulcers as is the case of BGC, Dermacol and Promogran as demonstrated in Table 1.5.

Collagen -based hydrogels were already studied as biomaterials for wound dressing applications in general, as well as for DFU treatment, in particular. As reported in Table 1.4, 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 (Adhirajan *et al.*, 2009, Singh *et al.*, 2011, Arul *et al.*, 2012, Manizate *et al.*, 2012). Different approaches tested so far include the incorporation of glucose oxidase in a collagen matrix in order to achieve 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 antimicrobial agent (Kawai *et al.*, 2005, Arul *et al.*, 2012, Kanda *et al.*, 2012)

Some formulations to stimulate healing in DFU are presently in clinical trials. For example, a randomized, prospective and comparative study has been done to analyze the effectiveness of a new collagen-oxidized regenerated cellulose antimicrobial dressing on DFUs treatment. In other study and with the purpose to analyze the most efficient and most cost-effective application, silver-impregnated collagen dressings (*Biostep* dressing with *Allevyn* Foam) will be used for 2 weeks to promote healing of non-infected DFU.

Table 1.3: Main characteristics of some natural based polymers usually applied to prepare wound dressings for DFUs.

	Natural Polymers	References
Chitin/Chitosan	- biocompatible, biodegradable, non-toxic, anti-bacterial, cheap, accessible - soluble in weak organic acids and dissolves at low pH - chitin is difficult to dissolve- use of chitosan - accelerates wound healing and skin regeneration	(Azad *et al.*, 2004, Huang and Fu, 2010, Jayakumar *et al.*, 2010, Dai *et al.*, 2011, Censi *et al.*, 2012)
Collagen/Gelatin	- natural presence in human body (skin, bone, cartilage) - biodegradable, biocompatibility, low antigenicity - gelatin is derived from collagen - enhanced acute inflammation and skin cells recruitment	(Lee *et al.*, 2001a, Valenta and Auner, 2004, Cen *et al.*, 2008, Kanda *et al.*, 2012)

Santyl is a collagenase gel that possesses the ability to digest the collagen of necrotic tissue proposed to follow the use of *Santy*l daily, for up to 6 weeks, on diabetic foot wounds. To test the hypothesis that daily treatment of diabetic foot wounds with *Santyl* will result in more rapid healing, in healthier wounds and decreasing sharp debridements over the study period.

Integra™ Flowable Wound Matrix (Integra LifeSciences Corp, USA) is an advanced 3-D porous matrix constituted by granulated cross-linked bovine tendon collagen and glycosaminoglycan. It provides a scaffold for cellular invasion and capillary growth. In this clinical trial study, the mixture of collagen and glycosaminoglycan is hydrated with saline and applied on the wound bed for DFUs treatment.

Incorporation of antibiotics to reduce infection is also a point of interest in dressing development. Gentamicin-collagen sponges are safe and effective dressings to treat mildly infected DFUs when compared to the treatment with an oral antibiotic (levofloxacin) or to standard daily wound care. In this study, patients were treated daily with gentamicin-collagen sponge treatment or ingest oral levofloxacin.

Table 1.4: Recently natural and synthetic based dressings studied for DFUs application.

Polymers		Bioactive substance	Models used	Results	References
Chitosan and derivatives	Chitosan-crosslinked collagen	Recombinant human aFGF	STZ diabetic rats	Accelerated wound healing promoting a faster tissue collagen deposition, higher TGF-β1 expression and dermal cell proliferation.	Wang *et al.*, 2008
	Chitosan with different degrees of deacetylation	Acetylglucosamine oligomers	Human diabetic	Decreased wound size and stimulated angiogenesis and reepithelialization after seven days.	Ben-shalom *et al.*, 2009
	Thiolated chitosan-oxidized dextran hydrogel	–	STZ diabetic mice	Showed to be non-cytotoxic, resistant to degradation and capable of stimulate tissue regeneration.	Zhang *et al.*, 2011
	Chitosan, alginate, and poly(γ-glutamic acid) hydrogel	–	STZ diabetic rats	Enhanced wound healing. Stimulated collagen deposition, hydroxyproline levels and promoted skin epithelialization.Showed antibacterial properties.	Lee *et al.*, 2012
Collagen	Colagen dressing	–	Human patients with DFUs	60% of them healed after two weeks of treatment. A decrease of infection by bacteria and an augment of granulation tissue were also observed.	Singh *et al.*, 2011
	Collagen matrix	Glucose oxidase	STZ diabetic rats	Increased cellular proliferation and stimulated a faster wound contraction.	Arul *et al.*, 2012
	Collagen–gelatin foam	bFGF	db/db mice	Accelerated dermis tissue formation and increased the number of new capillaries.	Kanda *et al.*, 2012

Table 1.5: Commercial collagen based-dressings commonly used for the treatment of DFUs.

Commercial dressing	Fabricant	Composition	Main characteristics
Unite® Biomatrix	Synovis Orthopedic and WoundCare, Inc.	non-reconstituted collagen	• Collagen dressing helps maintain wound bed in healing phase • Allows for healthy granulation tissue and wound closure • Absorbs excess exudate allowing few dressing changes • Easily conforms to the wound bed • Strong and durable
BGC Matrix®	Mölnlycke Health Care US, LLC	collagen and the advanced carbohydrate beta-glucan	• Protects underlying tissue from external contamination • Provides structural support for new cell growth • Adherent, flexible and conformable • Minimizes protein and water loss • Collagen aids in hemostasis • Minimizes pain
Promogran Prisma® Matrix	Systagenix	collagen, ORC and silver-ORC matrix	• In presence of exudate, the matrix transforms into a biodegradable gel • Provides protection from infection and optimal healing environment • Designed to "kick start" the healing process in stalled wounds • Biodegradable gel is soft and conformable • Can be used under compression therapy • Non-toxic and non-irritating • Easy to use
DERMACOL COLLAGEN MATRIX	DermaRite Industries	collagen, sodium alginate, carboxyl methyl-cellulose, ethylenediamine-tetraacetic acid (EDTA) and silver chloride	• Transforms into a soft gel sheet when in contact with wound exudates • Maintains a moist wound environment, and creates ideal conditions for healing • Antimicrobial silver chloride prevents colonization of the dressing • Easy to use
Fibracol® Plus Collagen Wound Dressing w/ Alginate	Systagenix	collagen and calcium alginate fibers wound	• Structural support of collagen with gel forming properties of alginates • Maintains a moist wound environment, and creates ideal conditions for healing. • Adherent, flexible and conformable • Sterile and soft

1.8 References

(2006) Diagnosis and classification of diabetes mellitus. Diabetes care 29 Suppl 1:S43-48.

Abu-Al-Basal MA (2010) Healing potential of Rosmarinus officinalis L. on full-thickness excision cutaneous wounds in alloxan-induced-diabetic BALB/c mice. Journal of Ethnopharmacology 131:443-450.

Acosta JB, del Barco DG, Vera DC, Savigne W, Lopez-Saura P, Guillen Nieto G, Schultz GS (2008) The pro-inflammatory environment in recalcitrant diabetic foot wounds. Int Wound J 5:530-539.

Adhirajan N, Shanmugasundaram N, Shanmuganathan S, Babu M (2009) Collagen-based wound dressing for doxycycline delivery: in-vivo evaluation in an infected excisional wound model in rats. Journal of Pharmacy and Pharmacology 61:1617-1623.

Ahmed I, Glodstein B (2006) Diabetes mellitus. Clinics in Dermatology 24:237-246.

Alberti KG, Zimmet PZ (1998) Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabetic medicine : a journal of the British Diabetic Association 15:539-553.

Ali S, Dornhorst A (2011) Diabetes in pregnancy: health risks and management. Postgrad Med J 57:417-427.

Altman AM, Yan Y, Matthias N, Bai X, Rios C, Mathur AB, Song Y, Alt EU (2009) IAFTS Collection: Human adipose-derived stem cells seeded on a silk fibroin-chitosan scaffold enhance wound repair in a murine soft tissue injury model. Stem Cells 27:250-258.

Amos PJ, Kapur SK, Stapor PC, Shang H, Bekiranov S, Khurgel M, Rodeheaver GT, Peirce SM, Katz AJ (2010) Human adipose-derived stromal cells accelerate diabetic wound healing: impact of cell formulation and delivery. Tissue Eng Part A 16:1595-1606.

Antonio F, Guillem R, Sonia T, Clara M, Piergiorgio G, Valeria C, Gianluca C, Tzanov T (2011) Cross-linked collagen sponges loaded with plant polyphenols with inhibitory activity towards chronic wound enzymes. Biotechnol J 6:1208-1218.

Aring AM, Jones DE, Falko JM (2005) Evaluation and prevention of diabetic neuropathy. Am Fam Physician 71:2123-2128.

Arul V, Kartha R, Jayakumar R (2007) A therapeutic approach for diabetic wound healing using biotinylated GHK incorporated collagen matrices. Life Sci 80:275-284.

Arul V, Masilamoni JG, Jesudason EP, Jaji PJ, Inayathullah M, Dicky John DG, Vignesh S, Jayakumar R (2012) Glucose oxidase incorporated collagen matrices for dermal wound repair in diabetic rat models: a biochemical study. J Biomater Appl 26:917-938.

Asai J, Takenaka H, Ii M, Asahi M, Kishimoto S, Katoh N, Losordo DW (2012) Topical application of ex vivo expanded endothelial progenitor cells promotes vascularisation and wound healing in diabetic mice. Int Wound J.

Ayodele OE, Alebiosu CO, Salako BL (2004) Diabetic Nephropathy- A Review of the Natural History, Burden, Risk Factors and Treatment. Journal of the National Medical Association 96:1445-1454.

Azad AK, Sermsintham N, Chandrkrachang S, Stevens WF (2004) Chitosan membrane as a wound-healing dressing: characterization and clinical application. J Biomed Mater Res B Appl Biomater 69:216-222.

Badillo AT, Chung S, Zhang L, Zoltick P, Liechty KW (2007) Lentiviral gene transfer of SDF-1alpha to wounds improves diabetic wound healing. J Surg Res 143:35-42.

Bagheri M, Jahromi BM, Mirkhani H, Solhjou Z, Noorafshan A, Zamani A, Amirghofran Z (2011) Azelnidipine, a New Calcium Channel Blocker, Promotes Skin Wound Healing in Diabetic Rats. Journal of Surgical Research 169:e101-e107.

Barcelos LS, Duplaa C, Krankel N, Graiani G, Invernici G, Katare R, Siragusa M, Meloni M, Campesi I, Monica M, Simm A, Campagnolo P, Mangialardi G, Stevanato L, Alessandri G, Emanueli C, Madeddu P (2009) Human CD133+ progenitor cells promote the healing of diabetic ischemic ulcers by paracrine stimulation of angiogenesis and activation of Wnt signaling. Circ Res 104:1095-1102.

Basic-Kes V, Zavoreo I, Rotim K, Bornstein N, Rundek T, Demarin V (2011) Recommendations for diabetic polyneuropathy treatment. Acta Clin Croat 50:289-302.

Berscht PC, Nies B, Liebendorfer A, Kreuter J (1994) Incorporation of basic fibroblast growth factor into methylpyrrolidinone chitosan fleeces and determination of the in vitro release characteristics. Biomaterials 15:593-600.

Bhattarai N, Gunn J, Zhang M (2010) Chitosan-based hydrogels for controlled, localized drug delivery. Adv Drug Deliv Rev 62:83-99.

Bitto A, Minutoli L, Altavilla D, Polito F, Fiumara T, Marini H, Galeano M, Calo M, Lo Cascio P, Bonaiuto M, Migliorato A, Caputi AP, Squadrito F (2008) Simvastatin enhances VEGF production and ameliorates impaired wound healing in experimental diabetes. Pharmacol Res 57:159-169.

Blakytny R, Jude EB (2009) Altered molecular mechanisms of diabetic foot ulcers. Int J Low Extrem Wounds 8:95-104.

Bloomgarden ZT (2008) The diabetic foot. Diabetes care 31:372-376.

Boateng JS, Matthews KH, Stevens HN, Eccleston GM (2008) Wound healing dressings and drug delivery systems: a review. Journal of pharmaceutical sciences 97:2892-2923.

Bottcher-Haberzeth S, Biedermann T, Reichmann E (2010) Tissue engineering of skin. Burns : journal of the International Society for Burn Injuries 36:450-460.

Boulton AJ, Malik RA, Arezzo JC, Sosenko JM (2004) Diabetic somatic neuropathies. Diabetes care 27:1458-1486.

Brain SD (1997) Sensory neuropeptides: their role in inflammation and wound healing. Immunopharmacology 37:133-152.

Breen AM, Dockery P, O'Brien T, Pandit AS (2008) The use of therapeutic gene eNOS delivered via a fibrin scaffold enhances wound healing in a compromised wound model. Biomaterials 29:3143-3151.

Brem H, Tomic-Canic M (2007) Cellular and molecular basis of wound healing in diabetes. J Clin Invest 117:1219-1222.

Brun P, Mastrotto C, Beggiao E, Stefani A, Barzon L, Sturniolo GC, Palu G, Castagliuolo I (2005) Neuropeptide neurotensin stimulates intestinal wound healing following chronic intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 288:G621-629.

Carraway R, Leeman SE (1973) The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalami. The Journal of biological chemistry 248:6854-6861.

Castagliuolo I, Wang C, Valenick L, Pasha A, Nikulasson S, Carraway RE, Pothoulakis C (1999) Neurotensin is a proinflammatory neuropeptide in colonic inflammation. J Clin Invest 103:843-849.

Cavanagh PR, Lipsky BA, Bradbury AW, Botek G (2005) Treatment for diabetic foot ulcers. Lancet 366:1725-1735.

Cen L, Liu W, Cui L, Zhang W, Cao Y (2008) Collagen tissue engineering: development of novel biomaterials and applications. Pediatr Res 63:492-496.

Censi R, Di Martino P, Vermonden T, Hennink WE (2012) Hydrogels for protein delivery in tissue engineering. Journal of controlled release : official journal of the Controlled Release Society 161:680-692.

Chang J, Liu W, Han B, Liu B (2008) The evaluation on biological properties of carboxymethyl-chitosan and carboxymethyl-chitin. Journal of Ocean University of China 7:404-410.

Chatelet C, Damour O, Domard A (2001) Influence of the degree of acetylation on some biological properties of chitosan films. Biomaterials 22:261-268.

Chen R, Wang G, Chen C, Ho H, Shen M (2006) Development of a new N-O-(Carboxymethyl)/chitosan /collagen matrixes as a wound dressing. Biomacromolecules 7:1058-1064.

Chen ZG, Wang PW, Wei B, Mo XM, Cui FZ (2010) Electrospun collagen-chitosan nanofiber: a biomimetic extracellular matrix for endothelial cell and smooth muscle cell. Acta biomaterialia 6:372-382.

Cheng CF, Sahu D, Tsen F, Zhao Z, Fan J, Kim R, Wang X, O'Brien K, Li Y, Kuang Y, Chen M, Woodley DT, Li W (2011) A fragment of secreted Hsp90alpha carries properties that

enable it to accelerate effectively both acute and diabetic wound healing in mice. J Clin Invest 121:4348-4361.
Chittleborough CR, Grant JF, Phillips PJ, Taylor AW (2007) The increasing prevalence of diabetes in South Australia: the relationship with population ageing and obesity. Public Health 121:92-99.
Clark RA, Ghosh K, Tonnesen MG (2007) Tissue engineering for cutaneous wounds. The Journal of investigative dermatology 127:1018-1029.
Cochrane DE, Boucher W, Bibb P (1986) Neurotensin Stimulates Histamine Release in in vivo Skin 'Blisters' in Rats: An Effect Inhibited by Cromolyn or Somatostatin. Int Arch Allergy Immunol 80:225-230.
Dai T, Tanaka M, Huang YY, Hamblin MR (2011) Chitosan preparations for wounds and burns: antimicrobial and wound-healing effects. Expert review of anti-infective therapy 9:857-879.
Dai YN, Li P, Zhang JP, Wang AQ, Wei Q (2008) A novel pH sensitive N-succinyl chitosan/alginate hydrogel bead for nifedipine delivery. Biopharmaceutics & drug disposition 29:173-184.
Daneman D (2006) Type 1 diabetes. Lancet 367:847-858.
Dash M, Chiellini F, Ottenbrite RM, Chiellini E (2011) Chitosan-a versatile semi-synthetic polymer in biomedical. Progress in Polymer Science 678.
Davis MD (1992) Diabetic retinopathy. A clinical overview. Diabetes care 15:1844-1874.
De la Fuente M, Garrido JJ, Arahuetes RM, Hernanz A (1993) Stimulation of phagocytic function in mouse macrophages by neurotensin and neuromedin N. J Neuroimmunol 42:97-104.
Delavary BM, Veer WM, Egmond M, Niessen FB, Beelen RHJ (2011) Macrophages in skin injury and repair. Immunobiology 216:753-762.
Dias AM, Seabra IJ, Braga MM, Gil MH, de Sousa HC (2010) Supercritical solvent impregnation of natural bioactive compounds in N-carboxybutyl chitosan membranes for the development of topical wound healing applications. Journal of controlled release : official journal of the Controlled Release Society 148:e33-35.
DiPietro LA (1995) Wound healing: the role of the macrophage and other immune cells. Shock 4:233-240.
Donelan J, Boucher W, Papadopoulou N, Lytinas M, Papaliodis D, Dobner P, Theoharides TC (2006) Corticotropin-releasing hormone induces skin vascular permeability through a neurotensin-dependent process. Proc Natl Acad Sci U S A 103:7759-7764.
Dumville JC, Deshpande S, O'Meara S, Speak K (2012a) Hydrocolloid dressings for healing diabetic foot ulcers. Cochrane Database Syst Rev 2:CD009099.
Dumville JC, Soares MO, O'Meara S, Cullum N (2012b) Systematic review and mixed treatment comparison: dressings to heal diabetic foot ulcers. Diabetologia 55:1902-1910.
Dwivedi VK, Chaudhary M (2012) Comparative wound healing efficacy of ampucare and becaplermin in diabetic rat. African Journal of Pharmacy and Pharmacology 6:883-892.
Dyck PJ, Kratz KM, Karnes JL, Litchy WJ, Klein R, Pach JM, Wilson DM, O'Brien PC, Melton LJ, 3rd, Service FJ (1993) The prevalence by staged severity of various types of diabetic neuropathy, retinopathy, and nephropathy in a population-based cohort: the Rochester Diabetic Neuropathy Study. Neurology 43:817-824.
Edwards J, Stapley S (2010) Debridment of diabetic foot ulcers. Cochrane Database System Reviews 20:1-44.
Enoch S, Leaper DJ (2008) Basic Science of wound healing. Surgery 26:31-37.
Evers BM (2006) Neurotensin and growth of normal and neoplastic tissues. Peptides 27:2424-2433.
Falanga V (2005) Wound healing and its impairment in the diabetic foot. Lancet 366:1736-1743.
Fletcher J, Moore Z, Anderson I, Matsuzaki K (2011) Pressure ulcers and hydrocolloids. Wound International 2:1-6.
Fonder MA, Lazarus GS, Cowan DA, Aronson-Cook B, Kohli AR, Mamelak AJ (2008) Treating the chronic wound: A practical approach to the care of nonhealing wounds and wound care dressings. Journal of the American Academy of Dermatology 58:185-206.

Fowler MJ (2008) Microvascular and Macrovascular Complications of Diabetes. Clinical Diabetes • Volume 26, Number 2, 2008 26:77-82.

Fujita N, Sagaguchi I, Kobayashi H, Ikeda N, Kato Y, Minamino M (2003) An Extract of the Root of Lithospermun erythrorhison Accelerates Wound Healing in Diabetic Mice. Biological and Pharmaceutical Bulletin 26:329-335.

Galiano RD, Tepper OM, Pelo CR, Bhatt KA, Callaghan M, Bastidas N, Bunting S, Steinmetz HG, Gurtner GC (2004) Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. The American journal of pathology 164:1935-1947.

Gallagher KA, Liu ZJ, Xiao M, Chen H, Goldstein LJ, Buerk DG, Nedeau A, Thom SR, Velazquez OC (2007) Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1 alpha. J Clin Invest 117:1249-1259.

Gavini E, Rassu G, Muzzarelli C, Cossu M, Giunchedi P (2008) Spray-dried microspheres based on methylpyrrolidinone chitosan

as new carrier for nasal administration of metoclopramide. 68:245-252.

Gibran NS, Jang YC, Isik FF, Greenhalgh DG, Muffley LA, Underwood RA, Usui ML, Larsen J, Smith DG, Bunnett N, Ansel JC, Olerud JE (2002) Diminished neuropeptide levels contribute to the impaired cutaneous healing response associated with diabetes mellitus. J Surg Res 108:122-128.

Giunchedi P, Genta I, Conti B, Muzzarelli RA, Conte U (1998) Preparation and characterization of ampicillin loaded methylpyrrolidinone chitosan and chitosan microspheres. Biomaterials 19:157-161.

Goldman R, Bar-Shavit Z, Romeo D (1983) Neurotensin modulates neutrophil locomotion and phagocytic capability FEBS Letter 159:63-67.

Guo S, Dipietro LA (2010) Factors affecting wound healing. J Dent Res 89:219-229.

Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008) Wound repair and regeneration. Nature 453:314-321.

Hamed S, Ullmann Y, Masoud M, Hellou E, Khamaysi Z, Teot L (2010) Topical erythropoietin promotes wound repair in diabetic rats. The Journal of investigative dermatology 130:287-294.

Han T, Nwe N, Furuike T, Tokura S, Tamura H (2012) Methods of N-acetylated chitosan scaffolds and its in vitro biodegradation by lysozyme. Journal of Biomedical Science and Engineering 5:15-23.

Harding KG, Jones V, Price P (2000) Topical treatment: which dressing to choose. Diabetes Metab Res Rev 16 Suppl 1:S47-50.

Hilton JR, Williams DT, Beuker B, Miller DR, Harding KG (2004) Wound dressings in diabetic foot disease. Clin Infect Dis 39 Suppl 2:S100-103.

Hong HJ, Jin SE, Park JS, Ahn WS, Kim CK (2008) Accelerated wound healing by smad3 antisense oligonucleotides-impregnated chitosan/alginate polyelectrolyte complex. Biomaterials 29:4831-4837.

Huang S, Fu X (2010) Naturally derived materials-based cell and drug delivery systems in skin regeneration. Journal of controlled release : official journal of the Controlled Release Society 142:149-159.

Ishihara M, Obara K, Nakamura S, Fujita M, Masuoka K, Kanatani Y, Takase B, Hattori H, Morimoto Y, Maehara T, Kikuchi M (2006) Chitosan hydrogel as a drug delivery carrier to control angiogenesis. J Artif Organs 9:8-16.

Jacobi J, Jang JJ, Sundram U, Dayoub H, Fajardo LF, Cooke JP (2002) Nicotine accelerates angiogenesis and wound healing in genetically diabetic mice. The American journal of pathology 161:97-104.

Jannesari M, Varshosaz J, Morshed M, Zamani M (2011) Composite poly(vinyl alcohol)/poly(vinyl acetate) electrospun nanofibrous mats as a novel wound dressing matrix for controlled release of drugs. Int J Nanomedicine 6:993-1003.

Jayakumar R, Prabaharan M, Nair SV, Tamura H (2010) Novel chitin and chitosan nanofibers in biomedical applications. Biotechnol Adv 28:142-150.

Jayakumar R, Prabaharan M, Sudheesh Kumar PT, Nair SV, Tamura H (2011) Biomaterials based on chitin and chitosan in wound dressing applications. Biotechnol Adv 29:322-337.

Jeffcoate WJ, Price P, Harding KG (2004) Wound healing and treatments for people with diabetic foot ulcers. Diabetes Metab Res Rev 20 Suppl 1:S78-89.

Kahn SE (2008) The relative contributions of insulin resistance and beta-cell dysfunction in the pathophysiology of type 2 diabetes. Diabetologia 46:3-19.

Kanda N, Morimoto N, Ayvazyan AA, Takemoto S, Kawai K, Nakamura Y, Sakamoto Y, Taira T, Suzuki S (2012) Evaluation of a novel collagen-gelatin scaffold for achieving the sustained release of basic fibroblast growth factor in a diabetic mouse model. J Tissue Eng Regen Med.

Kanitakis J (2002) Anatomy, histology and immunohistochemistry of normal human skin. European journal of dermatology : EJD 12:390-399; quiz 400-391.

Kato Y, Onishi H, Machida Y (2004) N-succinyl-chitosan as a drug carrier: water-insoluble and water-soluble conjugates. Biomaterials 25: 907–915.

Kawai K, Suzuki S, Tabata Y, Nishimura Y (2005) Accelerated wound healing through the incorporation of basic fibroblast growth factor-impregnated gelatin microspheres into artificial dermis using a pressure-induced decubitus ulcer model in genetically diabetic mice. Br J Plast Surg 58:1115-1123.

Keswani SG, Katz AB, Lim FY, Zoltick P, Radu A, Alaee D, Herlyn M, Crombleholme TM (2004) Adenoviral mediated gene transfer of PDGF-B enhances wound healing in type I and type II diabetic wounds. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 12:497-504.

Khoushab F, Yamabhai M (2010) Chitin research revisited. Mar Drugs 8:1988-2012.

Kim C, Newton KM, Knopp RH (2002) Gestational diabetes and the incidence of type 2 diabetes: a systematic review. Diabetes care 25:1862-1868.

Kim IY, Seo SJ, Moon HS, Yoo MK, Park IY, Kim BC, Cho CS (2008) Chitosan and its derivatives for tissue engineering applications. Biotechnol Adv 26:1-21.

Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, Nathan DM (2002) Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med 346:393-403.

Ko SH, Nauta A, Morrison SD, Zhou H, Zimmermann A, Gurtner GC, Ding S, Longaker MT (2011) Antimycotic ciclopirox olamine in the diabetic environment promotes angiogenesis and enhances wound healing. PloS one 6:e27844.

Koide SS (1998) Chitin-chitosan: properties, benefits and risks. Nutrition Research 13:1091-1101.

Kondo S, Niiyama H, Yu A, Kuroyanagi Y (2011) Evaluation of a wound dressing composed of hyaluronic acid and collagen sponge containing epidermal growth factor in diabetic mice. Journal of Biomaterials Science Polymer Edition.

Kumar MNVR (2000) A review of chitin and chitosan applications. Reactive & Functional Polymers 46:1-27.

Kwon DS, Gao X, Liu YB, Dulchavsky DS, Danyluk AL, Bansal M, Chopp M, McIntosh K, Arbab AS, Dulchavsky SA, Gautam SC (2008) Treatment with bone marrow-derived stromal cells accelerates wound healing in diabetic rats. Int Wound J 5:453-463.

Lammers G, Tjabringa GS, Schalkwijk J, Daamen WF, van Kuppevelt TH (2009) A molecularly defined array based on native fibrillar collagen for the assessment of skin tissue engineering biomaterials. Biomaterials 30:6213-6220.

Lau TW, Lam FF, Lau KM, Chan YW, Lee KM, Sahota DS, Ho YY, Fung KP, Leung PC, Lau CB (2009) Pharmacological investigation on the wound healing effects of Radix Rehmanniae in an animal model of diabetic foot ulcer. J Ethnopharmacol 123:155-162.

Lee CH, Singla A, Lee Y (2001a) Biomedical applications of collagen. Int J Pharm 221:1-22.

Lee CR, Grodzinsky AJ, Spector M (2001b) The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis. Biomaterials 22:3145-3154.

Lee KB, Choi J, Cho SB, Chung JY, Moon ES, Kim NS, Han HJ (2011) Topical embryonic stem cells enhance wound healing in diabetic rats. J Orthop Res 29:1554-1562.

Lee Y, Chang J, Yang M, Chien C, Lai W (2012) Aceleration of wound dressing in diabetic rats by layered hydrogel dressing. Carbohydr Polym 88:809-819.

Leung PC (2007) Diabetic foot ulcers – a comprehensive review. The Surgeon 5:219-231.

Li H, Fu X, Zhang L, Huang Q, Wu Z, Sun T (2008) Research of PDGF-BB Gel on the WoundHealing of Diabetic Rats and Its Pharmacodynamics. Journal of Surgical Research 145:41-48.

Li J, Chen J, Kirsner R (2007) Pathophysiology of acute wound healing. Clin Dermatol 25:9-18.

Lima MH, Caricilli AM, de Abreu LL, Araujo EP, Pelegrinelli FF, Thirone AC, Tsukumo DM, Pessoa AF, dos Santos MF, de Moraes MA, Carvalheira JB, Velloso LA, Saad MJ (2012) Topical insulin accelerates wound healing in diabetes by enhancing the AKT and ERK pathways: a double-blind placebo-controlled clinical trial. PloS one 7:e36974.

Lin Y, Tan F, Marra KC, Jan S, Liu D (2009) Synthesis and characterization of collagen/hyaluronan/chitosan composite sponges for potential biomedical applications. Acta biomaterialia 5:2591-2600.

Liu Y, Min D, Bolton T, Nube V, Twigg SM, Yue DK, McLennan SV (2009) Increased Matrix Mettaloproteinase-9 predicts poor wound healing in diabetic foot ulcers. Diabetes care 32:e137.

Lloyd LL, Kennedy JK, Methacanon P, Paterson M, Knill CJ (1998) Carbohydrate polymers as wound management aids. Carbohydr Polym 37:315-322.

Lobmann R, Zemlin C, Motzkau M, Reschke K, Lehnert H (2006) Expression of matrix metalloproteinases and growth factors in diabetic foot wounds treated with a protease absorbent dressing. Journal of Diabetes and Its Complications 20:329-335.

Lotti T, Hautmann G, Panconesi E (1995) Neuropeptides in skin. Journal of the American Academy of Dermatology 33:482-496.

Madihally SV (2011) Processing chitosan for tissue regeneration. Current Trends in Polymer Science 15:83-88.

Malafaya PB, Silva GA, Reis RL (2007) Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. Adv Drug Deliv Rev 59:207-233.

Manizate F, Fuller A, Gendics C, Lantis J (2012) A Prospective, Single-Center, Nonblinded, Comparative, Postmarket Clinical Evaluation of a Bovine-Derived Collagen With Ionic Silver Dressing versus a Carboxymethylcellulose and Ionic Silver Dressing for the Reduction of Bioburden in Variable-Etiology, Bilateral Lower-Extremity Wounds. Advances in Skin & Wound Care 25:220-225

Mano JF, Silva GA, Azevedo HS, Malafaya PB, Sousa RA, Silva SS, Boesel LF, Oliveira JM, Santos TC, Marques AP, Neves NM, Reis RL (2007) Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends. Journal of the Royal Society Interface 4:999-1030.

Matsumoto Y, Kuroyanagi Y (2010) Development of a Wound Dressing Composed of Hyaluronic Acid Sponge Containing Arginine and Epidermal Growth Factor. Journal of Biomaterials Science Polymer Edition 21:715-726.

Mattioli-Belmonte M, Gigante A, Muzzarelli RAA, Politano R, Benedittis A, Specchia N, Buffa A, Biagini G, Greco F (1999) N-N-dicarboxymethyl chitosan as delivery agent for bone morphogenetic protein in the repair of articular cartilage. Medical and Biological Engineering and Computing 37:130-134.

McCance DR (2011) Pregnancy and diabetes. Best Pract Res Clin Endocrinol Met 25:945-958.

McIntosh C (2007) Are hydrocolloid dressings suitable for diabetic foot ulcers? Wound Essencials 2:170-172.

McLaughlin PJ, Pothering CA, Immonen JA, Zagon IS (2011) Topical treatment with the opioid antagonist naltrexone facilitates closure of full-thickness wounds in diabetic rats. Experimental Biology and Medicine 236:1122-1132.

Meinel A, Germershaus O, Luhmann T, Merkle HP, Meinel L (2012) Electrospun matrices for localized drug delievery:Current technologies and selected biomedical applications. European Journal of Pharmaceutics and Biopharmaceutics 81:1-13.

Mohebali K, Ro P, Young DM, Boudreau N, Hansen SL (2008) Transfer of Hox B3 gene accerelates wound healing in diabetic mice. Journal of American College of Surgeons 207:S58.

Monaco JL, Lawrence WT (2003) Acute wound healing: and overview. Clin Plastic Surg 30:1-12.

Morin RJ, Tomaselli NL (2007) Interactive Dressings and Topical Agents. Clin Plast Surg 34:643-658.

Morton LM, Phillips TJ (2012) Wound healing update. Seminars in Cutaneous Medicine and Surgery 31:33-37.

Mourya VK, Inamdara NN, Tiwarib A (2010) Carboxymethyl chitosan and its applications. Advanced Materials Letters 1:11-33.

Mulder G, Armstrong D, Seaman S (2003) Diabetic foot ulcerations: dressings. Wounds 15:4-15.

Mulder M (2011) The selection of wound care products for wound bed preparation. Professional Nursing Today 30:15-21.

Muller M, Trocme C, Lardy B, Morel F, Halimi S, Benhamou PY (2008) Matrix metalloproteinases and diabetic foot ulcers: the ratio of MMP-1 to TIMP-1 is a predictor of wound healing. Diabetic medicine 25:419–426.

Muzzarelli R (1992) Depolymerization of methyl pyrrolidinone chitosan by lysozyme. Carbohydrate Polymers 19:29-34.

Muzzarelli R, Weckx M, Filippini O, Lough C (1989) Characteristic properties of N-Carboxybutyl chitosan. 11:307–320.

Muzzarelli RA, Ilari P, Petrarulo M (1994) Solubility and structure of N-carboxymethylchitosan. International journal of biological macromolecules 16:177-180.

Muzzarelli RAA (2009) Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone. Carbohydr Polym 76:167-182.

Muzzarelli RAA, Ilari P, Tomasetti M (1993) Preparation and characteristic properties of 5-methyl pyrrolidinone chitosan. Carbohydrate Polymers 20:99-105.

Muzzarelli RAA, Muzzarelli C (2005) Chitosan chemistry: relevance to the biomedical sciences. Advances in Polymer Science 186:151-209.

Nabzdyk LP, Kuchibhotla S, Guthrie P, Chun M, Auster ME, Nabzdyk C, Deso S, Andersen N, Gnardellis C, LoGerfo FW, Veves A (2013) Expression of neuropeptides and cytokines in a rabbit model of diabetic neuroischemic wound healing. Journal of Vascular Surgery.

Nisbet L (2012) Wound dressing materials. US Patent 20120135062 A1.

Nussinovitch A, Ben-Zion OM (2011) Depolymerized polysaccharide-based hydrogel adhesive and methods of use therefore. US Patent 20110190401 A1.

O´Donnell TF, Lau J (2006) A systematic review of randomized controlled trials of wound dressings for chronic venous ulcer. J Vasc Surg 44:1118-1125.

O'Loughlin A, O'Brien T (2011) Topical Stem and Progenitor Cell Therapy for Diabetic Foot Ulcers. Stem Cells in Clinic and Research 978:579-604.

Oyibo SO, Jude EB, Tarawneh I, Nguyen HC, Harkless LB, Boulton AJM (2001) A Comparison of Two Diabetic Foot Ulcer Classification Systems The Wagner and the University of Texas wound classification systems. Diabetes care 24:84-88

Papini R (2004) Management of burn injuries of various depths. BMJ 329:158-160.

Parenteau-Bareil R, Gauvin R, Berthod F (2010) Collagen-Based Biomaterials for Tissue Engineering Applications. Materials 3:1863-1887.

Pereira RF, Barrias CC, Granja PL, Bartolo PJ (2013) Advanced biofabrication strategies for skin regeneration and repair. Nanomedicine (Lond) 8:603-621.

Pérez RA, Won J, Knowles JC, Kim H (2012) Naturally and synthetic smart composite biomaterials for tissue regeneration. Adv Drug Deliv Rev.

Ponrasu T, Suguna L (2012) Efficacy of Annona squamosa on wound healing in streptozotocin-induced diabetic rats. Int Wound J.

Pradhan L, Cai X, Wu S, Andersen ND, Martin M, Malek J, Guthrie P, Veves A, Logerfo FW (2011) Gene expression of pro-inflammatory cytokines and neuropeptides in diabetic wound healing. J Surg Res 167:336-342.

Pradhan l, Nabzdyk C, Andersen N, LoGerfo F, Veves A (2009a) Inflammation and neuropeptides: the connection in diabetic wound healing. Expert Reviews in Molecular Medicine 11:1-24.

Pradhan L, Nabzdyk C, Andersen ND, LoGerfo FW, Veves A (2009b) Inflammation and neuropeptides: the connection in diabetic wound healing. Expert Rev Mol Med 11:e2.

Rathur HM, Bloulton AJM (2005) Recent advances in the diagnosis and management of diabetic neuropathy. The Journal of Bone and Joint Surgery 87:1605-1610.

Rinaudo M (2006) Chitin and chitosan: properties and applications. Progress in Polymer Science 31:603-632.

Ritz E (1999) Nephropathy in type 2 diabetes. Journal of Internal Medicine 245:111-126.

Rodero MP, Khosrotehrani K (2010) Skin wound healing modulation by macrophages. Int J Cli Exp Pathol 3:643-653.

Roosterman D, Goerge T, Schneider SW, Bunnett NW, Steinhoff M (2006) Neuronal Control of Skin Function:The Skin as a Neuroimmunoendocrine Organ. Physiol Rev 86:1309–1379.

Rossi S, Marciello M, Sandri G, Ferrari F, Bonferoni MC, Papetti A, Caramella C, Dacarro C, Grisoli P (2007) Wound Dressings Based on Chitosans and Hyaluronic Acid for the Release of Chlorhexidine Diacetate in Skin Ulcer Therapy. Pharmaceutical Development and Technology 12:415-422.

Sachs DL, Voorhees JJ (2011) Age-reversing drugs and devices in dermatology. Clinical pharmacology and therapeutics 89:34-43.

Santos KSCR, Silva HSRC, Ferreira EI, Bruns RE (2005) 32 Factorial design and response surfasse analysis optimization of N-carboxybutylchitosan synthesis. Carbohydr Polym 59:37-42.

Saranya N, Moorthi A, Saravanan S, Devi MP, Selvamurugan N (2011) Chitosan and its derivatives for gene delivery. International Journal of Biological Macromolecules 48:234-238.

Schaffer M, Beiter T, Becker HD, Hunt TK (1998) Neuropeptides: mediators of inflammation and tissue repair? Arch Surg 133:1107-1116.

Schreml S, Szeimies R, Prantl L, Landthaler M, Babilas P (2010) Wound healing in 21st century. Journal of the American Academy of Dermatology 63:866-881.

Scott JR, Tamura RN, Muangman P, Isik FF, Xie C, Gibran NS (2008) Topical substance P increases inflammatory cell density in genetically diabetic murine wounds. Wound Repair and Regeneration 16:529-533

Seetharaman S, Shanmugasundaram N, Stowers RS, Mullens C, Baer DG, Suggs LJ, Christy RJ (2011) A PEGylated fibrin-based wound dressing with antimicrobial and angiogenic activity. Acta biomaterialia 7:2787-2796.

Seino Y, Nanjo K, Tajima N, Kadowaki T, Kashiwagi A, Araki E, Ito C, Inagaki N, Iwamoto Y, Kasuga M, Hanafusa T, Haneda M, Ueki K (2010) Report of the Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus. Journal of Diabetes Investigation 1:212-228.

Sell SA, Wolfe PS, Garg K, McCool JM, Rodriguez IA, Bowlin GL (2010) The use of natural polymers in tissue engineering: a focus on electrospun extracellular matrix analogues. Polymers for Advanced Technologies 2:522-553.

Shaw JE, Sicree RA, Zimmet PZ (2010a) Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Research and Clinical Pratice 87:4-14.

Shaw JE, Sicree RA, Zimmet PZ (2010b) Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes research and clinical practice 87:4-14.

Shaw TJ, Martin P (2009) Wound repair at a glance. Journal of cell science 122:3209-3213.

Shevchenko RV, James SL, James SE (2010) A review of tissue-engineered skin bioconstructs available for skin reconstruction. Journal of the Royal Society, Interface / the Royal Society 7:229-258.

Sidhu GS, Mani H, Gaddipati JP, Singh AK, Seth P, Banaudha KK, Patnaik GK, Maheshwari RK (1999) Curcumin enhances wound healing in streptozotocin induced diabetic rats and genetically diabetic mice. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 7:362-374.

Silva L, Carvalho E, Cruz MT (2010) Role of neuropeptides in skin inflammation and its involvement in diabetic wound healing. Expert Opin Biol Ther 10:1427-1439.

Singer AJ, Clark RA (1999) Cutaneous wound healing. N Engl J Med 341:738-746.

Singh O, Gupta SS, Soni M, Moses S, Shukla S, Mathur RK (2011) Collagen Dressing Versus Conventional Dressings in Burn and Chronic Wounds: A Retrospective Study. Journal of Cutaneous and Aesthetic Surgery 4:12-16.

Sionkowska A (2011) Current research on the blends of natural and synthetic polymers as new biomaterials: Review. Progress in Polymer Science 36:1254-1276.

Skorkowska-Telichowska K, Czemplik M, Kulma A, Szopa J (2011) The local treatment and available dressings for chronic wounds. Journal of American Academy of Dermatology 5:1-11.

Slaughter BV, Shahana SK, Fisher OZ, Khademhosseini A, Peppas NA (2009) Hydrogels in Regenerative Medicine. Advanced Materials 21:3307–3329.

Slominski A, Wortsman J (2000) Neuroendocrinology of the skin. Endocrine reviews 21:457-487.

Snyder BJ, Waldman BJ (2009) Venous thromboembolism prophylaxis and wond healing in patients undergoing major orthopedic surgery. Advances in skin and wound care 22:311-315.

Srinivasan A, Sehgal PK (2010) Characterization of Biocompatible Collagen Fibers—A Promising Candidate for Cardiac Patch. Tissue Engineering Part C: Methods 16:895-903.

St-Gelais F, Jomphe C, Trudeau LE (2006) The role of neurotensin in central nervous system pathophysiology: what is the evidence? Journal of psychiatry & neuroscience : JPN 31:229-245.

Suganya S, Ram TS, Lakshmi BS, Giridev VR (2011) Herbal drug incorporated antibacterial nanofibrous mat fabricated by electrospinning: An excellent matrix for wound dressings. Journal of Applied Polymer Science 121:2893-2899.

Tabata Y (2009) Biomaterial technology for tissue engineering applications. Journal of the Royal Society Interface S311-S324.

Tajima M, Izume M, Fukuhara T, Kimura T, Kuroyanagi Y (2000) Development of new wound dressing composed of N-succinyl chitosan and gelatin. Journal of Japanese Society for Biomaterials 18:220-228.

Takei T, Nakahara H, Ijima H, Kawakami K (2012) Synthesis of a chitosan derivative soluble at neutral pH and gellable by freeze-thawing, and its application in wound care. Acta biomaterialia 8:686-693.

Tan Y, Han F, Ma S, Yu W (2011) Carboxymethyl chitosan prevents formation of broad-spectrum biofilm. Carbohydr Polym 84:1365-1370.

Tellechea A, Leal E, Veves A, Carvalho E (2009) Inflammatory and angiogenic abnormalities in diabetic wound healing: role of neuropeptides and therapeutic perspectives. The Open Circulation and Vascular Journal 3:43-55.

Tessmar JK, Gopferich AM (2007) Matrices and scaffolds for protein delivery in tissue engineering. Adv Drug Deliv Rev 58:274-291.

Tsirogianni AK, Moutsopoulos NM, Moutsopoulos HM (2006) Wound healing: Immunological aspects. Injury 37:S5-S22.

Uchi H, Igarashi A, Urabek, Koga T, Nakayama J, Kawamori R, Tamaki K, Hirakata H, Ohura T, Furue M (2009) Clinical efficacy of basic fibroblast growth factor (bFGF) for diabetic ulcer. European journal of dermatology : EJD 19:461-468.

Valenta C, Auner BG (2004) The use of polymers for dermal and transdermal delivery. European Journal of Pharmaceutics and Biopharmaceutics 58:279-289.

Vanichvattanadecha C, Supaphol P, Nagasawa N, Tamada M, Tokura S, Furuike T, Tamura H, Rujiravanit R (2010) Effect of gamma radiation on dilute aqueous solutions and thin films of N-succinyl chitosan. Polymer Degradation and Stability 95:244-254.

Vehik K, Dabelea D (2011) The changing epidemiology of type 1 diabetes: why is it going through the roof? Diabetes Metab Res Rev 27:3-13.

Vincent JP, Mazella J, Kitabgi P (1999) Neurotensin and neurotensin receptors. Trends Pharmacol Sci 20:302-309.

Vinik A, Ullal J, Parson HK, Casellini CM (2006) Diabetic neuropathies: clinical manifestions and current treatment options. Nature Reviews Endocrinology 2:269-281.

Vinik AI, Holland MT, Le Beau JM, Liuzzi FJ, Stansberry KB, Colen LB (1992) Diabetic neuropathies. Diabetes care 15:1926-1975.

Vinik AI, Vinik E (2003) Prevention of the Complications of Diabetes. The American Journal of Managed Care 9:S63-S80.

Wang J, Luben R, Khaw KT, Bingham S, Wareham NJ, Forouhi NG (2008) Dietary energy density predicts the risk of incident type 2 diabetes: the European Prospective Investigation of Cancer (EPIC)-Norfolk Study. Diabetes care 31:2120-2125.

Weller C, Sussman G (2006) Wound Dressings Update. Journal of Pharmacy Practice Research 36:318-324.

Weng L, Romanov A, Rooney J, Chen W (2008) Non-cytotoxic, in siu gelable hydrogels composed of N-carboxyethyl chitosan and oxidized dextran. Biomaterials 29:3905-3913.

Whiting DR, Guariguata L, Weil C, Shaw J (2011) IDF Diabetes Atlas: Global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes research and clinical practice 84:311-321.

Wilgus TA (2008) Immune cells in the healing skin wound: Influential players at each stage of repair. Pharmacological Research 58:112-116.

Wittaya-areekul S, Prahsarn C (2006) Development and in vitro evaluation of Chitosan-polysaccharides composite wound dressings. International Journal of Pharmaceutics 313:123-128.

Yang Y, Xia T, Zhi W, Wei L, Weng J, Zhang C, Li X (2011) Promotion of skin regeneration in diabetic rats by electrospun core-sheath fibers loaded with basic fibroblast growth factor. Biomaterials 32:4243-4254.

Yildirimer L, Thanh NT, Seifalian AM (2012) Skin regeneration scaffolds: a multimodal bottom-up approach. Trends in biotechnology 30:638-648.

Yin L, Fei L, Cui F, Tang C, Yin C (2007) Superporous hydrogels containing poly(acrylic acid-co-acrylamide)/o-carboxymethyl chitosan interpenetrating polymer networks. Biomaterials 28:1258-1266.

Zahedi P, Rezaeian I, Ranaei-Siadat S, Jafari S, Supaphol P (2010) A review on wound dressings with an emphasis on electrospun nanofibrous polymeric bandages. Polymers Advanced Technologies 21:77-95.

Zeugolis DI, Paul GR, Attenburrow G (2009) Cross-linking of extruded collagen fibers—A biomimetic three-dimensional scaffold for tissue engineering applications. Journal of Biomedical Materials Research Part A 89A:895-908.

Zhang C, Ping Q, Zhang H, Shen J (2003) Synthesis and characterization of water-soluble O-succinyl-chitosan. European Polymer Journal 39:1629-1634.

Zhao D, Zhan Y, Zeng H, Koon HW, Pothoulakis C (2005) Neurotensin stimulates interleukin-8 expression through modulation of IκBα phosphorylation and p65 transcriptional activity: involvement of protein kinase Cα Molecular Pharmacology 67:2025-2031.

Zhong SP, Zhang YZ, Lim CT (2010) Tissue scaffolds for skin wound healing and dermal reconstruction. Nanomedicine and Nanobiotechnologies 2:510-525.

Zoher K, Kolli ME, Riahi F, Doufnoune R (2009) Preparation and characterization of hydrocolloid biopolymer-based films for dressings application. International Journal of Polymeric Materials 58:665-680.

Chapter 2

In vitro study

Neurotensin modulates the migratory and inflammatory response of macrophages under hyperglycemic conditions

This Chapter comprises the work accepted in

BioMed Research International (2013) by

Moura, L. I. F., Silva, L., Leal, E.C., Tellechea, A., Cruz, M. T., Carvalho, E.

2.1 Abstract

Diabetic foot ulcers (DFU) are characterized by an unsatisfactory inflammatory and migratory response. Skin inflammation involves the participation of many cells and particularly macrophages. Macrophage function can be modulated by neuropeptides, however, little is known regarding the role of neurotensin (NT) as a modulator of macrophages under inflammatory and hyperglycemic conditions. Raw 264.7 cells were maintained at 10/30mM glucose, stimulated with/without LPS (1μg/ml) and treated with/without NT (10nM). The results show that NT did not affect macrophage viability. However, NT reverted the hyperglycemia-induced impair in the migration of macrophages. The expression of IL-6 and IL-1β was significantly increased under 10mM glucose in the presence of NT, while IL-1β and IL-12 expression significantly decreased under inflammatory and hyperglycemic conditions. More importantly, high glucose modulates NT and NT receptor expression, under normal and inflammatory conditions.

These results highlight the effect of NT on cell migration, which is strongly impaired under hyperglycemic conditions, as well as its effect in decreasing the pro-inflammatory status of macrophages under hyperglycemic and inflammatory conditions. These findings provide new insights into the potential therapeutic role of NT in chronic wounds, such as in DFU, characterized by a deficit in the migratory properties of cells and a chronic pro-inflammatory status.

Keywords: wound healing, inflammation, macrophages, neuropeptides, neurotensin, hyperglycemia

2.2 Introduction

Diabetes mellitus is characterized by an impaired blood glucose homeostasis and it affects millions of people in the world (Shaw *et al.*, 2010). Diabetes can cause poor circulation in the extremities, particularly in people with neuropathy and long-term diabetes can compromise the immune system increasing the incidence of infections in the patients. One of the most debilitating and costly

complications of diabetes is the development of chronic foot ulcers. This disease affects approximately 15% of the diabetic population (Brem and Tomic-Canic, 2007, Lan *et al.*, 2008, Tellechea *et al.*, 2010). It can diminish physical activity and in extreme cases, diabetic foot ulcerations (DFU) can lead to lower-limb amputations (Pradhan *et al.*, 2009). Chronic inflammation is a major characteristic of diabetic cutaneous wounds. Wound inflammation has a fundamental role in tissue regeneration (Kampfer *et al.*, 2005) while leukocyte dysfunction to the wound site has been shown to contribute to the development of non-healing wounds (Koh and DiPietro, 2011). Indeed, diabetic patients show impaired leukocyte function which has been correlated with hyperglycemia (Bagdade *et al.*, 1974). Studies performed in diabetic patients revealed that normalization of blood glucose levels through insulin administration can improve and ultimately restore the functional activity of neutrophils (Alba-Loureiro, 2007).

It is also well known that an imbalance between pro-inflammatory and anti-inflammatory cytokines in the diabetic wound tissue compromises the time resolution of inflammation and consequently the healing process (Khanna *et al.*, 2010). Macrophages play a crucial role in the modulation of the inflammatory response since they can be phenotypically polarized to the classical activated macrophages, that stimulate the inflammatory process, or to the alternatively activated macrophages that play role in resolution of inflammation (Martinez *et al.*, 2008). Recent results demonstrated that in a diabetic mouse model, impairment in glucose metabolism can cause changes in the macrophage response to lipopolysaccharide (LPS), namely increased secretion of interleukin 12 (IL-12) and TNF-α (de Souza *et al.*, 2008).

In addition to the involvement of inflammation in wound repair responses, various studies suggest that the neuroendocrine system also modulates wound healing (Elenkov, 2008), specifically through neuropeptides, such as substance P (SP) and neurotensin (NT) (Brun *et al.*, 2005, Delgado *et al.*, 2005, Toda *et al.*, 2008, Pradhan *et al.*, 2011, Jiang *et al.*, 2012). NT is a bioactive tridecapeptide that is widely distributed through the brain and the gastrointestinal tract (Lazarus *et al.*, 1977, Brun *et al.*, 2005). It regulates a wide range of biological functions, such as the gastric system and inflammatory processes in the lung (Brun *et al.*, 2005, Jiang *et al.*, 2012). Furthermore, NT modulates the immune response, as it interacts with leukocytes, peritoneal mast cells and

dendritic cells, stimulating cytokine release and chemotaxis (Zhao *et al.*, 2005, Kim *et al.*, 2006, da Silva *et al.*, 2011). In particular, neuropeptides such as NT are important in modulating macrophage function, due to its direct interaction with macrophages that leads to suppression of the production of pro-inflammatory cytokines and iNOS expression, showing a protective effect in inflammatory conditions (Hartung, 1988, Ganea and Delgado, 2001).

NT mediates its functions through its two G-protein coupled receptors: neurotensin receptor 1 (NTR1) and neurotensin receptor 2 (NTR2) (high and low affinity receptors, respectively). A third receptor, the neurotensin receptor 3 (NTR3), is an intracellular, non G-protein coupled receptor (Vincent *et al.*, 1999, Pradhan *et al.*, 2009). Although NT has been implicated in modulating immune responses and macrophage function, its molecular mechanisms of action, under either hyperglycemic or inflammatory conditions or both, remain unclear.

Therefore, this study aims to determine the effect of NT in macrophages function under hyperglycemic and inflammatory conditions.

2.3 Materials and methods

2.3.1 Materials

LPS from Escherichia coli (serotype 026:B6) was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and NT was obtained from Bachem (Weil am Rhein, Germany). Fetal calf serum was purchased from Invitrogen (Paisley, UK). The protease and phosphatase inhibitor cocktails were obtained from Roche (Mannheim, Germany).

The antibodies against phospho (p), p-p44/42MAPK, p-p38 MAPK, IkBα and total AKT were purchased from Cell Signaling Technologies (Danvers, MA, USA). The antibodies against pAKT (Ser 473) and the NT receptors were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA) and the antibodies against total p38 MAPK and p44/42MAPK were purchased from Biolegend (San Diego, CA, USA). The antibody against actin was purchased from Millipore Corporation (Bedford, MA).

All primers were obtained from IDT (Ebersberg, Germany). SYBR green was obtained from BioRAD (Hercules, CA, USA) and High Capacity cDNA Reverse Transcription kit was obtained from Applied Biosystems (Carlsbad, CA, USA).

The alkaline phosphatase-linked secondary antibodies and the enhanced chemifluorescence (ECF) reagent were obtained from GE Healthcare (Chalfont St. Giles, UK), and the polyvinylidene difluoride (PVDF) membranes were from Millipore Corporation (Bedford, MA). TRIzol® reagent was purchased from Invitrogen (Barcelona, Spain). All other reagents were from Sigma Chemical Co. (St. Louis, Mo, USA) or from Merck (Darmstadt, Germany).

2.3.2 Macrophage cell culture

Raw 264.7 (mouse leukaemic monocyte macrophage cell line) cells were cultured in DMEM medium, pH 7.4, supplemented with 10% heat inactivated fetal bovine serum (FBS), 3.02 g/l sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, at 37ºC in a humidified incubator containing 5% CO_2. Sub-culturing was performed according to ATCC recommendations. The Raw 264.7 cell line was purchased from ATCC (number TIB-71).

2.3.3Treatments of macrophages

The cells were incubated in 10mM (normal glucose) or 30mM (high glucose) D-glucose, for 15 days, before the beginning of the experiments.

For the viability assay, we used the cells incubated with high glucose for 15 days. Macrophages were treated with 1 µg/ml of LPS or with 10, 50 or 100 nM of NT alone or a combination of both treatments (NT plus LPS) for 1, 3, 5 and 7 days. These dose and treatment protocol were selected to evaluate the role of NT in macrophage cell viability after exposure to an acute (1 and 3 days) and a chronic (5 and 7 days) hyperglycemic and inflammatory state

For the migration assay, Raw 264.7 cells were incubated with 1 µg/ml of LPS alone or with 10 nM of NT alone or a combination of both treatments (10 nM NT plus1 µg/ml LPS) in DMEM medium containing 2 % of inactivated fetal calf serum.

In order to study the signal transduction pathways, macrophages were incubated with 1 µg/ml of LPS alone or with 10 nM of NT alone or a combination of both treatments (10 nM NT plus1 µg/ml LPS) for 5, 15, 30, 60 min. The cells were incubated with the same treatments for 24h to evaluate the levels of NT receptors and for 6h in the real-time PCR studies.

2.3.4 MTT viability assay

Raw 264.7 (8×10^4 cells/well) cells were plated in 48-well plates in 430 µL of DMEM. After cell treatment, as described previously, 43µl of MTT solution (5 mg/ml) was added to each well. The plates were further incubated at 37ºC for 1h, in a humidified incubator containing 5 % CO_2. 300 µl of acidic isopropanol (0.04 N HCl in isopropanol) were then added to each well and mixed in order to dissolve the dark blue crystals of formazan. Acidic isopropanol was collected to an ELISA microplate and formazan quantification was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm.

2.3.5 *In vitro* scratch migration assay

Raw 264.7 ($4x10^5$ cells/well) cells were ressuspended in 3 mL of DMEM medium in µ-Dish$^{35mm,\ high}$ (Ibidi). After 24h, a ‘‘scratch’’ was made, with a pipette tip, in the cell monolayer in a straight line to create an area without cells. The medium was removed and cells were washed two times with PBS. DMEM medium containing 2 % of inactivated fetal calf serum was then added to the cells to diminish cell proliferation. The cells were incubated as described above and allowed to migrate for 24 h. Photographs were captured with a coupled AxioCamMR3 camera with PALM reflector and 5X objective, using an inverted Axiovert 200. A specific numbered/lettered area was chased to permit later recognition of the photographed area. After the incubation period, photographs were taken in the same area where the first photograph was taken. Photographs were analysed and the number of cells in the scratch area was counted. For the analysis, the number of cells in the zero point was taken into account.

2.3.6 Western blotting

Raw 264.7 ($1.5x10^6$ cells/well) cells were seeded in 6-well plates and treated as described before. Cells were then washed twice with ice-cold PBS buffer and lysed with RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1 % NP-40 (Nonidet P-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 bicinchoninic acid method and cell lysates were denatured at 95ºC, for 5 min, in sample buffer (0.125 mM Tris pH 6.8; 2% w/v SDS; 100 mM DTT; 10% glycerol and bromophenol blue) for its use in western blot analysis. 30µg of total protein were resolved on 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% (w/v) 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 and washing, membranes were incubated overnight at 4ºC with the primary antibodies against the different proteins of interest: p-p38 MAPK (1:1000), IκB-α (1:1000); p- p44/42MAPK (1:1000), p-AKT (1:500), NTR1 (1:500), NTR2 (1:500) or NTR3 (1:500). After incubation, membranes were washed and incubated for 1 h at room temperature, with alkaline phosphatase-conjugated anti-rabbit antibody (1:5000), or alkaline phosphatase-conjugated anti-mouse antibody (1:5000). The membranes were exposed to ECF reagent followed by scanning for blue excited fluorescence on the VersaDoc (Bio-Rad Laboratories, Amadora, Portugal). To test whether similar amounts of protein for each sample were loaded, the membranes were stripped and reprobed with antibodies for total, p38 MAPK, p44/42MAPK and AKT or with an anti-actin antibody, and blots were developed with alkaline phosphatase-conjugated secondary antibodies and visualized by enhanced chemifluorescence. The generated signals were analyzed using the Image-Quant TL software.

2.3.7 Real time RT-PCR

Cells (2×10^6 cells/well) were seeded in 6-well plates and treated as described before. Total RNA was isolated from cells with the TRIzol reagent according to the manufacturer's instructions and concentration was determined by OD260 measurement using the NanoDrop spectrophotometer

(Thermo Scientific, USA). First strand cDNA was synthesized using High Capacity cDNA Reverse Transcription. Briefly, 2µl of 10X RT Buffer, 0.8 µl of 25X dNTP Mix, 2 µl of 10X RT random primers, 1 µl of MultiscribeTM Reverse Transcriptase and 4.2 µl of nuclease free H_20 were added to 10 µl of RNA (1 µg) sample. Then, real-time RT-PCR was performed in a Bio-Rad My Cycler iQ5. For each reaction 10 µl volume was used containing 2.5 µl cDNA, 5 µl 2X Syber Green Supermix, 1 µl of each primer (250 nM) and 0.5 µl of H_2O PCR grade. Primer sequences are given in Table 2.1. Gene expression changes were analyzed using iQ5Optical system software v2. The software enables analysis of the results with the Pfaffl method (Pfaffl, 2001). The results were normalized using a reference gene, hypoxanthine phosphoribosyltransferase 1 (HPRT-1) that was selected based on our previous results demonstrating that it does not changed under these conditions.

Table 2.1: Forward and reverse primers sequences used in RT-PCR.

Primer	5'-3'Sequence (Forward; Reverse)
NT	For: AATGTTTGCAGCCTCATAAATAAC Rev: TGCCAACAAGGTCGTCATC
NTR1	For: GGCAATTCCTCAGAATCCATCC Rev: ATACAGCGGTCACCAGCAC
NTR2	For: GCCATTACTAACAGTCTAAGC Rev: GCAATTCGTCCTATTCTACAC
NTR3	For: ATGGCACAACTTCCTTCTG Rev: AGAGACTTGGAGTAGACAATG
IL-6	For: TTCCATCCAGTTGCCTTC Rev: TTCTCATTTCCACGATTTCC
TNF-α	For: CAAGGGACTAGCCAGGAG Rev: TGCCTCTTCTGCCAGTTC
Il-10	For: CCCTTTGCTATGGTGTCCTTTC Rev: ATCTCCCTGGTTTCTCTTCCC
IL-1β	For: ACCTGTCCTGTGTAATGAAAG Rev: GCTTGTGCTCTGCTTGTG
IL-12	For:CAGAAGCTAACCATCTCCTGGTTTG Rev:TCGGGAGTAATTTGGTGCTTCACAC
HPRT1	For: GTTGAAGATATAATTGACACTG Rev: GGCATATCCAACAACAAAC

2.3.8 Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA

followed by Tukey's multiple comparison tests or through the unpaired t student´s test using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). p values less than 0.05 were considered statistically significant.

2.4 Results

All experiments were performed using Raw 267.4 cells incubated with either 10mM glucose (normal glucose) or 30mM glucose (high glucose), for a period of 15 days.

2.4.1 Cell viability under hyperglycemic conditions

NT treatment did not change significantly the viability of macrophages under hyperglycemic conditions either in the absence or presence of LPS (Figure 2.1 A and B, respectively). Since no major differences were observed between the different doses of NT used (10, 50 or 100nM), the following experiments were performed using 10nM of NT.

2.4.2 Migration of macrophages, under normal or hyperglycemic conditions

Our results show that, under normoglycemic conditions (10mM glucose), NT treatment did not stimulate macrophage migration, either in the absence or in the presence of LPS (Figures 2.1 and 2.3). However, under hyperglycemic conditions (30mM glucose), NT significantly increase cell migration compared to control ($p<0.05$) as shown in figure 2.2 and 2.3. Moreover, high glucose alone ($p<0.01$) or in combination with LPS treatment ($p<0.05$) significantly decreased macrophage migration when compared with normoglycemic conditions (Figure 2.2 and 2.3).

These results demonstrated that macrophage migration is impaired under hyperglycemic conditions. Moreover, this impairment is partially reverted by NT treatment.

2.4.3 Inflammatory cytokine expression under normal and hyperglycemic conditions

In order to address the pattern of cytokine gene expression that is involved in wound healing

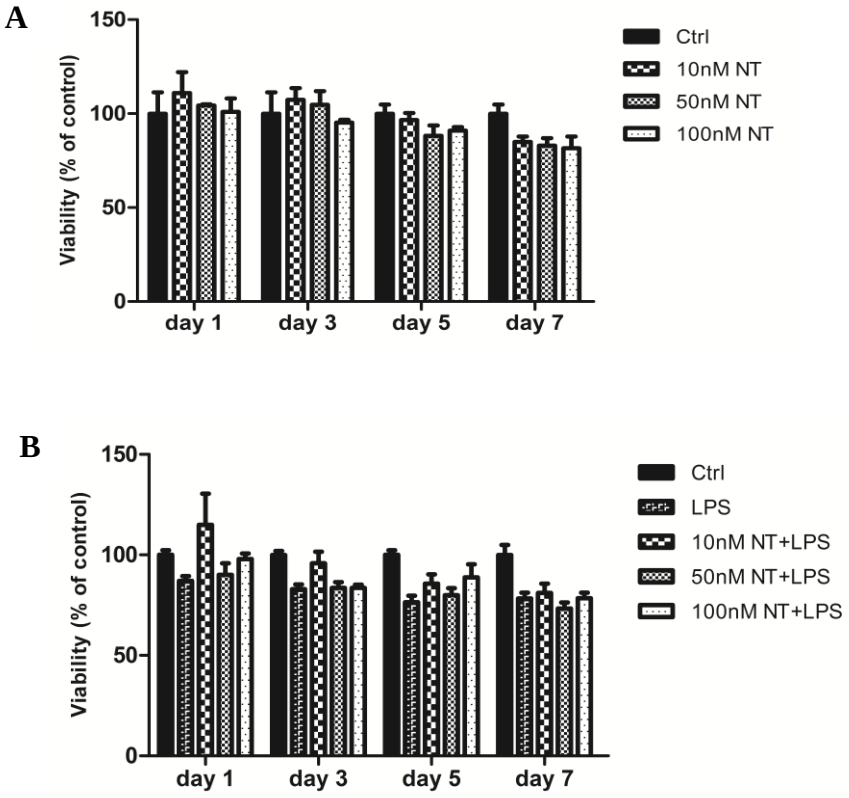

Figure 2.1: Viability of macrophages, under either 10 or 30mM glucose, by MTT assay. Raw 264.7 cells were plated at $8x10^4$ /well and were treated with 10, 50 or 100 nM of NT (Figure 2.1 A) or in combination with 1 µg/ml of LPS (Figure 2.1 B) for 7days. After, 1, 3, 5 or 7 days of incubation, MTT assay was performed as described in "Materials and Methods". Absorbance quantification was performed using a microplate reader at 570 nm, with a reference wavelength of 620 nm. Results are presented as mean ± SEM of three independent experiments.

processes, stimulated by NT alone or in the presence of LPS, we measured gene expression for the pro-inflammatory cytokines IL-6, TNF-α, IL-1β and IL-12 and for the anti-inflammatory cytokine IL-10, in macrophages, as indicated in Figure 2.3.

Under 10mM glucose, NT induced a significant over expression of IL-6 ($p<0.05$) and IL-1β ($p<0.05$). On the other hand, under 30mM glucose, NT significantly increased the expression of TNF-α ($p<0.05$) and IL-1β ($p<0.05$), as compared to high glucose alone (Figure 2.3).

Moreover, in LPS-treated cells, NT treatment significantly increased TNF-α ($p<0.05$) and IL-12 ($p<0.05$) expression, under 10mM glucose, when compared with LPS-treated cells (Figure 2.3). However, NT did not alter the expression of these genes under hyperglycemic conditions.

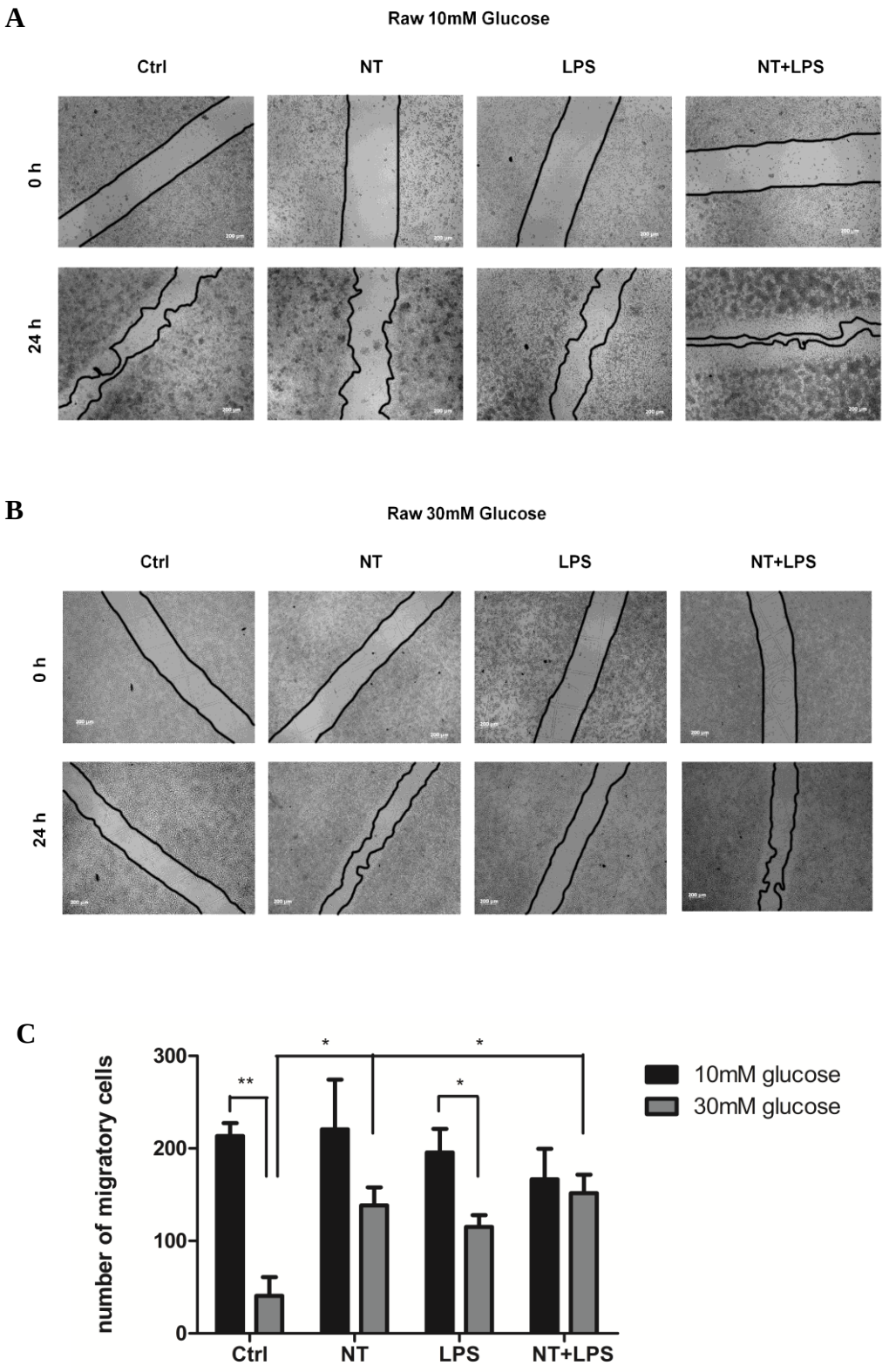

Figure 2.2: Migration of macrophages, at 10 (Figure 2.2 A) and 30mM (Figure 2.2 B) glucose, by *in vitro* scratch assay. Figure 2.2 C: Number of migrating cells (referred before). Cells were plated at $4x10^5$ / well and treated with 10nM NT or 1µM/ml LPS or both, during 24h. The images were acquired by transmission microscopy and photographs were taken before cell treatment (0 h) and 24 h after treatments. Magnification used 40x. Results are presented as mean ± SEM of three independent experiments. *$p<0.05$; **$p<0.01$

Interestingly, hyperglycemia alone increased the expression of IL-6 ($p<0.05$) and decreased the expression of IL-1β ($p<0.05$), when compared to normal glycemia under inflammatory conditions (Figure 2.3). Overall, these results show that NT modulates the inflammatory profile of macrophages, however, this effect was not observed under hyperglycemic conditions, as observed in Figure 2.3.

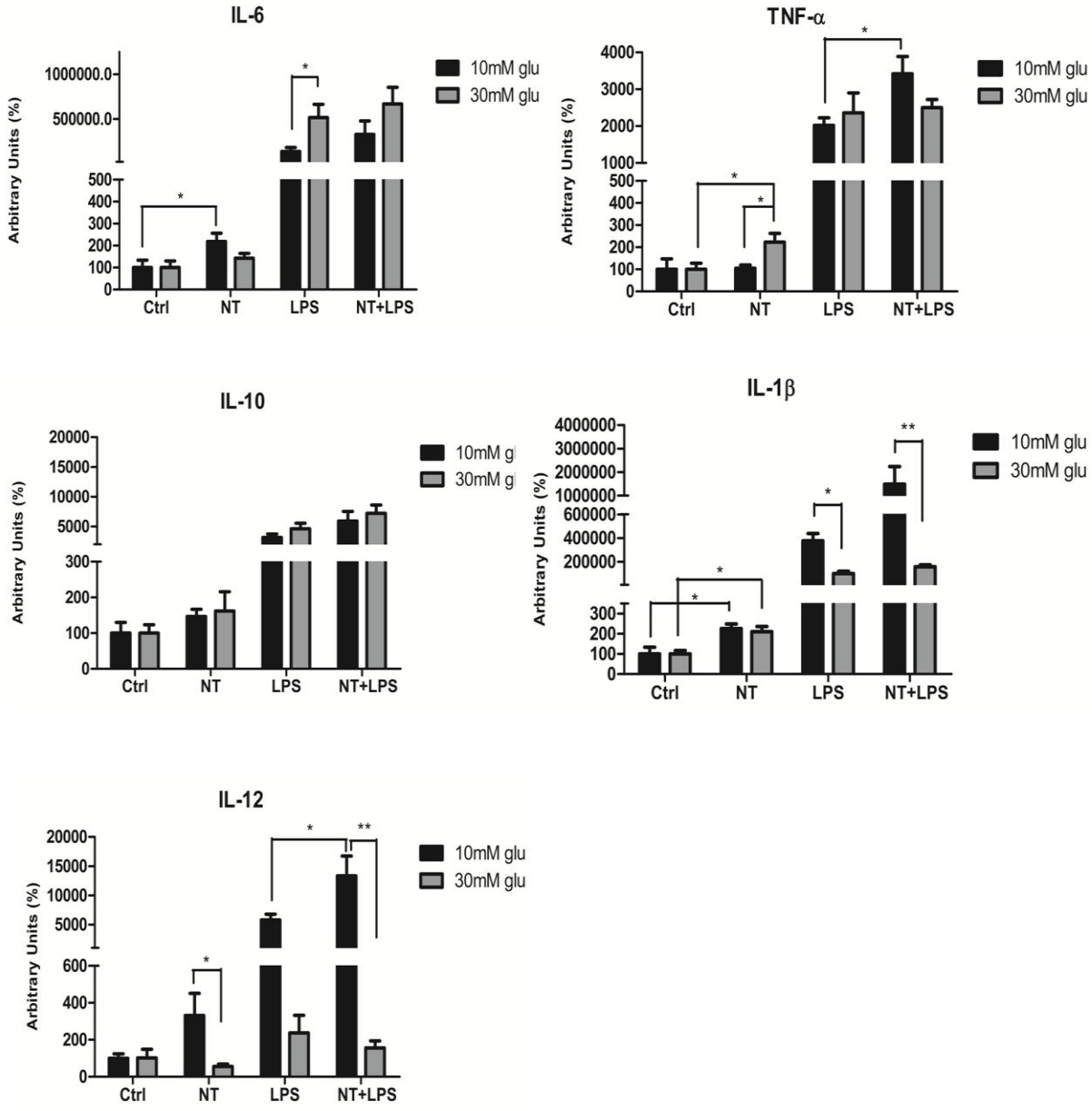

Figure 2.3: Expression of inflammatory cytokines IL-6, TNF-α, IL-10, IL-1β and IL-12 in macrophages, at 10 and 30mM glucose, by real-time PCR. Cells were plated at $2x10^6$ /well and treated with 10nM NT or 1 μg/ml LPS or both, during 6h. Total RNA was isolated as described in "Materials and Methods". The relative gene expression is indicated as arbitrary units and was obtained after normalization with the HPRT gene. Results are presented as mean ± SEM of six to ten independent experiments. *$p<0.05$; **$p<0.01$

2.4.4 Modulation of intracellular signaling pathways by NT, in LPS treated macrophages, under either 10 or 30 mM glucose

The expression of pro-inflammatory molecules is tightly regulated by several transcription factors and signaling pathways. Among these pathways, mitogen-activated proteins kinases (MAPKs) and the transcription factor NF-kB constitute signaling molecules that play critical roles in the orchestration of an inflammatory response. The effect of NT on LPS-induced molecular pathway activation, under either 10 or 30mM glucose, was assessed by measuring the levels of the phosphorylated forms of MAP kinases (p38 MAPK, p44/42 MAPK, SAPK/JNK), and PKB/AKT, by Western blot. The importance of the transcription factor NF-κB was also evaluated by determination of the protein levels of its inhibitory protein, IkB-α, as shown in Figures 2.4 A and 2.4 B. No significant differences were observed after NT treatment in the presence of LPS, in the presence of either 10 or 30 mM glucose, as compared to cells treated with LPS alone.

2.4.5 Modulation of NT receptors under normal and hyperglycemic conditions

Gene expression results showed that under hyperglycemic conditions, NTR1 was significantly decreased ($p<0.001$), while both NTR2 and endogenous NT were not changed, compared to normal glycemia in these cells. Interestingly, the expression of NTR3 was significantly increased under hyperglycemic conditions ($p<0.001$) when compared to normal glycemia. In addition, the NTR3 was the most expressed receptor in macrophages under either 10 or 30 mM glucose, as shown in Figure 2.5 A. Furthermore, we also evaluated how NT, LPS or the co-treatment of macrophages with both agents affect the expression of endogenous NT and its receptors (Figure 2.5 B). The endogenous NT gene expression is significantly increased under NT-treated cells at 10mM glucose ($p<0.05$), when compared to non-treated cells. This effect does not occur when the cells were incubated under hyperglycemia. In addition, NT-treated cells significantly increased ($p<0.01$) NTR1 expression under 10mM glucose, whereas in cells maintained under 30mM glucose, NTR1 expression was significantly decreased ($p<0.05$). Furthermore, in LPS-treated cells, NTR1

expression was significantly increased when compared to untreated cells, both under 10 and 30mM of glucose.

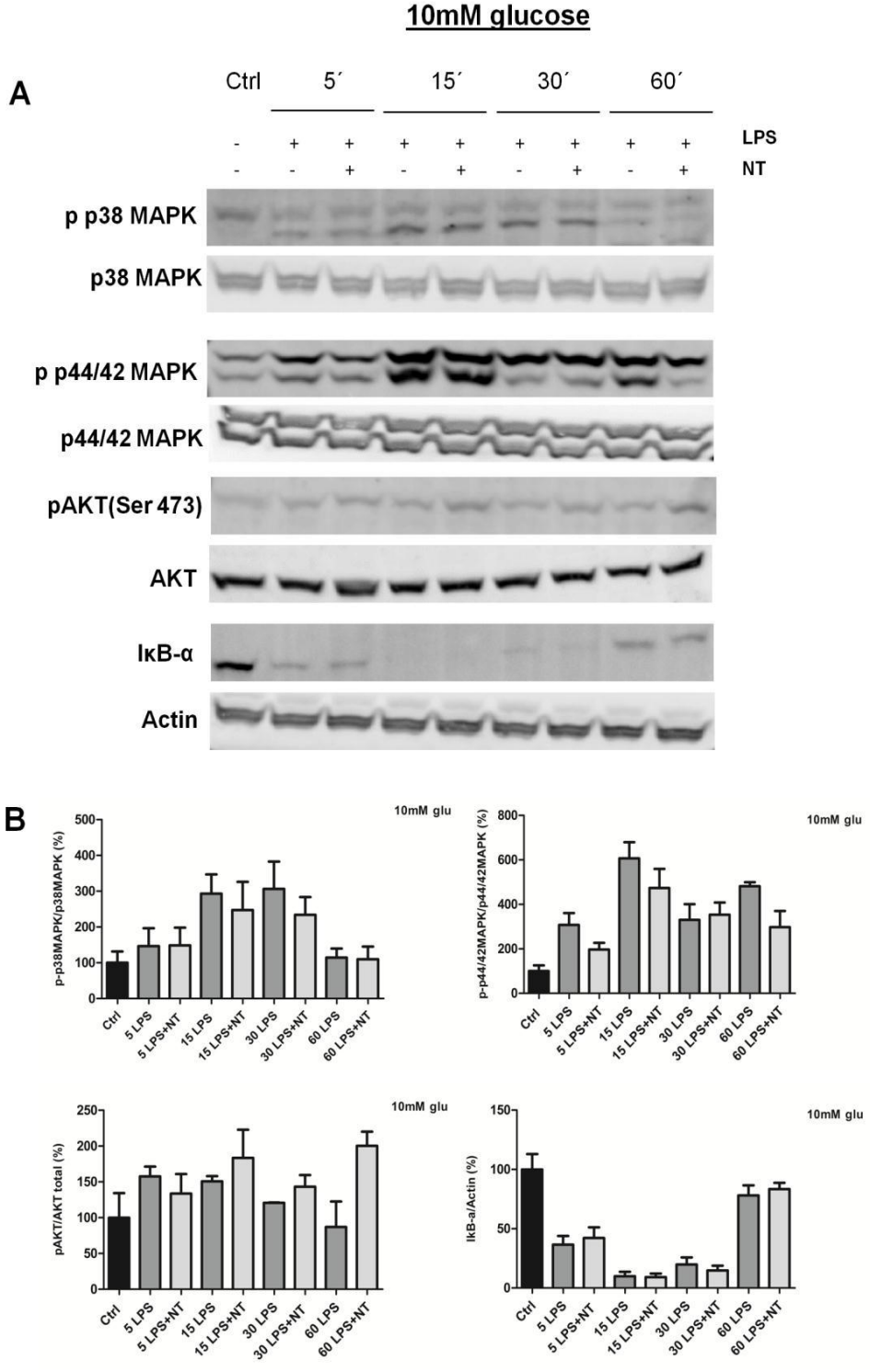

Figure 2.4: Modulation of LPS activated signaling pathways by NT at 10 and 30mM glucose (Figure 2.4), in macrophages, by Western Blot (A and C) and relative quantification (B and D). Cells were plated at $1.5x10^6$/well and treated simultaneously with 10nM NT and 1µg/ml LPS during 5, 15, 30 or 60 minutes. The lysates were probed for phospho p38MAPK, phospho p44/42 MAPK, phospho pAKT (Ser437) and inhibitory protein for NF-kB activation, IkB-α antibodies. Equal amounts of protein were evaluated with total p38MAPK, p44/42MAPK, AKT and actin antibodies. The results shown are representative of four to six independent experiments with similar results. (cont.)

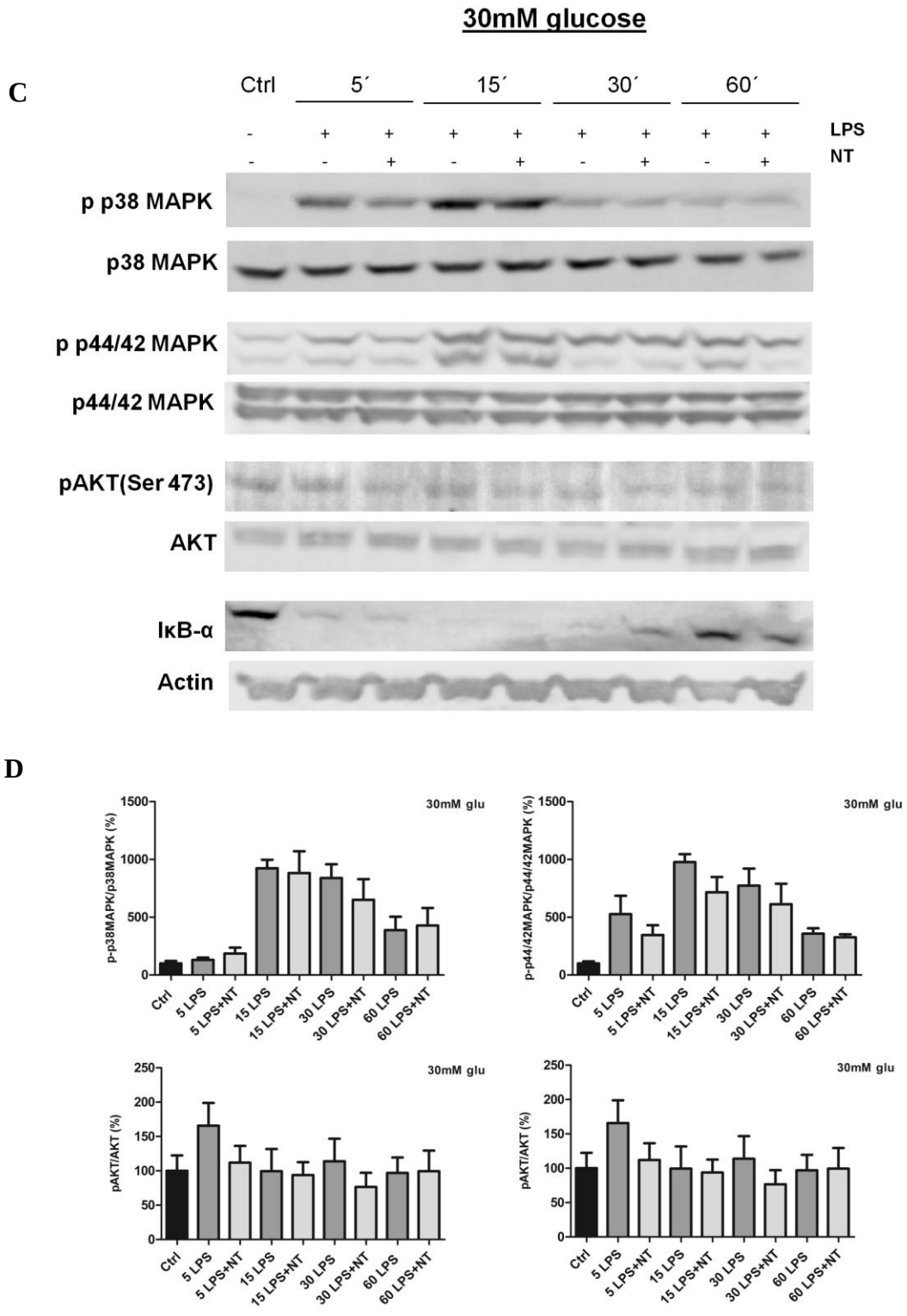

Figure 2.4: Modulation of LPS activated signaling pathways by NT at 10 and 30mM glucose (Figure 2.4), in macrophages, by Western Blot (A and C) and relative quantification (B and D). Cells were plated at $1.5x10^6$/well and treated simultaneously with 10nM NT and 1µg/ml LPS during 5, 15, 30 or 60 minutes. The lysates were probed for phospho p38MAPK, phospho p44/42 MAPK, phospho pAKT (Ser437) and inhibitory protein for NF-kB activation, IkB-α antibodies. Equal amounts of protein were evaluated with total p38MAPK, p44/42MAPK, AKT and actin antibodies. The results shown are representative of four to six independent experiments with similar results.

However, NT significantly decreased NTR1 expression in 30mM glucose ($p<0.05$) (Figure 2.5 B). A similar pattern of expression was observed for NTR2. In NT-treated cells, NTR2 expression was increased ($p<0.05$) when compared to untreated cells, in 10mM glucose but not in hyperglycemic condition, similarly as previously observed for both NTR1 and endogenous NT expression. In the presence of LPS, NTR2 expression was significantly increased in 30mM glucose ($p<0.05$), and this effect was not observed in NT-treated cells. Moreover, hyperglycemia did not change NTR3 expression but in the presence of NT, NTR3 expression was significantly increased (Figure 2.5 B). However, under inflammatory conditions, NTR3 gene expression was decreased compared to untreated cells, and no further changes were observed in the presence of NT, as shown in Figure 2.5 B. Interestingly, after macrophage treatment with exogenous NT, the expression of endogenous NT and its two extracellular receptors, NTR1 and NTR2 were significantly increased compared to untreated cells under 10 mM glucose. However, this effect of NT-treatment was not found in hyperglycemic condition. The opposite effect was observed for the intracellular receptor, NTR3, where hyperglycemia significantly increased NTR3 expression but not in the presence of exogenous NT.

At the protein level, however, no differences were observed in NTR1 or NTR3 levels, in 10mM or 30mM glucose, either in the presence or absence of LPS (Figure 2.5 C), while NTR2 was undetectable in these cells, as we have shown previously (da Silva *et al.*, 2011).

2.5 Discussion

Platelets, neutrophils, fibroblasts and macrophages contribute to wound healing by releasing cytokines, interleukins and growth factors. These important cellular mediators modulate the inflammatory phase of healing (Bagdade *et al.*, 1974, Brem and Tomic-Canic, 2007). Macrophages, in particular, play an important role in inflammatory and immune processes. Physiological and pathophysiological events can be activated and ultimately regulated by neuropeptides, such as SP and/or NT (Jeon *et al.*, 1999, Yaraee *et al.*, 2003, Jiang *et al.*, 2012). It is

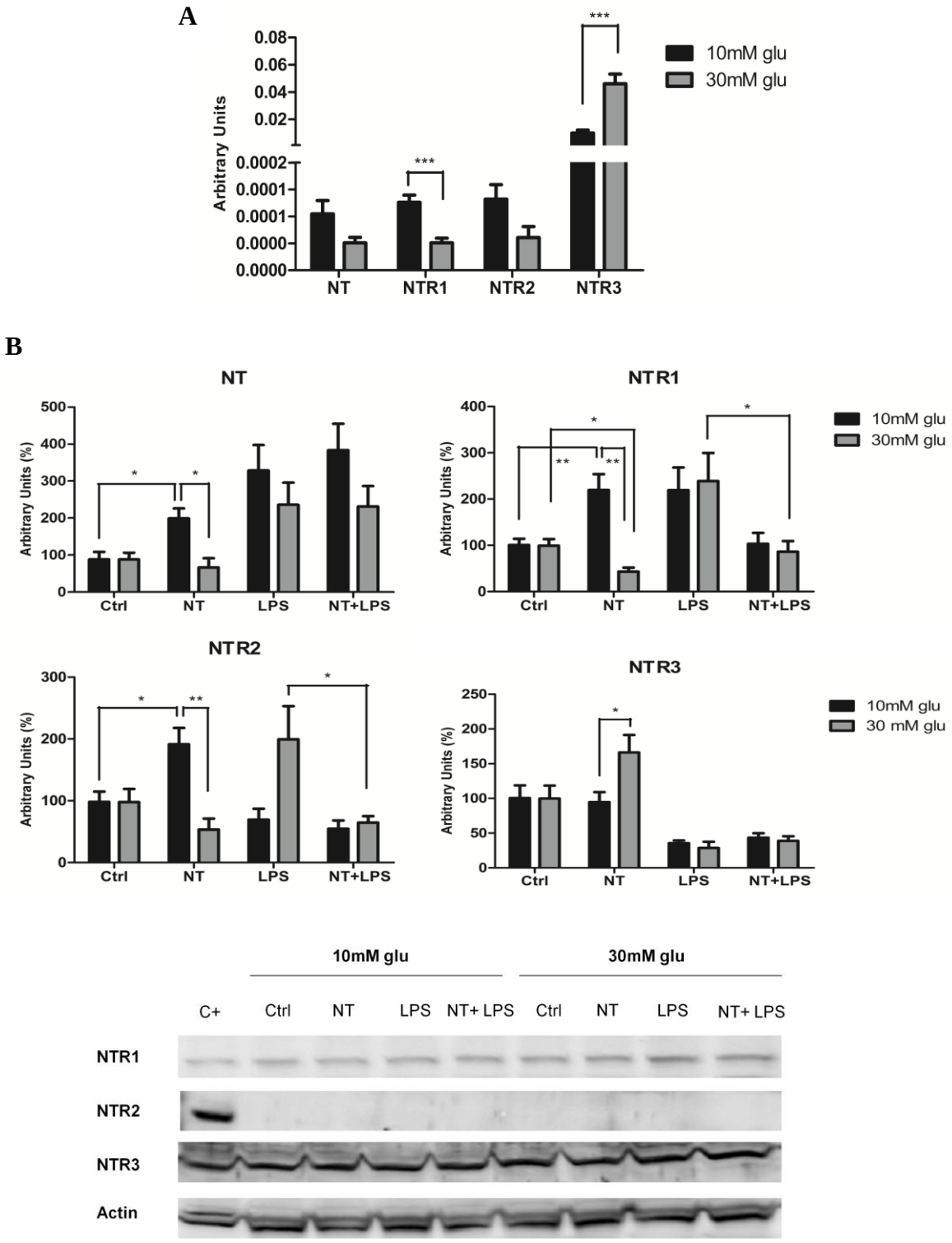

Figure 2.5: A) Expression of neurotensin and neurotensin receptors, NTR1, NTR2 and NTR3 in macrophages at 10 and 30 mM glucose, by real-time PCR. B) Expression of neurotensin and neurotensin receptors, NTR1, NTR2 and NTR3 in macrophages at 10 and 30 mM glucose, by real-time PCR. Cells were plated at $2x10^6$ / well and treated with 10nM NT or 1µM/ml LPS or both, during 6h. The relative gene expression is indicated as arbitrary units and was obtained after normalization with the HPRT gene. Results are presented as mean ± SEM of six to nine independent experiments. *$p<0.05$; **$p<0.01$
C) Neurotensin receptor protein levels in macrophages, at 10 and 30 mM glucose, by Western Blot. Cerebral cortex lysate (C+) was used as the positive control. Raw 264.7 cells were plated at $1.5x10^6$ /well and maintained at the indicated conditions. The lysates were probed for NTR1, NTR2 and NTR3 and actin antibodies. Three independent experiments were done for each antibody.

known that local acute inflammation and migration are crucial events for proper wound healing and that chronic low-grade inflammation contributes to the impaired healing observed in diabetes (Pradhan *et al.*, 2009, Tellechea *et al.*, 2010). Our results demonstrate that, under hyperglycemia, NT decreases the inflammatory response of macrophages and stimulates their capacity of migration. This is, to the best of our knowledge, the first study that evaluates the effect of NT in macrophages under either inflammatory or hyperglycemic conditions or both. These findings highlight the potential therapeutic role of NT in compromised wound healing conditions, such as diabetic foot ulcers, characterized by a pathological pro-inflammatory status and impaired cell migration. Accordingly, in an *in vitro* cerebral wound healing model, NT was shown to play an important role in response to inflammation or lesions in the central nervous system through the NTR3 (Martin *et al.*, 2003). Moreover, Brun et al, 2005 (Brun *et al.*, 2005) verified that NT, through NT receptor 1, stimulates epithelial restitution in intestine mucosa through a COX-2 dependent pathway, in chronic inflammation of the intestine.

In addition, we observed a reduction in the macrophage migratory profile under hyperglycemic conditions, when compared to normal glycaemia. However, NT was able to highly improve the migratory capacity of these cells, either under normal or inflammatory conditions. Accordingly, Martin et al, 2003 (Martin *et al.*, 2003) observed that NT stimulates migration of a human microglial cell line C13NJ in normoglycemic conditions. Furthermore, NT significantly stimulates the phagocytic process of peritoneal macrophages from BALB/c mice (De la Fuente *et al.*, 1993). Moreover, these results show that, NT increases the migratory capacity of macrophages but not cell proliferation, since the MTT assay did not show any proliferative differences either in the presence or in the absence of NT. These results suggest that NT stimulates the migratory response of macrophages in the diabetic state.

The pattern of inflammatory cytokines expressed by macrophages is affected under high glucose conditions. It is known that diabetes induces the expression of various cytokines, such as TNF-α and IL-6, by immune cells (Devaraj, 2005, Hatanaka, 2006, Dasu *et al.*, 2008). IL-6 is secreted by T cells and macrophages and acts as a pro-inflammatory cytokine to stimulate the immune response (Cavaillon, 1994). TNF-α is one of the major inflammatory mediators secreted by macrophages

upon a pro-inflammatory stimulation and is expressed constitutively at a low level in monocytic cells. This basal level expression of TNF-α has been shown to be altered by the inflammatory milieu leading to either its upregulation or downregulation (Silswal *et al.*, 2005). Particularly, TNF-α gene expression is increased in the presence of NT in 30 mM glucose but not in 10 mM glucose. However, in the presence of LPS, TNF-α expression is increased and this effect was even more pronounced in the presence of NT, as observed in 10 mM glucose, but not in 30 mM glucose. IL-1β is produced by activated macrophages and is an important mediator of the inflammatory response, it is involved in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis (Cavaillon, 1994). IL-12, a cytokine produced mainly by monocytes/macrophages is a central inducer of cell-mediated immunity that promotes the development, proliferation and function of T helper 1 (Th1) cells (Silswal *et al.*, 2005). Specifically, IL-1β and IL-12 gene expression were markedly decreased when the cells were treated with NT and LPS in 30 mM glucose. Hill and colleagues (Hill *et al.*, 1998) demonstrated that hyperglycemia inhibits IL-1 release from LPS-activated macrophages, a key mediator of the immune response against infection. Thus, different glucose concentrations can change the phenotype of macrophages leading to a switch from a pro-inflammatory to an anti-inflammatory profile after cell treatment with normal or high glucose concentration, respectively, as observed in other cells such as lymphocytes. This imbalance in the Th1/Th2 homeostasis contributes to the onset and progression of diabetes (Sia, 2005). This may justify the prevalence of infections in poorly controlled diabetics. Our results show that NT inhibits the inflammatory response of macrophages under hyperglycemic conditions. NT induces cytokine/chemokine expression, such as macrophage inflammatory protein-2, monocyte chemotactic protein (MCP)-1, IL-1β, and TNF-α through p44/42MAPK and PI-3K-associated pathways, in a murine microglial cell line (Dicou *et al.*, 2004). We, on the other hand, have demonstrated that NT does not activate p38 MAPK, p44/42MAPK and PKB/AKT signaling pathways under either 10mM or 30mM glucose. More importantly, we observed a significant decrease in endogenous NT and NTR expression in hyperglycemic conditions which correlates with the high glucose-induced decrease in macrophage migration. Understanding which of these

receptors might be involved in the inflammatory response of macrophages induced by NT will be important in order to better delineate the mechanisms involved in the effects of NT.

Our results demonstrate that the NTR3 was the most expressed receptor in macrophages. Similar results were obtained by Martin et al, 2003 (Martin *et al.*, 2003) in a human microglial cell line. A downregulation of the VPAC2 (receptor for vasoactive intestinal peptide) expression has also been shown, after 4 weeks of diabetes, as observed by Dvoráková et al, 2006 (Dvorakova *et al.*, 2006), indicating that hyperglycemia may impair signal transduction through these receptors.

Furthermore, under 10mM glucose, NT significantly stimulated endogenous NT, NTR1 and NTR2 expression, while no changes were observed for NTR3. On the other hand, in cells under 30mM glucose, NT highly increased NTR3 expression. However, NT and NTR2 protein expression were not detected in these cells (data not shown for NT). Similar results were observed previously by da Silva et al, 2011 (da Silva *et al.*, 2011), where the NTR3 was the most expressed receptor in a dendritic cell line under normoglycemic conditions. These differences in the protein expression of NT receptors could be due to the NTR3 localization, since it is an intracellular receptor and its responses can be mostly mediated by endogenous NT. These results suggest that hyperglycemia causes the decrease in the levels of cell surface receptors, increasing the number of receptors in the light vesicle fraction, without changes in the binding affinity for the peptide, and consequently internalization of receptor 3 (Hermans and Maloteaux, 1998, Mazella, 2001). Furthermore, under inflammatory conditions, endogenous NT is highly expressed in either the presence or absence of exogenous NT, while NTR1 and NTR2 are greatly expressed in the presence of LPS but when NT is present their expression returns to control levels. On the other hand, the expression of NTR3 is decreased compared to non-inflammatory conditions. It is known that, in peripheral tissues, such as gastrointestinal tract, desensitization of NT receptors to NT seems to be frequent (Mule *et al.*, 1995, Hermans and Maloteaux, 1998). Furthermore, in hyperglycemia the loss of G-coupled protein receptor function is mainly caused by reduced affinity for the neurotensin (Hashim *et al.*, 2006). These results indicate that the effect of NT is masked by high glucose and/or reduction of the NT affinity to the receptors, as observed for other neuropeptides in similar conditions

(Akabayashi *et al.*, 1993). Further studies to better understand the role of NT receptors in inflammatory and hyperglycemic conditions are needed.

Furthermore, and in agreement with our results, Matyal et al, 2011 (Matyal *et al.*, 2011) observed that in diabetic patients, atrial cardiac tissue neuropeptide Y expression is decreased and its receptors Y2 and Y5 mRNA levels are upregulated. Altered expression of neuropeptide Y and its receptors during hyperglycemia may contribute to coronary artery disease, due to decreased angiogenesis, increased apoptosis and increased vascular smooth muscle proliferation. Under these conditions, NT promotes an earlier acute inflammatory response reflecting possible beneficial effects for diabetic wound healing.

2.6 Conclusions

These studies demonstrate that NT affects macrophage responses, both under inflammatory and hyperglycemic conditions, through the stimulation of cell migration and regulation of cytokine expression. These *in vitro* results are the start point to find relevant molecules and signaling pathways triggered by NT under inflammatory and hyperglycemic conditions that are currently being confirmed both in *in vivo* models as well as in primary macrophage cultures. Based on the present results obtained, we suggest that NT administration, under normal glucose conditions, promotes an inflammatory response by macrophages, which may be important in the early phases of healing. When administered under hyperglycemic conditions, NT stimulates migration but inhibits the pro-inflammatory status of macrophages thus contributing to the resolution of inflammation and allowing the progression to the migration-remodeling phases of wound healing. These effects have the potential to be beneficial in a diabetic wound environment.

2.7 Acknowledgments

This work was financially supported by COMPETE and Fundação para a Ciência e Tecnologia (FCT-MES) under contracts, SFRH/BD/60837/2009, SFRH/BD/30563/2006, PTDC/SAU-BEB/71395/2006, PTDC/SAU-MII/098567/2008, PEst-C/SAU/LA0001/2011, by

EFSD/JDRF/Novo Nordisk European Programme in Type 1 Diabetes Research and Sociedade Portuguesa de Diabetologia.

2.8 References

Akabayashi A, Zaia CTBV, Silva I, Chae HJ, Leibowitz SF (1993) Neuropeptide Y in the arcuate nucleus is modulated by alterations in glucose utilization. Brain Res 621:343-348.

Alba-Loureiro TC (2007) Neutrophil function and metabolism in individuals with diabetes mellitus. Braz J Med Biol Res 40:1037-1044.

Bagdade JD, Root RK, Bulger RJ (1974) Impaired leukocyte function in patients with poorly controlled diabetes. Diabetes 23:9-15.

Brem H, Tomic-Canic M (2007) Cellular and molecular basis of wound healing in diabetes. J Clin Invest 117:1219-1222.

Brun P, Mastrotto C, Beggiao E, Stefani A, Barzon L, Sturniolo GC, Palu G, Castagliuolo I (2005) Neuropeptide neurotensin stimulates intestinal wound healing following chronic intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 288:G621-629.

Cavaillon JM (1994) Cytokines and macrophages. Biomedicine & Pharmacotherapy 48:445–453.

da Silva L, Neves BM, Moura L, Cruz MT, Carvalho E (2011) Neurotensin downregulates the pro-inflammatory properties of skin dendritic cells and increases epidermal growth factor expression. Biochim Biophys Acta 1813:1863-1871.

Dasu MR, Devaraj S, Zhao L, Hwang DH, Jialal I (2008) High glucose induces toll-like receptor expression in human monocytes: mechanism of activation. Diabetes 57:3090-3098.

De la Fuente M, Garrido JJ, Arahuetes RM, Hernanz A (1993) Stimulation of phagocytic function in mouse macrophages by neurotensin and neuromedin N. J Neuroimmunol 42:97-104.

de Souza LF, Jardim FR, Sauter IP, de Souza MM, Bernard EA (2008) High glucose increases RAW 264.7 macrophages activation by lipoteichoic acid from Staphylococcus aureus. Clin Chim Acta 398:130-133.

Delgado AV, McManus AT, Chambers JP (2005) Exogenous administration of Substance P enhances wound healing in a novel skin-injury model. Exp Biol Med (Maywood) 230:271-280.

Devaraj S, Venugopal, S. K., Singh, u., Jialal, I., (2005) Hyperglycemia Induces Monocytic Release of Interleukin-6 via Induction of Protein Kinase C-α and –β. Diabetes care 54:85-91.

Dicou E, Vincent JP, Mazella J (2004) Neurotensin receptor-3/sortilin mediates neurotensin-induced cytokine/chemokine expression in a murine microglial cell line. J Neurosci Res 78:92-99.

Dvorakova MC, Pfeil U, Kuncova J, Sviglerova J, Galvis G, Krasteva G, Konig P, Grau V, Slavikova J, Kummer W (2006) Down-regulation of vasoactive intestinal peptide and altered expression of its receptors in rat diabetic cardiomyopathy. Cell and tissue research 323:383-393.

Elenkov IJ (2008) Neurohormonal-cytokine interactions: implications for inflammation, common human diseases and well-being. Neurochem Int 52:40-51.

Ganea D, Delgado M (2001) Neuropeptides as Modulators of Macrophage Functions. Regulation of Cytokine Production and Antigen Presentation by VIP and PACAP. Archivum Immunologiae et Therapiae Experimentalis 49:101-110.

Hartung HP (1988) Activation of macrophages by neuropeptides. Brain Behav Immun 2:275-281.

Hashim S, Li Y, Anand-Srivastava MB (2006) G protein-linked cell signaling and cardiovascular functions in diabetes/hyperglycemia. Cell Biochem Biophys 44:51-64.

Hatanaka E (2006) Neutrophils and monocytes as potentially important sources of proinflammatory cytokines in diabetes. Clin Exp Immunol 146:443-447.

Hermans E, Maloteaux JM (1998) Mechanisms of regulation of neurotensin receptors. Pharmacology & therapeutics 79:89-104.

Hill JR, Kwon G, Marshall CA, McDaniel ML (1998) Hyperglycemic levels of glucose inhibit interleukin 1 release from RAW 264.7 murine macrophages by activation of protein kinase C. The Journal of biological chemistry 273:3308-3313.

Jeon HK, Jung NP, Choi IH, Oh YK, Shin HC, Gwag BJ (1999) Substance P augments nitric oxide production and gene expression in murine macrophages. Immunopharmacology 41:219-226.

Jiang MH, Chung E, Chi GF, Ahn W, Lim JE, Hong HS, Kim DW, Choi H, Kim J, Son Y (2012) Substance P induces M2-type macrophages after spinal cord injury. Neuroreport 23:786-792.

Kampfer H, Schmidt R, Geisslinger G, Pfeilschifter J, Frank S (2005) Wound inflammation in diabetic ob/ob mice: functional coupling of prostaglandin biosynthesis to cyclooxygenase-1 activity in diabetes-impaired wound healing. Diabetes 54:1543-1551.

Khanna S, Biswas S, Shang Y, Collard E, Azad A, Kauh C, Bhasker V, Gordillo GM, Sen CK, Roy S (2010) Macrophage dysfunction impairs resolution of inflammation in the wounds of diabetic mice. PloS one 5:e9539.

Kim HS, Yumkham S, Choi JH, Lee SH, Him T, Ryu SH, Suh P (2006) Neurotensin enhances nitric oxide generation via the JAK2-STAT1 pathway in murine macrophage Raw 264.7 cells during costimulation with LPS and IFNγ. Neuropeptides 40:221-229.

Koh TJ, DiPietro LA (2011) Inflammation and wound healing: the role of the macrophage. Expert Rev Mol Med 13:e23.

Lan CC, Liu IH, Fang AH, Wen CH, Wu CS (2008) Hyperglycaemic conditions decrease cultured keratinocyte mobility: implications for impaired wound healing in patients with diabetes. Br J Dermatol 159:1103-1115.

Lazarus LH, Brown MR, Perrin MH (1977) Distribution, localization and characteristics of neurotensin binding sites in the rat brain. Neuropharmacology 16:625-629.

Martin S, Vincent JP, Mazella J (2003) Involvement of the neurotensin receptor-3 in the neurotensin-induced migration of human microglia. J Neurosci 23:1198-1205.

Martinez FO, Sica A, Mantovani A, Locati M (2008) Macrophage activation and polarization. Frontiers in bioscience : a journal and virtual library 13:453-461.

Matyal R, Mahmood F, Robich M, Glazer H, Khabbaz K, Hess P, Bianchi C, Hagberg R, Hu SX, Sellke FW (2011) Chronic type II diabetes mellitus leads to changes in neuropeptide Y receptor expression and distribution in human myocardial tissue. Eur J Pharmacol 665:19-28.

Mazella J (2001) Sortilin/neurotensin receptor-3: a new tool to investigate neurotensin signaling and cellular trafficking? Cellular signalling 13:1-6.

Mule F, Serio R, Postorino A (1995) Motility pattern of isolated rat proximal colon and excitatory action of neurotensin. Eur J Pharmacol 275:131-137.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

Pradhan L, Cai X, Wu S, Andersen ND, Martin M, Malek J, Guthrie P, Veves A, Logerfo FW (2011) Gene expression of pro-inflammatory cytokines and neuropeptides in diabetic wound healing. J Surg Res 167:336-342.

Pradhan l, Nabzdyk C, Andersen N, LoGerfo F, Veves A (2009) Inflammation and neuropeptides: the connection in diabetic wound healing. Expert Reviews in Molecular Medicine 11:1-24.

Shaw JE, Sicree RA, Zimmet PZ (2010) Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Research and Clinical Pratice 87:4-14.

Sia C (2005) Imbalance in Th cell polarization and its relevance in type 1 diabetes mellitus. Rev Diabet Stud 2:182-186.

Silswal N, Singh AK, Aruna B, Mukhopadhyay S, Ghosh S, Ehtesham NZ (2005) Human resistin stimulates the pro-inflammatory cytokines TNF-alpha and IL-12 in macrophages by NF-kappaB-dependent pathway. Biochemical and biophysical research communications 334:1092-1101.

Tellechea A, Leal E, Veves A, Carvalho E (2010) Inflammatory and angiogenic abnormalities in diabetic wound healing: role of neuropeptides and therapeutic perspectives The Open Circulation and Vascular Journal 3:43-55.

Toda M, Suzuki T, Hosono K, Kurihara Y, Kurihara H, Hayashi I, Kitasato H, Hoka S, Majima M (2008) Roles of calcitonin gene-related peptide in facilitation of wound healing and angiogenesis. Biomed Pharmacother 62:352-359.

Vincent JP, Mazella J, Kitabgi P (1999) Neurotensin and neurotensin receptors. Trends Pharmacol Sci 20:302-309.

Yaraee R, Ebtekar M, Ahmadiani A, Sabahi F (2003) Neuropeptides (SP and CGRP) augment pro-inflammatory cytokine production in HSV-infected macrophages. Int Immunopharmacol 3:1883-1887.

Zhao D, Zhan Y, Zeng H, Koon HW, Pothoulakis C (2005) Neurotensin stimulates interleukin-8 expression through modulation of IkBα phosphorylation and p65 transcriptional activity: involvement of protein kinase Cα Molecular Pharmacology 67:2025-2031.

Chapter 3

In vitro study

The effect of neurotensin in human keratinocytes– implication on impaired wound healing in diabetes

This Chapter comprises the work submitted in the journal *Experimental Biology and Medicine* (2013) by **Moura, L. I. F.**, Cruz, M. T., Carvalho, E.

3.1 Abstract

Diabetic foot ulcers (DFU) are an important complication of diabetes mellitus characterized by chronic, non-healing ulcers resulting from poor proliferation and migration of fibroblasts and keratinocytes, thus impairing a correct re-epithelialization of wounded tissues. This healing process can be modulated by neuropeptides released from peripheral nerves, however little is known regarding the role of neurotensin (NT) as a modulator of human keratinocyte function under hyperglycemic conditions. Therefore, this work is focused on the effect of NT in human keratinocytes, under normal and hyperglycemic conditions at different functional levels, namely NT receptors, cytokine and growth factor expression, as well as, proliferation and migration.

HaCaT cells were maintained at 10/30mM glucose and treated with or without NT (10nM). The results show that NT did not affect keratinocyte viability. In addition, hyperglycemia reduced significantly NT and all NT receptor expression; however NT treatment stimulated expression of NT and NTR2 while NTR1 and NTR3 expression levels were unchanged. Keratinocyte proliferation was not affected by NT and hyperglycemia, while cell migration was reduced by NT treatment.

These results demonstrated that hyperglycemic conditions strongly impaired endogenous NT and NTR2 expression in keratinocytes. Despite the addition of exogenous NT to stimulate the endogenous NT and NTR2 expression, these changes do not translate into functional modifications on keratinocytes, particularly in terms of migration, proliferation and production of cytokines or growth factors. These results suggest that NT production by keratinocytes may exert a paracrine effect on other skin cells, namely fibroblasts, macrophages and dendritic cells for correct wound healing.

Keywords: wound healing, keratinocytes, hyperglycemia, neuropeptides, neurotensin

3.2 Introduction

Diabetes mellitus is one of the most complicated chronic diseases that affect millions of people

worldwide (Shaw *et al.*, 2010). Patients with diabetes are susceptible to develop complications such as chronic, non-healing diabetic foot ulcers (DFU) that cause pain, suffering, decrease in quality of life and, in extreme cases, culminate with lower extremity amputations (Pradhan *et al.*, 2009b, Tellechea *et al.*, 2010).

An important phase during the wound healing process is the re-epithelialization of wounded tissues. Correct re-epithelialization is an essential feature for the restoration of an intact epidermal layer and the migration and proliferation of keratinocytes are critical steps in this process (Raja *et al.*, 2007, Lan *et al.*, 2008). After injury, keratinocytes not only migrate and proliferate to cover the wound but also express cytokines and growth factors that regulate the wound healing process (Kawai *et al.*, 2008).Furthermore, the peripheral nervous system also plays an important role in the inflammatory, proliferative and reparative processes after skin injury (Song et al, 2000). The interaction between peripheral nerves and the immune system is mediated by different types of cutaneous nerve fibers that release neuropeptides, such as Substance P and Neuropeptide Y, which in turn activate specific receptors on target cells in the skin, such as keratinocytes, mast cells, Langerhans cells, microvascular endothelial cells, fibroblasts and macrophages (Steinhoff *et al.*, 2001, Steinhoff *et al.*, 2003, Dallos *et al.*, 2006, Silva *et al.*, 2010). In response to neuropeptides, these skin cells produce and release cytokines and growth factors (Schaffer *et al.*, 1998, Dallos *et al.*, 2006). These neuro-skin interactions influence a variety of physiologic and pathophysiologic functions including cellular development, growth, differentiation, immunity, vasoregulation, leukocyte recruitment and wound repair (Legat *et al.*, 2002, Silva *et al.*, 2010).

Neurotensin (NT) is a bioactive trideca-neuropeptide that is widely distributed through the brain, cardiovascular system and the gastrointestinal tract (Lazarus *et al.*, 1977, Vincent *et al.*, 1999b, Brun *et al.*, 2005). In addition, NT regulates inflammatory processes in the lung and gastric system (Brun *et al.*, 2005, Zhao *et al.*, 2005). NT functions are mediated through its binding to two G-protein coupled receptors: neurotensin receptor 1 (NTR1) and neurotensin receptor 2 (NTR2) (high and low affinity, coupled receptor (Vincent *et al.*, 1999b, Pradhan *et al.*, 2009a). Since the effect of NT on keratinocyte has never been addressed before, this study aims to determine how NT modulates keratinocyte function under hyperglycemic conditions.

3.3 Material and methods

3.3.1 Materials

NT was obtained from Bachem (Weil am Rhein, Germany). All primers were obtained from IDT (Ebersberg, Germany). The TRIzol® reagent was purchased from Invitrogen (Barcelona, Spain). SYBR green was obtained from BioRAD (Hercules, CA, USA) and the High Capacity cDNA Reverse Transcription kit was obtained from Applied Byosistems (Carlsbad, CA, USA).

The antibodies against the NT receptors were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA) and the antibody against β-actin was purchased from the Millipore Corporation (Bedford, MA, USA).

3.3.2 Cell culture

The human keratinocyte cell line (HaCaT) was purchased from CLS (number 300493) and was cultured in DMEM medium, pH 7.4, supplemented with 10% heat inactivated fetal bovine serum (FBS), 3.02 g/l sodium bicarbonate, 100 U/ml penicillin, 100 µg/ml streptomycin, at 37ºC in a humidified incubator containing 5% CO_2. Sub-culturing was performed according to CLS recommendations. The cells were maintained in either 10mM (normal glucose) or 30mM (high glucose) D-glucose, for more than two weeks, before starting the experiments.

3.3.3 MTT viability assay

HaCaT (4×10^4 cells/well) cells, at 30mM glucose conditions, were plated in 48-well plates in 430 µL of DMEM. Cells were treated with 10, 50 or 100 nM of NT for 7days. After, either 1, 3, 5 or 7 days of incubation, 43µl of MTT solution (5 mg/ml) were added to each well. The plates were further incubated at 37ºC for 1h, in a humidified incubator containing 5 % CO_2. 300 µl of acidic isopropanol (0.04 N HCl in isopropanol) were then added to each well and mixed in order to dissolve the dark blue crystals of formazan. Acidic isopropanol was collected into an ELISA microplate and formazan quantification was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm.

3.3.4 Real time RT-PCR

HaCaT cells (5×10^5) were seeded in 6-well plates and treated with 10nM NT during either 6 h or 24 h. Total RNA was isolated from cells with the TRIzol reagent according to the manufacturer's instructions and concentration was determined by OD260 measurement using NanoDrop spectrophotometer (Thermo Scientific, USA). The first cDNA strand was synthesized using High Capacity cDNA Reverse Transcription. Briefly, 2µl of 10X RT Buffer, 0.8 µl of 25X dNTP Mix, 2 µl of 10X RT random primers, 1 µl of MultiscribeTM Reverse Transcriptase and 4.2 µl of nuclease free H_20 were added to 10 µl of RNA (1 µg) sample. Then, real-time RT-PCR was performed in a Bio-Rad My Cycler iQ5. For each reaction 10 µl were used containing 2.5 µl cDNA, 5 µl 2X Syber Green Supermix, 1 µl of each primer (250 nM) and 0.5 µl of H_2O PCR grade. Primer sequences are given in Table 3.1. Gene expression changes were analyzed using iQ5Optical system software v2. The results were normalized using a reference gene, hypoxanthine phosphoribosyltransferase 1 (HPRT-1), selected based on our previous results demonstrating that it does not change under these conditions.

3.3.5 Western blotting

HaCaT ($5x10^5$ cells/well) cells were seeded in 6-well plates and treated with 10nM of NT for 24 h. Cells were then washed twice with ice-cold PBS buffer and lysed with RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1 % NP-40 (Nonidet P-40), 0.5 % Sodium Deoxycholate, 0.1 % SDS, 2 mM EDTA, proteases inhibitor cocktail, phosphatases inhibitor cocktail and 1 mM DTT). Protein concentration was determined using the bicinchoninic acid method and the cell lysates were denatured at 95ºC, for 5 min, in sample buffer (0.125 mM Tris pH 6.8; 2% w/v SDS; 100 mM DTT; 10% glycerol and bromophenol blue) for its use in western blot analysis. 30µg of total protein were resolved on 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% (w/v) 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 and washing, membranes were incubated

Table 3.1: Primer sequences for target cDNAs.

Primer	5'-3'Sequence (Forward; Reverse)
NT	For: GCATACATCAAAGATTAGT Rev: TAAAGCAGTAGGAAGTTT
NTR1	For: CCATCCACACTGCCACCGTCA Rev: TGAATGTGCTGTGCTCGCCC
NTR2	For: TCCAAGTCTTTATCCAGGTG Rev: TACGATGAAGCTGAGGAGAC
NTR3	For: TGGGTTGGAGATAGCACTGG Rev: ACGACTTCCTCCAGACACCT
IL-1β	For: GCTTGGTGATGTCTGGTC Rev: GCTGTAGAGTGGGCTTATC
IL-8	For: TTGGCAGCCTTCCTGATTTC Rev: AACTTCTCCACAACCCTCTG
EGF	For: AATCATGGCTGTACTCTTGGG Rev: CAGGACAGAAACATAAGGGAC
VEGF	For: CAGAATCATCACGAAGTG Rev: TCTGCATGGTGATGTTGGA
PDGF	For: CAGAAGCTAACCATCTCCTGG Rev: TCGGGAGTAATTTGGTGCTTC
HPRT1	For: TGACACTGGCAAAACAATG Rev: GGCTTATATCCAACACTTCG

overnight at 4ºC with the primary antibodies against NT receptors (1:500). After incubation, membranes were washed and incubated for 1 h at room temperature with alkaline phosphatase-conjugated anti-rabbit antibody (1:5000), or alkaline phosphatase-conjugated anti-mouse antibody (1:5000). The membranes were exposed to the ECF reagent followed by scanning for blue excited fluorescence on the VersaDoc (Bio-Rad Laboratories, Amadora, Portugal). To test whether similar amounts of protein for each sample were loaded, the membranes were stripped and reprobed with an anti-actin antibody and blots were developed with an alkaline phosphatase-conjugated secondary antibody and visualized by enhanced chemifluorescence. The generated signals were analyzed using the Image-Quant TL software.

3.3.6 Proliferation

HaCaT ($4x10^5$ cells/well) cells were seeded in 6-well plates and treated with 10nM of NT for 24 h.

Cells were ressuspended and 0.1ml of 0.4% trypan blue stock solution in PBS were added to 1ml of cells. The number of blue staining cells and the number of total cells were counted in a Zeiss Primo Vert Microscope (Carl Zeiss, Gottingen, Germany).

3.3.7 *In vitro* scratch assay - migration

HaCaT ($4x10^5$) cells were ressuspended in 3 mL of DMEM medium in µ-Dish$^{35mm, high}$ (Ibidi). After 24h, a ‘‘scratch’’ was made in the cell monolayer, with a pipette tip, in a straight line to create an area without cells. The medium was removed and cells were washed two times with PBS 1X. Following, DMEM medium containing 2 % of inactivated fetal calf serum was added to the cells to diminish cell proliferation. Photographs were captured with a coupled AxioCamMR3 camera with PALM reflector and 5X objective, using an inverted Axiovert 200. A specific number/letter marked area was chased to permit later recognition of the photographed area. HaCaT cells were then incubated with 10nM of NT or maintained in DMEM medium containing 2 % of inactivated fetal calf serum (control) and allowed to migrate during 24 h. After the incubation period, photographs were taken in the same area where the first photograph was taken. Photographs were analyzed and the number of cells in the scratched area was counted. For the analysis, the number of cells in the zero point was taken into account.

3.3.8 Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using either one-way ANOVA or the unpaired t student´s test by GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). p values less than 0.05 were considered statistically significant.

3.4 Results

All experiments were performed using HaCaT cells incubated with either 10mM glucose (normal glucose conditions) or 30mM glucose (high glucose conditions), for a period of 15 days.

3.4.1 Cell viability under hyperglycemic conditions

Different NT concentration treatments did not affect significantly the viability of keratinocytes under hyperglycemic conditions (Figure 3.1). Since no major differences were observed between the concentrations of NT used (10, 50 or 100 nM), the following experiments were performed with 10nM of NT.

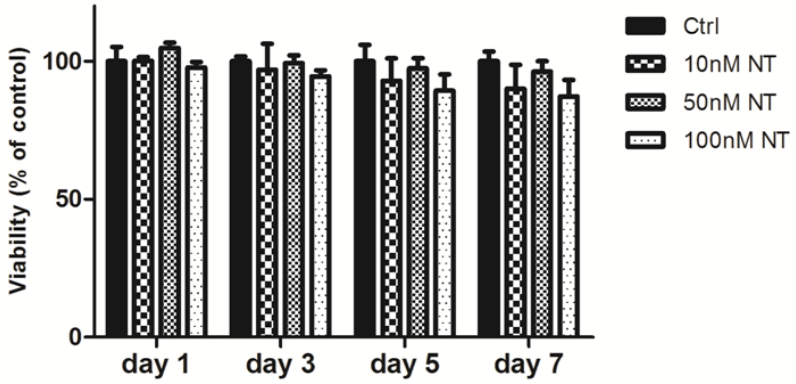

Figure 3.1: Viability of HaCaT cells, under either 10 or 30mM glucose, by the MTT assay. HaCaT cells were plated at $4x10^4$ /well and were treated with 10, 50 or 100 nM of NT for 7days. Absorbance quantification was performed using a microplate reader at 570 nm, with a reference wavelength of 620 nm. Results are presented as mean ± SEM of three independent experiments.

3.4.2 Expression of NT receptors in HaCaT cells under normal and hyperglycemic conditions

Gene expression results showed that in endogenous conditions, hyperglycemia strongly reduced the expression of NT and all NT receptors (Figure 3.2 A-D). When stimulated with NT, total NT expression significantly increased ($p<0.001$) either in normal and hyperglycemic conditions. However, in the same conditions, no differences were observed for NT receptors expression. Interestingly, only NTR2 expression showed a significant increase ($p<0.05$) in response to NT treatment, under hyperglycemia. owever, at the protein level no differences were observed for all NT receptors after keratinocytes treatment with either 10mM or 30mM glucose (Figure 3.2-E).

3.4.3 Proliferation and migration of HaCaT cells under normal or hyperglycemic conditions

After NT treatment, no statistical differences were observed in HaCaT cells proliferation under normal or hyperglycemic conditions, during 24h (Figure 3.3-A). However, migration studies revealed that under 30mM glucose, NT decreased HaCaT migration when compared to 10mM glucose treatment (Figure 3.3 B-C).

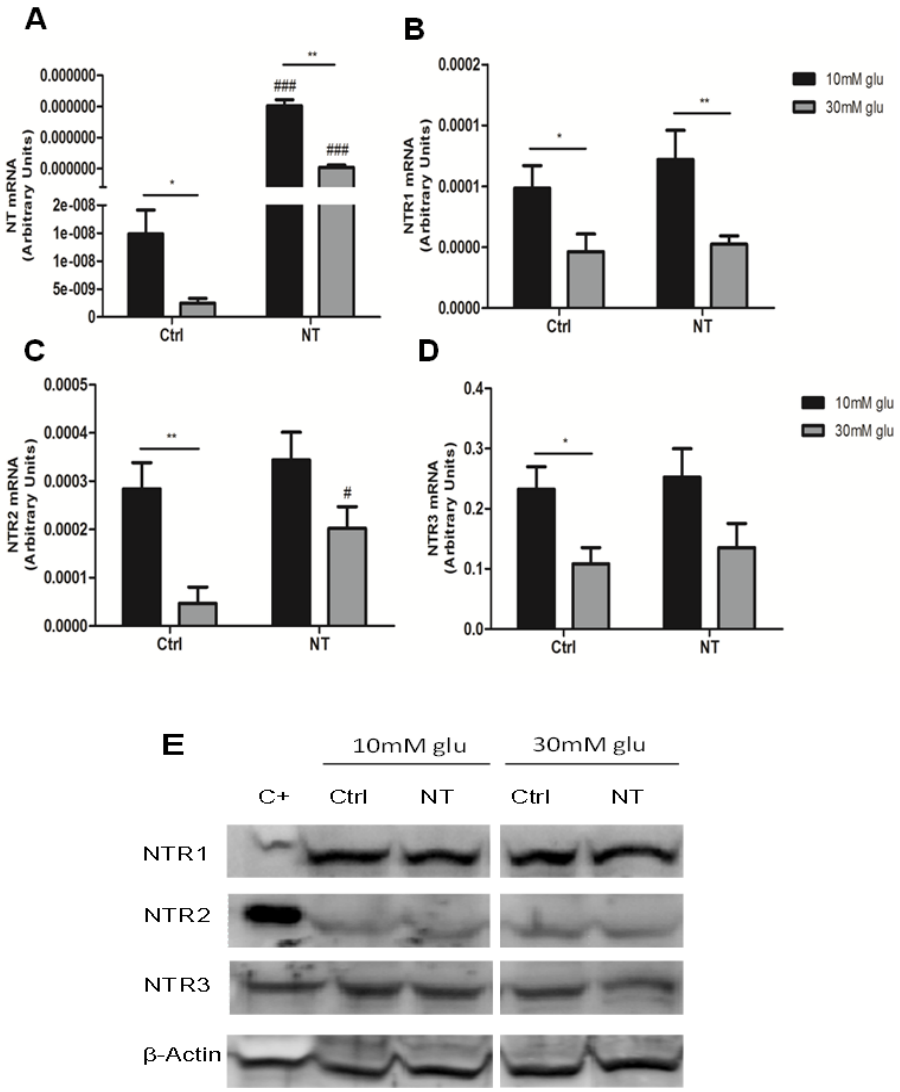

Figure 3.2: Expression of NT and NT receptors, NTR1, NTR2 and NTR3 in HaCaT cells under either 10 or 30 mM glucose, by real-time PCR (Figure 3.2 A-D) and Western Blot (Figure 3.2 E). Cells were plated at $5x10^5$ / well and treated with 10nM NT during 6h or 24h. RT-PCR results are presented as mean ± SEM of six to nine independent experiments. * p<0.005; p<0.01;***p<0.001; ### p<0.001 compared to respective control. In the Western Blots, cerebral cortex lysates (C+) were used as positive controls. Three independent experiments were performed for each antibody.

3.4.4 Cytokine and growth factors expression by HaCaT cells under normal and hyperglycemic conditions

NT decreased IL-1β expression under normoglycemic conditions, however at 30mM glucose, no differences were observed compared to normoglycemia (Figure 3.4-A).

IL-8 and growth factors (VEGF, EGF and PDGF) were not affected by NT treatment either in normo or hyperglycemic conditions (Figure 3.4 B-E).

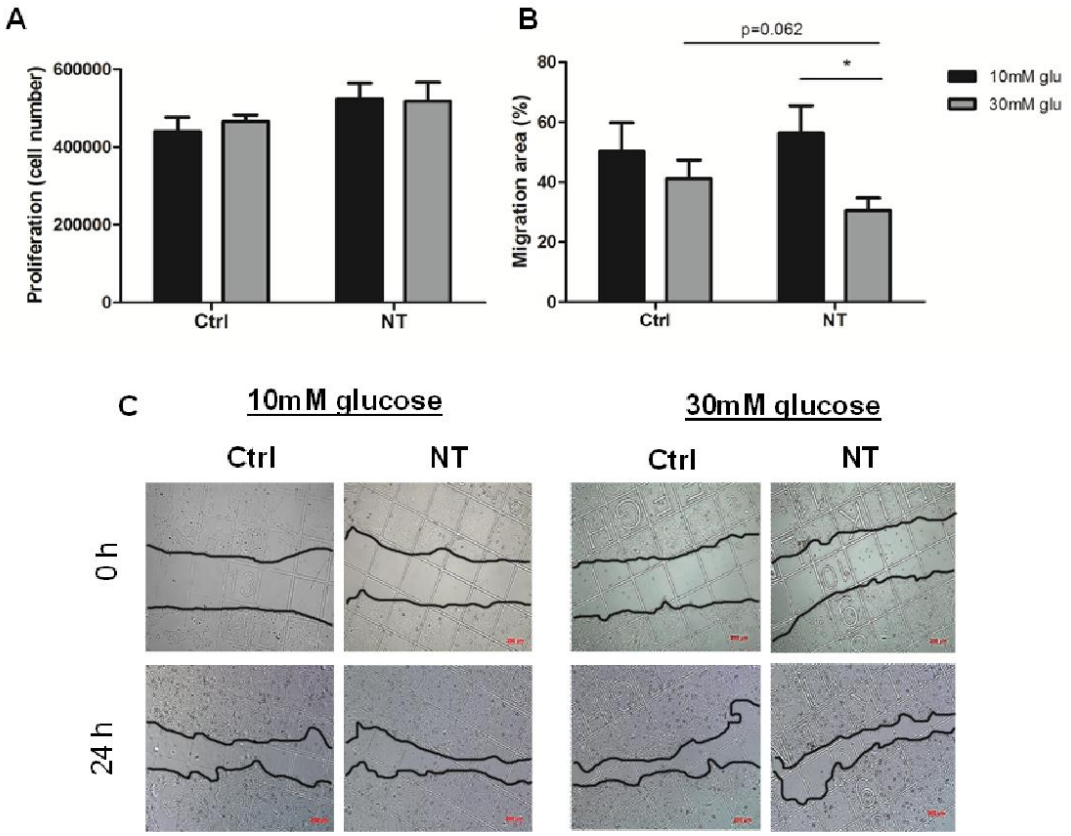

Figure 3.3: Proliferation of HaCaT cells under either 10 or 30mM glucose, by the trypan blue assay (Figure 3A). Migration of HaCaT cells under either 10 or 30mM glucose, by the *in vitro* scratch assay (Figure 3B-C). In both experiments, cells were plated at $4x10^5$ / well and treated with 10nM NT during 24h. The images were acquired by transmission microscopy and photographs were taken before cell treatment (0 h) and 24 h after treatments. Magnification used 40x. Results are presented as mean ± SEM of three independent experiments.*$p<0.05$.

3.5 Discussion

Skin is the outermost layer of the body, with a protective barrier against the external environment (Kawai *et al.*, 2008, Lan *et al.*, 2008). However, skin is susceptible to become injured and the healing process must be highly controlled and organized for correct repair (Tellechea *et al.*, 2010).

Keratinocytes are important cells in the regulation of homeostasis and pathophysiological processes through proliferation, migration and cytokines/growth factors secretion (Dallos *et al.*, 2006). Keratinocytes from the wound edges are the main responsible for the re-epithelialization phase of wound healing. They migrate across the wound site, proliferate in its edges and differentiate to restore the functionality of the epidermis (Raja *et al.*, 2007). Alterations in this process are associated withchronic, non-healing ulcers, such as DFU. Neuropeptides are produced in the skin by peripheral nerves such as autonomic or sensory ones. In addition to neuronal cells, immunocompetent cells, as well as, epithelial cells, such as keratinocytes, are able to produce neuropeptides (Luger and Lotti, 1998, Dallos *et al.*, 2006, da Silva *et al.*, 2011), which can exert

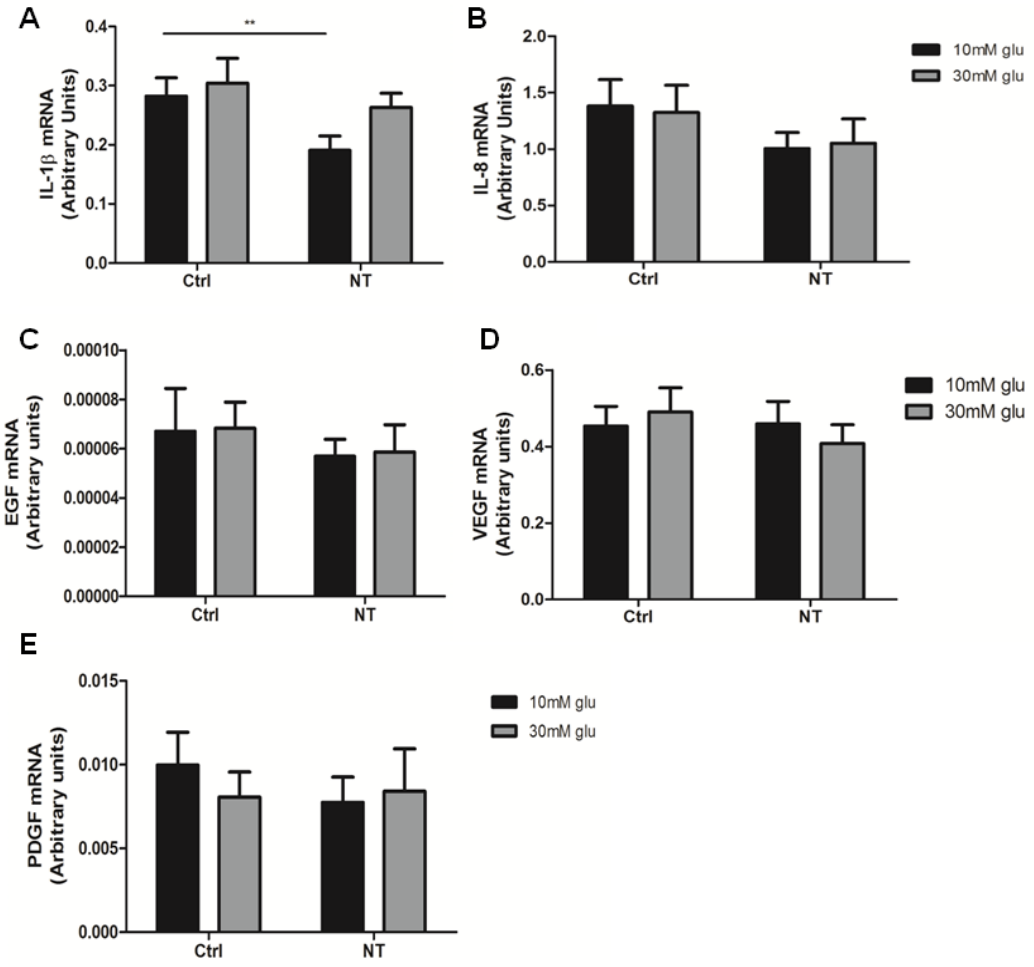

Figure 3.4: Expression of inflammatory cytokines (IL-1β and IL-8) and growth factors (EGF, VEGF, PDGF) in HaCaT cells, under either 10 or 30mM glucose, by real-time PCR. Cells were plated at $5x10^5$ /well and treated with 10nM NT during 6h or 24h. The relative gene expression is indicated as arbitrary units and was obtained after normalization with the HPRT gene. Results are presented as mean ± SEM of six to nine independent experiments. **p<0.01

mitogenic actions and modulate the functions of other different cell types in the skin (Dallos *et al.*, 2006). However, no studies have investigated the effects of NT in human keratinocytes under hyperglycemic conditions. Our results demonstrated that hyperglycemia reduced significantly NT and all NT receptors expression in human keratinocytes. In agreement with our results, previous studies have demonstrated that neuropeptides, such as substance P and neuropeptide Y expression are downregulated in skin of diabetic rabbits, correlating with a suppression of a proper inflammatory response at the injury site (Pradhan *et al.*, 2011). These results highlight the crucial role of neuropeptides in wound healing and a dysfunctional expression of these molecules under hyperglycemic conditions could be correlated with the physiopathology of diabetic foot ulcers. In addition, we verified that under hyperglycemic conditions, NT treatment significantly stimulated the expression of NT and NTR2 while NTR1 and NTR3 expression levels were unchanged. NTR1 and NTR2 are part of the high and low affinity G protein–coupled receptor family, respectively, while the NTR3 is a sortilin type I receptor with a single transmembrane domain (Vincent *et al.*, 1999a, Martin *et al.*, 2002). NTR2 is internalized inside the cell after NT binding with a lower affinity (30-40%) compared to NTR1 (60%). In the end, NTR2 is efficiently recycled to the cell surface (Mazella and Vincent, 2006). Our results may suggest that under hyperglycemia, these mechanisms could be modified. The NTR2 cellular coupling functions remain to be clarified, however various studies refer its involvement in the analgesic effect of the neuropeptide (Hermans and Maloteaux, 1998, Mazella and Vincent, 2006). In addition, NT fulfills the function of a growth factor in various human cancer cell lines; however the trophic effect of NT on these cells has always been attributed to the NTR1 and NTR3. Martin et al, 2002 (Martin *et al.*, 2002) showed that the structurally different receptors NTR1 and NTR3 were co-expressed in several human cancer cells on which NT exerts proliferative effects. We may speculate that high glucose conditions also induce structural modifications in NTR2 rendering keratinocytes unresponsive to NT. Indeed, and excluding a slight effect on migration, exogenous addition of NT did not modulate keratinocytes function under hyperglycemia, despite the increase in NT and NTR2 expression. To understand the role of NT in the important process of re-epithelialization in diabetics, we performed proliferation and migration assays. We verified that neither proliferation or migration are affected by NT. Only

hyperglycemia decreased keratinocyte migration after NT treatment. Moreover, since neuropeptides can stimulate cytokine and growth factors expression, IL-1β, IL-8, EGF, VEGF and PDGF were analyzed. Under normoglycemia, NT significantly decreased IL-1β expression while the expression of the other cytokines and growth factors were not affected by the NT stimulus. Proliferation and migration are important steps in re-epithelialization wound healing process that need the recruitment of cytokines and growth factors, such as TNF-α, IL1, EGF, VEGF and FGF (Schaffer *et al.*, 1998, Raja *et al.*, 2007). As these factors were not affected either by high glucose or by the NT stimulus, no direct effect where observed in migration and proliferation. However, it is known that the neuropeptides SP and CGRP require the production of IL-1β to support nociceptive sensitization in REKs (keratinocyte cell line) (Shi *et al.*, 2011).

We hypothesized that neuropeptides and specifically NT increase, in keratinocytes under hyperglycemic conditions, could have a paracrine effect on other skin cells, namely on macrophages and dendritic cells. In accordance, a previous study in our group demonstrated that NT promoted a pro-inflammatory status in a dendritic cell line (FSDC) under hyperglycemic conditions (da Silva *et al.*, 2011). Furthermore, in a macrophage cell line (Raw 264.7), we showed that NT stimulates migration and inhibits the pro-inflammatory status of macrophages contributing to the resolution of inflammation and allowing the progression to the migration-remodeling phases of wound healing (unpublished results). On the other hand, NT stimulated the phagocytic process in murine peritoneal macrophages (De la Fuente *et al.*, 1993). In addition, Jain et al, 2011 (Jain *et al.*, 2011) observed that hyperglycemia impairs dermal endothelial cell proliferation and tube formation, and these effects were mitigated by SP treatment.

3.6 Conclusions

Taken together, our results demonstrate that NT and all receptors are decreased under hyperglycemia and NT upregulates the expression of total NT and NT receptor 2 in human keratinocytes. However, NT did not affect proliferation, migration and expression of inflammatory

cytokines and growth factors in keratinocytes, under these conditions, reinforcing a potential paracrine effect of NT.

Further studies to analyze the function of NT and specifically NTR2 on human keratinocytes, under normal and high glucose conditions, are necessary to understand all the mechanisms involved.

3.7 Acknowledgments

This work was financially supported by COMPETE, FEDER and Fundação para a Ciência e Tecnologia (FCT-MES) under contracts, SFRH/BD/60837/2009, SFRH/BD/30563/2006, PTDC/SAU-BEB/71395/2006, PTDC/SAU-MII/098567/2008, PEst-C/SAU/LA0001/2013-2014 by EFSD/JDRF/Novo Nordisk European Programme in Type 1 Diabetes Research and Sociedade Portuguesa de Diabetologia.

3.8 References

Brun P, Mastrotto C, Beggiao E, Stefani A, Barzon L, Sturniolo GC, Palu G, Castagliuolo I (2005) Neuropeptide neurotensin stimulates intestinal wound healing following chronic intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 288:G621-629.

da Silva L, Neves BM, Moura L, Cruz MT, Carvalho E (2011) Neurotensin downregulates the pro-inflammatory properties of skin dendritic cells and increases epidermal growth factor expression. Biochim Biophys Acta 1813:1863-1871.

Dallos A, Kiss M, Polyanka H, Dobozy A, Kemeny L, Husz S (2006) Effects of the neuropeptides substance P, calcitonin gene-related peptide, vasoactive intestinal polypeptide and galanin on the production of nerve growth factor and inflammatory cytokines in cultured human keratinocytes. Neuropeptides 40:251-263.

De la Fuente M, Garrido JJ, Arahuetes RM, Hernanz A (1993) Stimulation of phagocytic function in mouse macrophages by neurotensin and neuromedin N. J Neuroimmunol 42:97-104.

Hermans E, Maloteaux JM (1998) Mechanisms of Regulation of Neurotensin Receptors. . Pharmacol Ther 79:89–104.

Jain M, LoGerfo FW, Guthrie P, Pradhan L (2011) Effect of hyperglycemia and neuropeptides on interleukin-8 expression and angiogenesis in dermal microvascular endothelial cells. J Vasc Surg 53:1654-1660 e1652.

Kawai K, Kageyama A, Tsumano T, Nishimoto S, Fukuda K, Yokoyama S, Oguma T, Fujita K, Yoshimoto S, Yanai A, Kakibuchi M (2008) Effects of adiponectin on growth and differentiation of human keratinocytes--implication of impaired wound healing in diabetes. Biochemical and biophysical research communications 374:269-273.

Lan CC, Liu IH, Fang AH, Wen CH, Wu CS (2008) Hyperglycaemic conditions decrease cultured keratinocyte mobility: implications for impaired wound healing in patients with diabetes. Br J Dermatol 159:1103-1115.

Lazarus LH, Brown MR, Perrin MH (1977) Distribution, localization and characteristics of neurotensin binding sites in the rat brain. Neuropharmacology 16:625-629.

Legat FJ, Armstrong CA, Ansel JC (2002) The cutaneous neurosensory system in skin disease. Adv Dermatol 18:91-109.

Luger TA, Lotti T (1998) Neuropeptides: role in inflammatory skin diseases. J Eur Acad Dermatol Venereol 10:207-211.

Martin S, Navarro V, Vincent JP, Mazella J (2002) Neurotensin receptor-1 and -3 complex modulates the cellular signaling of neurotensin in the HT29 cell line. Gastroenterology 123:1135-1143.

Mazella J, Vincent JP (2006) Internalization and recycling properties of neurotensin receptors. Peptides 27:2488-2492.

Pradhan L, Cai X, Wu S, Andersen ND, Martin M, Malek J, Guthrie P, Veves A, Logerfo FW (2011) Gene expression of pro-inflammatory cytokines and neuropeptides in diabetic wound healing. J Surg Res 167:336-342.

Pradhan l, Nabzdyk C, Andersen N, LoGerfo F, Veves A (2009a) Inflammation and neuropeptides: the connection in diabetic wound healing. Expert Reviews in Molecular Medicine 11:1-24.

Pradhan L, Nabzdyk C, Andersen ND, LoGerfo FW, Veves A (2009b) Inflammation and neuropeptides: the connection in diabetic wound healing. Expert Rev Mol Med 11:e2.

Raja, Sivamani K, Garcia MS, Isseroff RR (2007) Wound re-epithelialization: modulating keratinocyte migration in wound healing. Frontiers in bioscience : a journal and virtual library 12:2849-2868.

Schaffer M, Beiter T, Becker HD, Hunt TK (1998) Neuropeptides: mediators of inflammation and tissue repair? Arch Surg 133:1107-1116.

Shaw JE, Sicree RA, Zimmet PZ (2010) Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes research and clinical practice 87:4-14.

Shi X, Wang L, Li X, Sahbaie P, Kingery WS, Clark JD (2011) Neuropeptides contribute to peripheral nociceptive sensitization by regulating interleukin-1beta production in keratinocytes. Anesth Analg 113:175-183.

Silva L, Carvalho E, Cruz MT (2010) Role of neuropeptides in skin inflammation and its involvement in diabetic wound healing. Expert Opin Biol Ther 10:1427-1439.

Steinhoff M, Brzoska T, Luger TA (2001) Keratinocytes in epidermal immune responses. Curr Opin Allergy Clin Immunol 1:469-476.

Steinhoff M, Stander S, Seeliger S, Ansel JC, Schmelz M, Luger T (2003) Modern aspects of cutaneous neurogenic inflammation. Arch Dermatol 139:1479-1488.

Tellechea A, Leal E, Veves A, Carvalho E (2010) Inflammatory and angiogenic abnormalities in diabetic wound healing: role of neuropeptides and therapeutic perspectives The Open Circulation and Vascular Journal 3:43-55.

Vincent J, Mazella J, Kitabgi P (1999a) Neurotensin and neurotensin receptors. TiPS 20:302-309.

Vincent JP, Mazella J, Kitabgi P (1999b) Neurotensin and neurotensin receptors. Trends Pharmacol Sci 20:302-309.

Zhao D, Zhan Y, Zeng H, Koon HW, Pothoulakis C (2005) Neurotensin stimulates interleukin-8 expression through modulation of IκBα phosphorylation and p65 transcriptional activity: involvement of protein kinase Cα Molecular Pharmacology 67:2025-2031.

Chapter 4

In vivo study

Chitosan-based dressings loaded with neurotensin- an efficient strategy to improve early diabetic wound healing

This Chapter comprises the work submitted in

Acta Biomaterialia (2013) by

Moura, L.I. F.,. Dias, A. M.A., Leal, E. C., Carvalho, L., de Sousa, H.C., Carvalho, E.

4.1 Abstract

One important complication of diabetes mellitus is the chronic, non-healing diabetic foot ulcer (DFU). This study aims to develop and use dressings based on chitosan derivatives for the sustained delivery of the neurotensin (NT), a neuropeptide that act as an inflammatory modulator in wound healing. Three different derivatives, namely N-carboxymethyl chitosan (CMC), 5-methyl pyrrolidinone chitosan (MPC) and N-succinyl chitosan (SC), are presented as potential biomaterials for wound healing applications. Our results showed that MPC has the best fluid handling capacities and delivery profile being also non-toxic to Raw 264.7 and HaCaT cells. NT-loaded and non-loaded MPC dressings were applied into control/diabetic wounds to evaluate their in vitro/in vivo performances and the results show that the first induced a faster healing (50% wound area reduction) in the early phases of wound healing in diabetic mice. NT-loaded MPC dressings also reduced inflammatory cytokines expression namely TNF-α ($p<0.001$) and decreased the inflammatory infiltrate at day 3. At day 10, MMP-9 is reduced in diabetic skin ($p<0.001$) increasing significantly fibroblasts migration and collagen (COL1A1, COL1A2 and COL3A1) expression and deposition. These results suggest that MPC-based dressings may work as an effective support for a NT sustained release to modulate DFU.

Keywords: Chitosan derivatives; wound dressings; diabetic foot ulcers; neurotensin; wound healing

4.2 Introduction

Diabetes mellitus is one of the most prevalent chronic diseases worldwide. Impaired wound healing is a complication of diabetes that results in the failure to completely heal diabetic foot ulcers (DFUs) (Moura *et al.*, 2013). Complications of DFUs lead to frequent hospitalizations and in extreme cases, to amputations that result in elevated hospital costs and poor quality of life for

patients (Tellechea *et al.*, 2010). DFU is a multifactorial complication that results particularly as a consequence of peripheral neuropathy, impaired vascular function, impaired angiogenesis and/or chronic inflammation(Silva *et al.*, 2010, Moura *et al.*, 2013).

Recently, it became evident that peripheral nerves and cutaneous neurobiology contributes to wound healing (Pradhan *et al.*, 2009). Loss of peripheral sensory and autonomic nerves reduces the production of neuropeptides that are important for proper wound healing (Silva *et al.*, 2010). Neurotensin (NT) is a bioactive neuropeptide that is widely distributed in the brain and in several peripheral tissues (Lazarus *et al.*, 1977, Sundler *et al.*, 1977). NT interacts with leukocytes, mast cells, dendritic cells and macrophages leading to cytokine release and chemotaxis that can modulate the immune response. In addition, NT affects microvascular tone, vessel permeability, vasodilation/vasoconstriction and new vessel formation which helps to improve angiogenesis during wound healing processes (Brain, 1997, Silva *et al.*, 2010, Kalafatakis and Triantafyllou, 2011).

Some studies demonstrated that topical application of neuropeptides, such as substance P and neuropeptide Y can improve wound healing in diabetes (Scott *et al.*, 2008, Pradhan *et al.*, 2011). However, the major problem of topical administration of peptides is their short half-life and loss of bioactivity in the peptidase-rich wound environment (Sweitzer *et al.*, 2006). An alternative strategy to overcome this problem is the use of biocompatible wound dressings for the sustained delivery of neuropeptides. Also, 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 (Malafaya *et al.*, 2007, Sell *et al.*, 2010).

Wound dressings based on natural polymers have been extensively applied to simulate extracellular matrix (ECM) regeneration after injury (Malafaya *et al.*, 2007, Sell *et al.*, 2010). One of the most used natural-based polymer for wound healing applications is chitosan (Malafaya *et al.*, 2007), which is a linear copolymer of D-glucosamine and *N*-acetyl-D-glucosamine (Rinaudo, 2006). Since it is derived from chitin, a polymer found in fungal cell walls and crustacean exoskeletons, it is a relatively inexpensive and abundant material (Park *et al.*, 2009). In addition, it has been proven to

be biodegradable, biocompatible, non-antigenic, non-toxic, bioadhesive, anti-microbial, bioactive and to have haemostatic capacity (Park *et al.*, 2009, Huang and Fu, 2010, Dai *et al.*, 2011). Furthermore, chitosan promotes tissue granulation and accelerates wound healing through the recruitment of inflammatory cells such as polymorphonuclear leukocytes (PMN) and macrophages to the wound site (Takei *et al.*, 2012).

To increase its poor solubility in water, chitosan functional groups can be chemically modified to originate water soluble chitosan derivatives such as *N*-carboxymethyl chitosan (CMC), 5-methyl pyrrolidinone chitosan (MPC) and *N*-succinyl chitosan (SC) (Berscht *et al.*, 1994, Dai *et al.*, 2008, Tan *et al.*, 2011). These chitosan derivatives are functional biomaterials that maintain the antibacterial and non-cytotoxic properties of parent chitosan. In addition, they stimulate extracellular lysozyme activity of skin fibroblasts (Chen *et al.*, 2002, Prabaharan, 2008).

The aim of this study was to develop and apply wound dressings, prepared from the chitosan derivatives referred above (CMC, MPC, SC), for a prolonged and efficient NT delivery into diabetic and non-diabetic wounds, and also confer wound protection and comfort. The progression of skin wound healing in diabetic and non-diabetic mice was also evaluated by the analysis of the inflammatory and angiogenic effects of NT when applied in skin wounds alone or loaded into MPC-based dressings.

4.3 Materials and Methods

4.3.1 Materials

Chitosan (medium molecular weight, degree of acetylation of 90% confirmed by ^{1}H-NMR), glyoxylic acid monohydrate (98%), sodium hydroxide, sodium borohydride (99.5%), levulinic acid (98%), succinic anhydride (97%), reduced GSH, DTNB, dialysis membranes (Spectra/Por (6)) with a MWCO of 8000 Da and methanol p.a., were obtained from Sigma-Aldrich (USA). Acetic acid was obtained from Panreac (Spain), and ethanol was purchased from Riedel-de-Haen (Germany). 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 antibodies against VEGF and actin were purchased from the Millipore Corporation (USA).

4.3.2 Synthesis of chitosan derivatives CMC, MPC, SC

Chitosan (2g) reacted with glyoxylic acid (1,16g), levulinic acid (5ml) or succinic anhydride (3g) to synthesize CMC, MPC and SC respectively (Muzzarelli *et al.*, 1993, Santos *et al.*, 2005), following by precipitation with ethanol and dialysis to obtain more purified samples. Dressings of CMC, MPC and SC were prepared by freeze-drying adding 1.5 ml of each solution in 12 multi well plates. The average thickness of the obtained materials was 250±15 μm. All samples were stored at −20 ^{0}C, away from light and humidity before usage. The degree of substitution of each of the derivatives was calculated by NMR using a Bruker Avance III 400 MHz spectrometer, with a 5-mm TIX triple resonance detection probe using D_2O acidified with acetic acid (10μl of acetic acid in 600μl of D_2O).

4.3.3 Scanning electron microscopy (SEM)

SEM micrographs were obtained at 25 kV (Jeol, model JSM-5310, Japan). Samples were coated with gold (approximately 300 Å) in an argon atmosphere.

4.3.4 Water vapor and water sorption capacities

Samples of CMC, MPC and SC, with 22mm of diameter, were dried at 37^{0}C for 72h until constant weight was achieved. Both water vapor and water sorption capacities were measured gravimetrically. In the first case, dried dressings were then exposed to a 95% relative humidity atmosphere, in a desiccator containing a saturated solution of potassium sulfate at 32°C accordingly to Dias et al, 2013. In the second case, samples were immersed into phosphate buffer pH 7 at 37^{0}C and weighted after removing the surface phosphate buffer using filter paper.

Samples were weighted at fixed time until they reach equilibrium. The water vapor and water sorption capacities were calculated as the ratio between sample weight at time *t* and sample initial dry weight. All the samples were measured in duplicate.

4.3.5 *In vitro* release kinetics

Kinetic release profiles of GSH were performed spectrophotometically (Jasco, model 630, Japan), at 412nm. Known amounts of a GSH solution (5mM) were loaded into previously weighted samples of each polymer. The GSH solution has been previously placed in an ultrasonic bath to avoid oxidation. After drying, samples were immersed in phosphate buffer at pH 6, 7 or 8 at 32ºC, under orbital stirring (100 rpm) during 8h. The quantification of released GSH was based on the Ellman's Test. This test is based on the addition of 5,5'-dithio-*bis*-(2-nitrobenzoic acid) (DTNB), a yellow water-soluble compound, that reacts with free sulfhydryl groups in peptide solution. At pre-determined time periods, an aliquot (100µl) of the released solution was removed and analyzed with a mixture of 1800µL of phosphate buffer and 100µl of DNTB stock solution (20mM). Fresh 100µL of phosphate buffer was added each time point to the medium. Each sample was analyzed in duplicate.

4.3.6 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), 3.02g/l sodium bicarbonate, 30mM glucose, 100U/ml penicillin, 100µg/ml streptomycin, at 37ºC 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 by ATCC (number TIB-71) and CLS (number 300493), respectively.

4.3.7 MTT assay

Raw 264.7 (8×10^4 cells/well) and HaCaT (4×10^4 cells/well) cells were plated individually in 12-

well_plates with 430 µL of DMEM, above the previously sterilized biomaterials (UV light for at least 30 minutes). After 24 and 48h of incubation, 43µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5mg/ml) was added to each well. The plates were further incubated at 37ºC for 1h, in a humidified incubator containing 5%CO_2. After this period, 300µl of acidic isopropanol (0.04 N HCl in isopropanol) was added. Quantification was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a reference wavelength of 620 nm. Each sample was analyzed in duplicate.

4.3.8 NO production – Griess Method

Raw 264.7 (8×10^4 cells/well) cells were plated in 12-well plates with 430µL of DMEM, above the previously sterilized biomaterials (UV light for at least 30 minutes). After 24 and 48 h after incubation, 170µl 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 minutes of incubation in the dark, the absorbance was measured at 550nm in a microplate reader (SLT, Austria). Nitrite concentration was calculated from a previously obtained nitrite standard curve.

4.3.9 *In vivo* wound closure

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

Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 150 mg/kg) in citrate buffer pH 4.5. Four days after diabetes induction, blood glucose levels were checked by Accu-Chek Aviva (Roche Diagnostics GmbH, Germany). The animals with blood glucose levels higher than 300 mg/dl were considered diabetic. Mice were anesthetized by intraperitoneal

injection of xylazine (13mg/kg) and ketamine (66.7mg/kg). The dorsal hair of diabetic mice was shaved and two full-thickness wounds of 25mm^2 were created with a biopsy punch.

C57BL/6 mice were randomly divided into six groups of treatments for control (non-diabetic) and diabetic mice – three groups for day 3 (d3) (I, II, III) and three similar groups for day 10 (d10) (IV, V, VI): groups I and IV were treated with MPC dressings alone, groups II and V with topical application of 50µg/ml NT and groups III and VI with 50µg/ml NT-loaded MPC dressings. In each animal one of the wounds served as control (PBS application only) and the other received treatment. The progress of wound healing was evaluated periodically by acetate tracing. Topical application of PBS or NT (alone or loaded into the prepared MPC dressing) was done daily. At day 3 or day 10, C57BL/6 mice were sacrificed and ulcerative tissue and skin surrounding the ulcer were harvested.

4.3.10 Real time RT-PCR

Total RNA was isolated from skin 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 MyCycler iQ5. Primer sequences are in table 4.1. Gene expression changes were analyzed using iQ5Optical system software v2. The results were normalized using a reference gene, TATA box binding protein (TBP).

4.3.11 Western Blotting

Skin tissue lysate was homogenized in RIPA buffer (50mM Tris HCl pH8, 150mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 2mM EDTA, proteases inhibitor cocktail, phosphatase inhibitor cocktail and 1mM DTT). Protein concentration was determined using the BSA method and the skin lysates were denatured at 95ºC, for 5min, in sample buffer. 40µg 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 1h,

at room temperature. After blocking, membranes were incubated with the primary antibodies against the TNF-α (1:500), VEGF (1:1000), MMP-9 (1:500), overnight at 4 ºC. After incubation, membranes were washed and incubated for 1h 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.

4.3.12 Hydroxyproline content

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

4.3.13 Histopathological analysis

For histological preparation, the skin was fixed in 10% neutral buffered formalin and then embedded in paraffin. Skin tissues were sectioned in 3µm thickness slices for histopathological examination by hematoxylin/eosin (H&E) and for collagen formation by Masson's trichrome staining, using standard procedures. 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.

4.3.14 Statistical analysis

Results are expressed as mean ± SEM (Structural equation modeling). Statistical analysis was

Table 4.1: RT-PCR primers used for analysis of gene expression..

Primer	5'-3'Sequence (Forward; Reverse)
TNF-α	For: CAAGGGACTAGCCAGGAG Rev:TGCCTCTTCTGCCAGTTC
IL-6	For: TTCCATCCAGTTGCCTTC Rev:TTCTCATTTCCACGATTTCC
KC	For: ATTAGGGTGAGGACATGTGTGGGA Rev: AATGTCCAAGGGAAGCGTCAACAC
IL-1β	For: ACCTGTCCTGTGTAATGAAAG Rev:GCTTGTGCTCTGCTTGTG
MMP-9	For: CATAGAGGAAGCCCATTACAG Rev: GATCCACCTTCTGAGACTTCA
EGF	For: ACGGCACAGTTTGTCTTCAATGGC Rev: TGTTGGCTATCCAAATCGCCTTGC
VEGF	For: CTTGTTCAGAGCGGAGAAAGC Rev: ACATCTGCAAGTACGTTGGTT
PDGF	For: AGATCTGCCACGCACTCATCCTT Rev: ACGCACACTGCACCTCTAATCCAT
TGFβ1	For: TCAATACGTCAGACATTCGGG Rev: CGTGGAGTTTGTTATCTTTGC
TGFβ3	For: ACTATGCCAACTTCTGCTCAG Rev: GGTCTGTCGCTTTGGTTTTC
COL1A1	For: AGGCTTCAGTGGTTTGGATG Rev: TTCACCCTTAGCACCAACTG
COL1A2	For: AAGGATACAGTGGATTGCAGG Rev: AACCAAAGTCATAGCCACCTC
COL3A1	For: ACCTAAAATTCTGCCACCCC Rev: GCACATCAACGACATCTTCAG
TBP	For: ACCCTTCACCAATGACTCCTATG Rev: TGACTGCAGCAAATCGCTTGG

performed using one-way ANOVA followed by Tukey's multiple comparison tests or through the unpaired or paired t test by GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) and p values lower than 0.05 were considered statistically significant.

4.4 Results

4.4.1 Degree of substitution and morphology of CMC, MPC and SC

The degree of substitution (amount of native chitosan amino groups substituted) of each chitosan

derivative was confirmed by ^{1}H-NMR and it was equal to 25.5%, 24% and 28.5% for CMC, MPC and SC, respectively (Figure 4.2). The schematic representation of each derivative is shown in Figure 4.1A

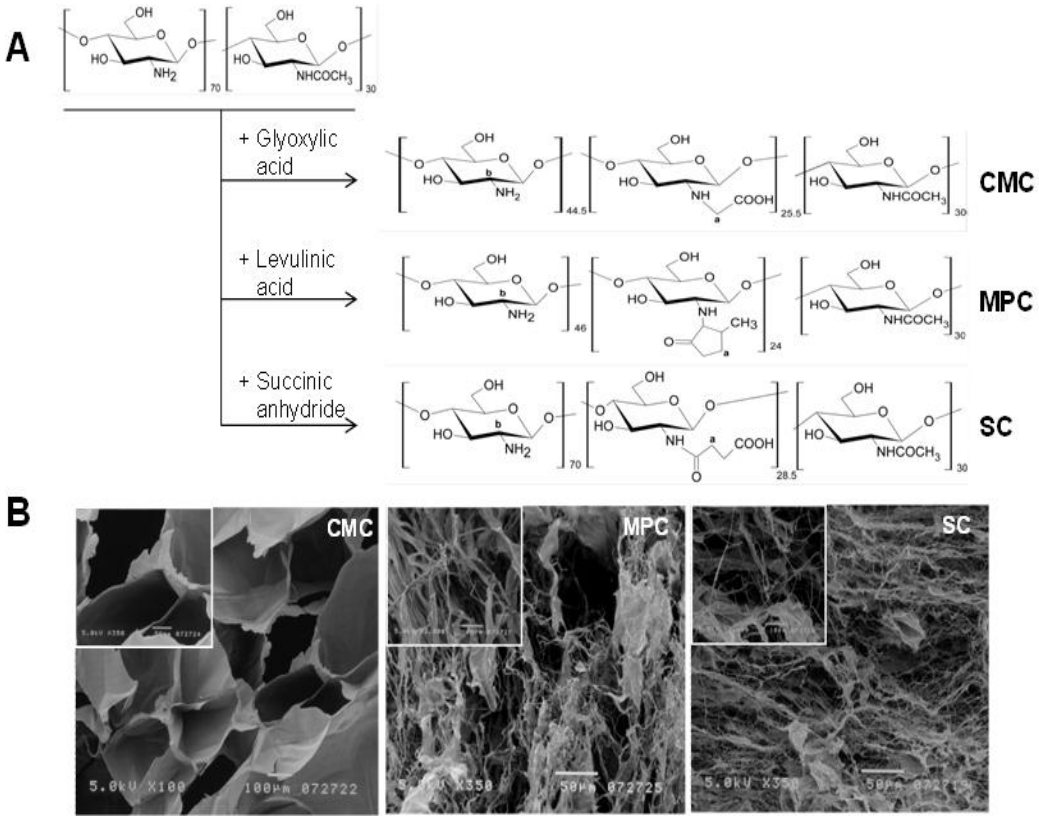

Figure 4.1. A) Chemical synthesis of chitosan derivatives: *N*-carboxymethyl chitosan (CMC), 5-methyl pyrrolidinone chitosan (MPC) and *N*-succinyl chitosan (SC). B) SEM micrographs for non-loaded chitosan derivatives CMC, MPC and SC representing the different structures obtained by freeze-drying. Inner images represent magnifications.

The different morphologies obtained for each of the prepared chitosan derivative dressings are shown in Figure 1B. CMC presents a honeycomb-like porous structure, with larger pores than MPC and SC, which presented an interlaced fiber-like pattern. The fiber-like structure of SC seems to be thinner than the one observed for MPC.

4.4.2 Water vapor and water swelling properties

Figure 4.2A shows the water vapor sorption behavior of CMC, MPC and SC dressings in

controlled humidity (95%) and temperature conditions (32ºC). Data shows that the hydrophilicity of the materials change in the sequence SC>MPC>CMC. All the samples achieved equilibrium

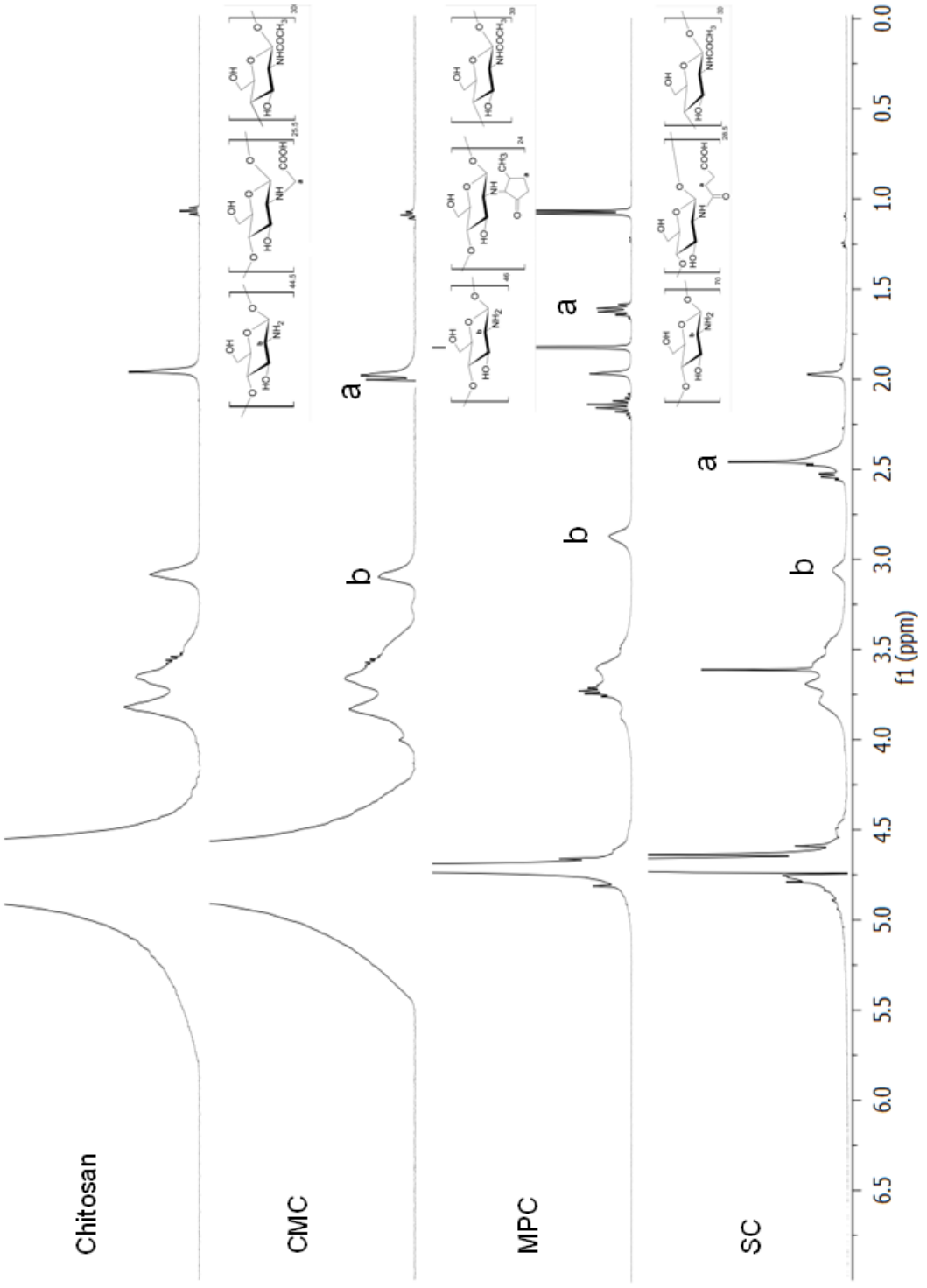

Figure 4.2. ^{1}H-RMN spectra of chitosan, CMC, MPC and SC dressings.

after approximately 8hours and at this point, SC adsorbed 35% of its weight in water vapor while MPC and CMC adsorbed 24% and 14%, respectively.

In terms of water swelling capacity, Figure 4.2 B shows that SC presents the fastest swelling rate, reaching its maximum (2438%) after 5 hours and it starts to dissolve after this period. On the other hand, CMC presented the lowest swelling capacity (163%) while MPC has an intermediate water swelling profile. Both MPC and SC dressings reach water swelling equilibrium after approximately 6 hours and both maintain their structure (macroscopically, at naked eye) until day 15, at the tested experimental conditions.

4.4.3 *In vitro* release kinetics

Glutathione (GSH) was used as a model peptide test molecule for *in vitro* release kinetics studies. The release of GSH from CMC, MPC and SC dressings was followed for a period of 8 hours at 3 different pHs (6, 7 and 8) (Figure 4.3 A, B and C, respectively), which is the pH range that can be observed during the wound healing process. The release profiles show that equilibrium is attained between 5 and 8h for all the samples and that the amount of GSH released from SC is significantly higher than for CMC and MPC (~9 and 4 times higher, respectively). When comparing the amount of GSH released after 8h with the total GSH loaded amount, the results show that ~50% was released from CMC and MPC while almost 100% was released from SC. Obtained results also show that the amount of GSH released from the chitosan derivatives is not significantly affected in the pH range studied and considering the experimental error, being average equal to (32.33±0.72), (67.65±6.77) and (287.18±14.92) $m_{GSH\ released}/(m_{GSH\ loaded} \times m_{polymer})$ for CMC, MPC and SC, respectively.

4.4.4 *In vitro* biocompatibility of CMC and MPC

There was no significant difference in the viability of the Raw and HaCaT cells exposed to CMC and MPC dressings during 24, 48 and 72h, when compared to control, as shown in Figure 4.4 (A and B, respectively). NO is produced by macrophages in response to an inflammatory stimuli. The

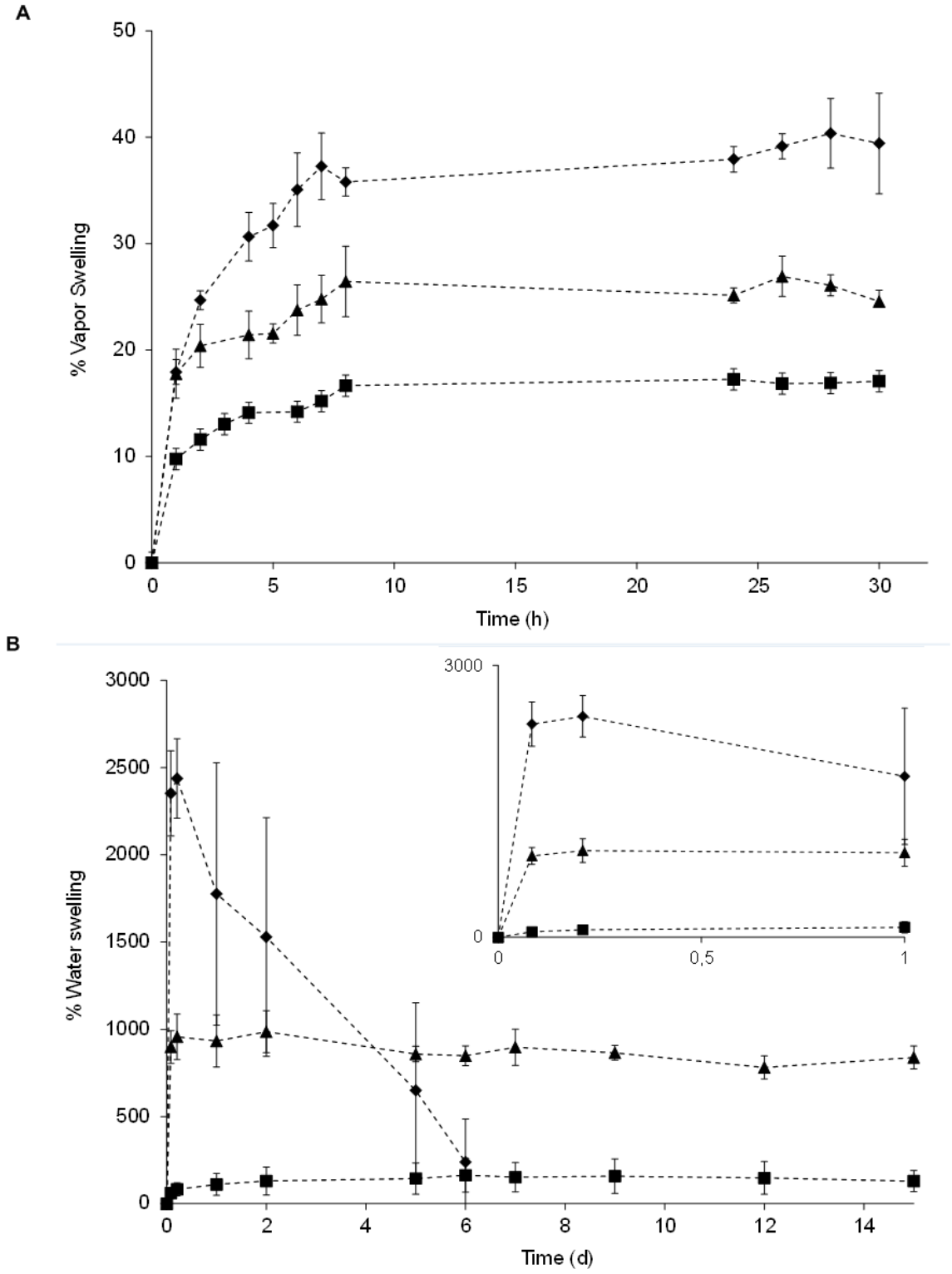

Figure 4.3. Water vapor (A) and water (B) swelling profiles observed for CMC (■), MPC (▲) and SC (♦) dressings. Lines serve only as guides for the eye. Results are presented as mean ± SEM of two independent experiments.

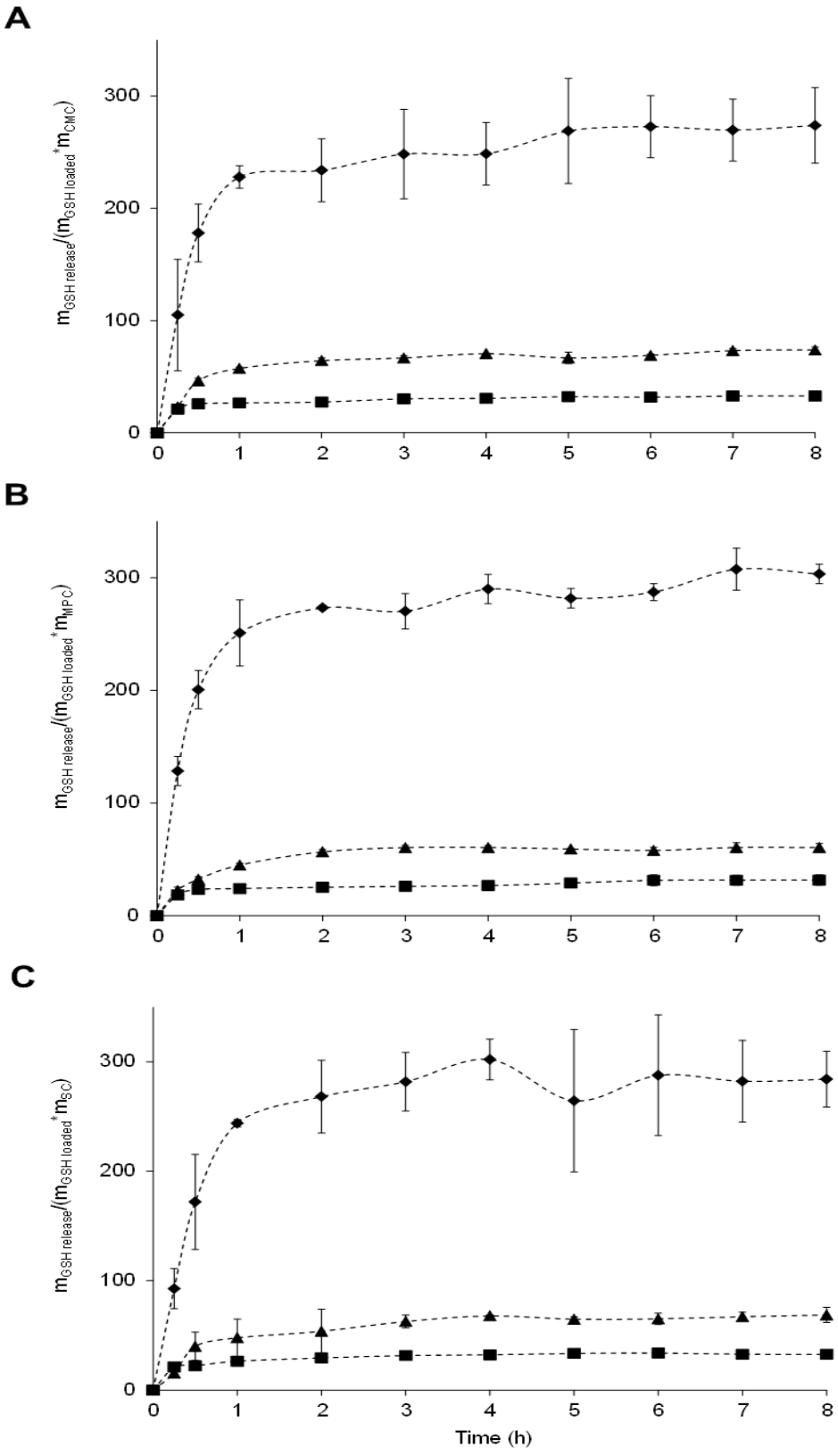

Figure 4.4. Release kinetic profiles for GSH from CMC (■), MPC (▲) and SC (♦) dressings at pH 6 (A), 7 (B) and 8 (C) measured for 8 h at 37 ºC. Lines serve only as guides for the eye. Results are presented as mean ± SEM of two independent experiments.

production of nitrites, final stable breakdown product of NO, measured after exposure of the cells to the chitosan derivatives (Figure 4.4C) was not also significantly affected, however, a slight increase in the nitrites produced after 72h was observed, which may be due to the stress to which cells are subjected after this exposure period.

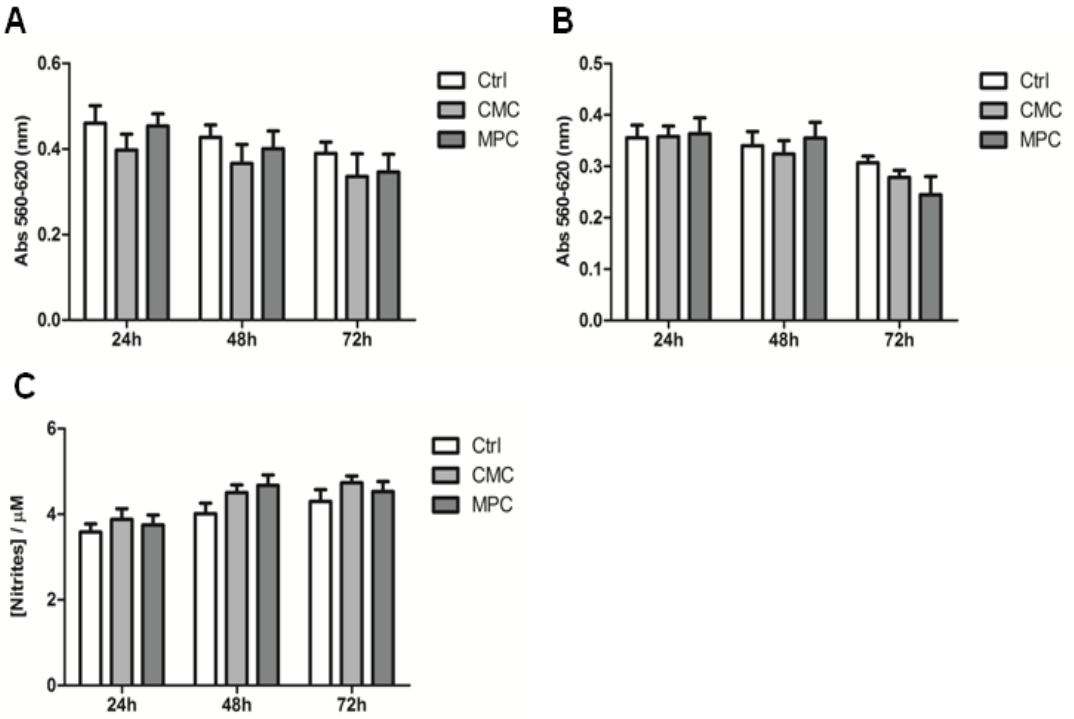

Figure 4.5. Cell viability of Raw (A) and HaCaT (B) cells in the presence of CMC or MPC dressings, during 24, 48 and 72 h. and NO production in Raw cells (C). Results are presented as mean ± SEM of three independent experiments.

4.4.5 Wound healing experiments – *in vivo*

Figure 4.5 shows the effect of the different topical treatments studied in this work: NT alone, MPC foam alone and NT-loaded MPC foam both in control (A and B) and diabetic (C and D) mice. PBS was applied as control. All treatments were shown to reduce significantly the wound area, as compared to PBS treated wounds, in both control and diabetic mice. In Figure 4.5 A and C, NT alone reduced significantly the wound size at day 3 post wounding, by 22% ($p<0.05$), compared to the PBS treated wounds, in control mice. In diabetic mice, the wound size of the NT treated wounds is also significantly reduced at day 3, and at day 5 by 29% ($p<0.01$) and 34% ($p<0.01$), respectively. A different healing profile is observed for the non-loaded and NT-loaded MPC treated

wounds either in control and diabetic mice. A significant decrease in the wound area is evident at day 1 post wounding in non-loaded MPC by 48% ($p<0.001$) and in NT-loaded MPC, by 43% ($p<0.001$), when compared with PBS-treated wounds (Figure 4.5A). In diabetic animals, the profile of wound closure was similar, however the NT-loaded MPC treatment was significantly more effective than MPC alone, with a wound reduction of 50% ($p<0.001$) instead of 35% ($p<0.001$) of closure for the non-loaded dressing (Figure 4.5C).

Throughout the entire monitored period (10 days), the treatments with non-loaded MPC and NT-loaded MPC were more effective in decreasing the wound size, when compared to the PBS, in control mice. However, in diabetic mice, the most significant treatment was the NT-loaded MPC ($p<0.001$) compared to PBS control (Figure 4.5 B and D). Finally, the effect was more pronounced for the NT-loaded MPC foam. After this period, the NT treatment alone induced a wound size reduction only in diabetic animals ($p<0.01$), as observed by the area under the curve.

4.4.6 Cytokine expression at the wound site

In order to address the pattern of cytokine gene expression in untreated or treated wounds at 0, 3 and 10 days post-wounding, the gene expression for inflammatory cytokines (TNF-α, IL-6, KC, IL-1β), MMP-9, growth factors (EGF, VEGF, PDGF), TGFβ1, TGFβ3 and several types of collagen genes (COL1A1, COL1A2, COL3A1) were measured and the results are presented in Figure 4.6 A-Z.

In unwounded skin (day 0, baseline), all the measured inflammatory cytokines were significantly increased in the skin of diabetic animals compared with the healthy controls (Figure 4.6 A-G). In other hand, TGFβ3 expression is significantly reduced ($p<0.01$) in diabetic unwounded skin as well as all types of collagen analyzed (Figure 6 S-Y, respectively). We observed a significant increase, at day 3 post-wounding, in the inflammatory stimulus, as one might expect, when compared to day 0 in controls. However, the same effect is not observed in diabetic mice. Furthermore, at day 3, in control mice, the MPC treatment alone reduced significantly the expression of TNF-α ($p<0.05$),

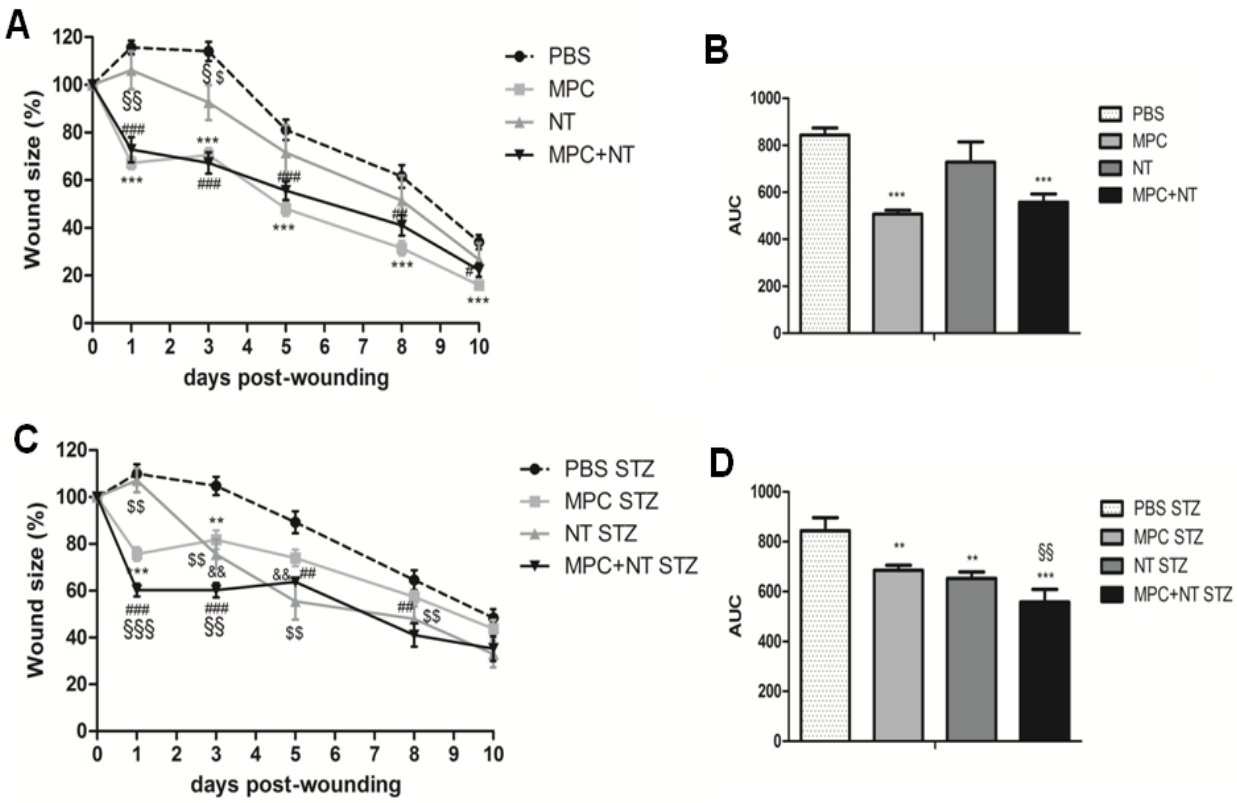

Figure 4.6 Wound size measurements for MPC, NT and NT-loaded MPC foam treatments in either control (A) or diabetic (C) mice. The wound size was determined at days 0, 1, 3, 5, 8 and 10 post-wounding. Area under the curve (AUC) was obtained from the wound closure with the different treatments in control (B) and diabetic (D) mice. Results are presented as mean ± SEM of seven to eighteen independent experiments. *p < 0.05 MPC compared to PBS, **p < 0.01 MPC compared to PBS, *** p < 0.001 MPC compared to PBS, # p < 0.05 MPC+NT compared to PBS, ## p < 0.01 MPC+NT compared to PBS, ### p < 0.001 MPC+NT compared to PBS, $ p < 0.05 compared to PBS, $$ p < 0.01 NT compared to PBS; §p < 0.05 NT compared to MPC+NT, §§ p < 0.01 NT compared to MPC+NT, && p < 0.01 MPC compared to MPC+NT

IL-6 ($p<0.05$) and IL-1β ($p<0.05$) while the NT alone decreased the expression of TNF-α ($p<0.05$) and IL-1β ($p<0.05$) (Figure 6 A, C and G, respectively).In addition, the NT-loaded MPC treatment reduced the TNF-α expression ($p<0.05$), however the IL-6 and KC expression significantly increased in the controls ($p<0.05$). In diabetic mice, the TNF-α expression was significantly higher for all treatments ($p<0.05$) but the IL-1β expression is reduced upon the NT-loaded MPC treatment ($p<0.05$) compared with PBS alone.

Moreover, at day 3, NT alone reduced the EGF expression in diabetic mice ($p<0.05$) and increased the VEGF expression ($p<0.05$) in the control (Figure 6 K). In addition, at day 3, while NT and NT-loaded MPC foam significantly induced TGFβ3 expression ($p<0.001$) in controls, no differences where observed in diabetic skin (Figure 6 S). Moreover, at day 3, the collagen genes were more

expressed in control skin and NT treatment significantly increased COL1A1, COL1A2 and COL3A1 expression in diabetic skin (Figure 6 U-Y, respectively).

At day 10, the expression of all the inflammatory cytokines was diminished to baseline levels in the controls, with the exception of TNF-α that increase ($p<0.05$) with NT and the NT-loaded MPC application, compared to PBS treated wounds. In diabetic mice, all the treatments reduced the expression of TNF-α, IL-6 and KC ($p<0.05$ in all cases) (Figure 4.6 B, D and F, respectively). The non-loaded and the NT-loaded MPC treatments caused a decrease in the MMP-9 expression in both control and diabetic mice ($p<0.05$) (Figure 6 J). In addition, the NT-loaded MPC treatment reduced EGF in diabetic mouse skin ($p<0.05$) (Figure 6 L).

NT and NT-loaded MPC foam significantly induced TGFβ1 and TGFβ3 expression ($p<0.001$) in controls at day 10, no differences where observed in diabetic skin. In diabetic skin, only NT treatment reduced significantly TGFβ3 ($p<0.05$) (Figure 4.6 R, T). In addition, NT and NT-loaded MPC foam highly stimulated an increase in COL1A1 and COL1A2 ($p<0.001$) in control mice while in diabetic mice only NT-loaded MPC significantly induced expression of all collagen genes (Figure 4.6 V, X, Z).

4.4.7 Protein expression in the wound site

To evaluate protein expression levels at the wound site, Western Blot analysis of skin tissue was performed (Figure 4.7). At day 0, only MMP-9 is significantly increased ($p<0.001$) in diabetic mice when compared to controls. At day 3, NT treatment induced a reduction of MMP-9 protein levels in control mice. Moreover, in diabetic wounds, MPC treatment increased TNF-α level. In contrast, NT and NT-loaded MPC foam significantly reduced MMP-9 ($p<0.05$) and TNF-α ($p<0.001$) protein levels, respectively.

4.4.8 Hydroxyproline content in the wound site

To evaluate collagen deposition in mouse skin, hydroxyproline levels were measured in unwounded and wounded treated and non-treated with NT (Figure 4.8). In unwounded skin,

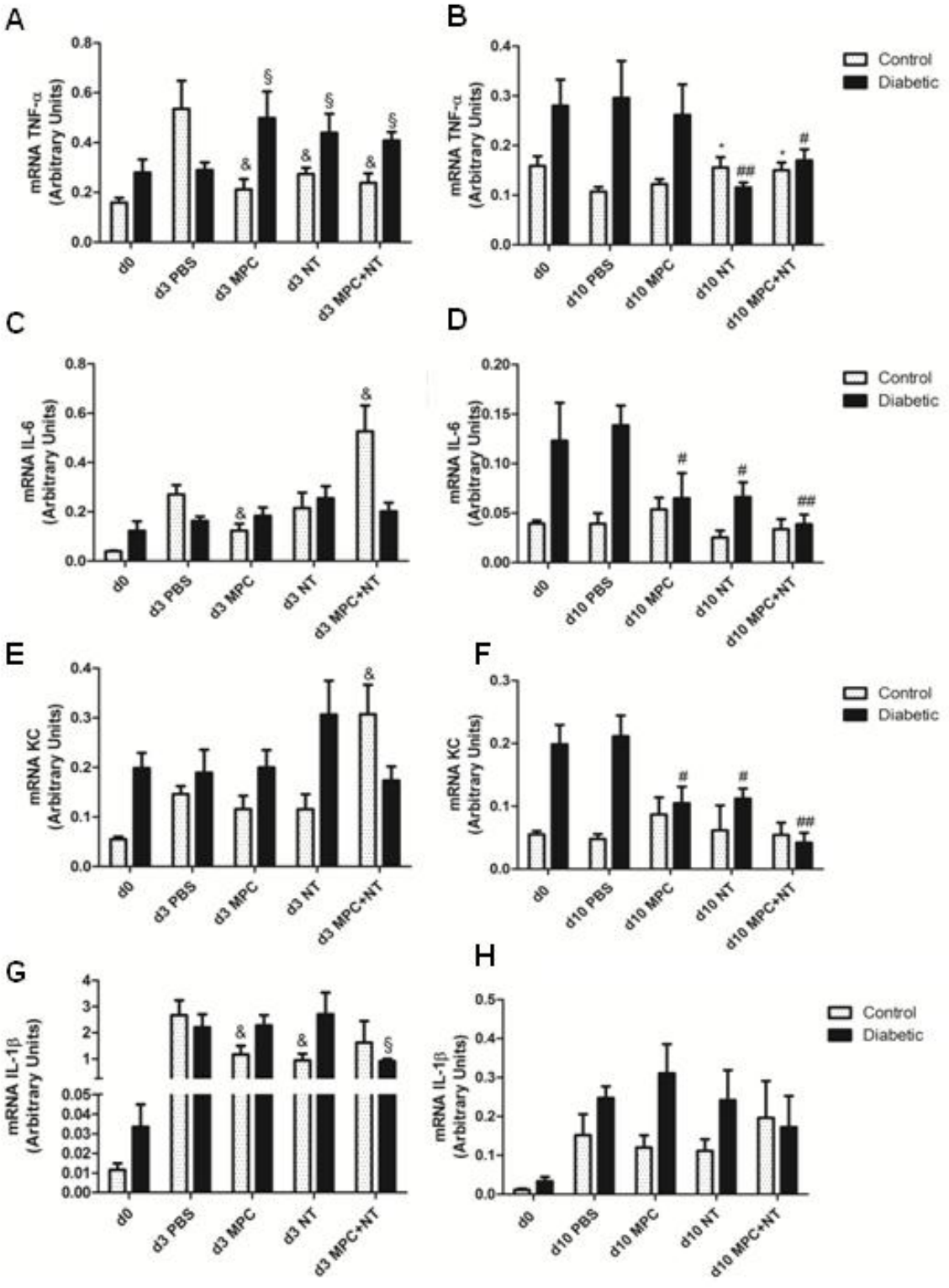

Figure 4.7. The gene expression profile for TNF-α, IL-6, KC, IL-1β, MMP-9, EGF, VEGF ,PDGF, TGFβ1, TGFβ3, COL1A1, COL1A2 and COL3A1 in skin biopsies before and after treatments, at either day 3 (A, C, E, G, I, K, M, O, Q, S, U, W and Y) or 10 (B, D, F, H, J, L, N, P, R, T, V, X and Z) post wounding. Results are presented as mean ± SEM of seven to eighteen independent experiments. & $p < 0.05$ compared with PBS d3, *$p < 0.05$ compared with PBS d10, **$p < 0.01$ compared with PBS d10§ $p < 0.05$ compared with diabetic PBS d3, # $p < 0.05$ compared with diabetic PBS d10, # #$p < 0.01$ compared with diabetic PBS d10 (cont.).

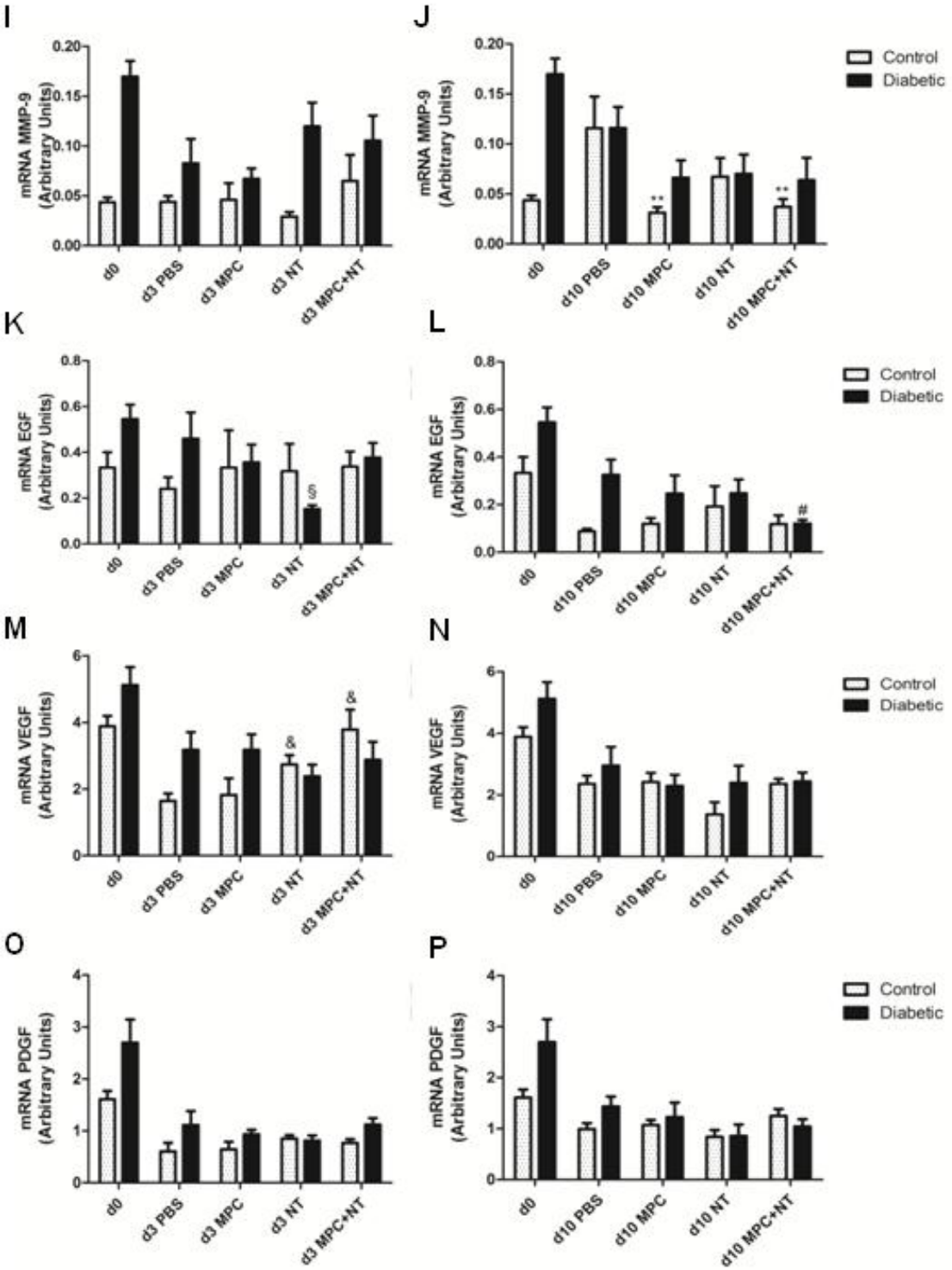

Figure 4.7. The gene expression profile for TNF-α, IL-6, KC, IL-1β, MMP-9, EGF, VEGF ,PDGF, TGFβ1, TGFβ3, COL1A1, COL1A2 and COL3A1 in skin biopsies before and after treatments, at either day 3 (A, C, E, G, I, K, M, O, Q, S, U, W and Y) or 10 (B, D, F, H, J, L, N, P, R, T, V, X and Z) post wounding. Results are presented as mean ± SEM of seven to eighteen independent experiments. & $p < 0.05$ compared with PBS d3, *$p < 0.05$ compared with PBS d10, **$p < 0.01$ compared with PBS d10§ $p < 0.05$ compared with diabetic PBS d3, # $p < 0.05$ compared with diabetic PBS d10, # #$p < 0.01$ compared with diabetic PBS d10 (cont.)

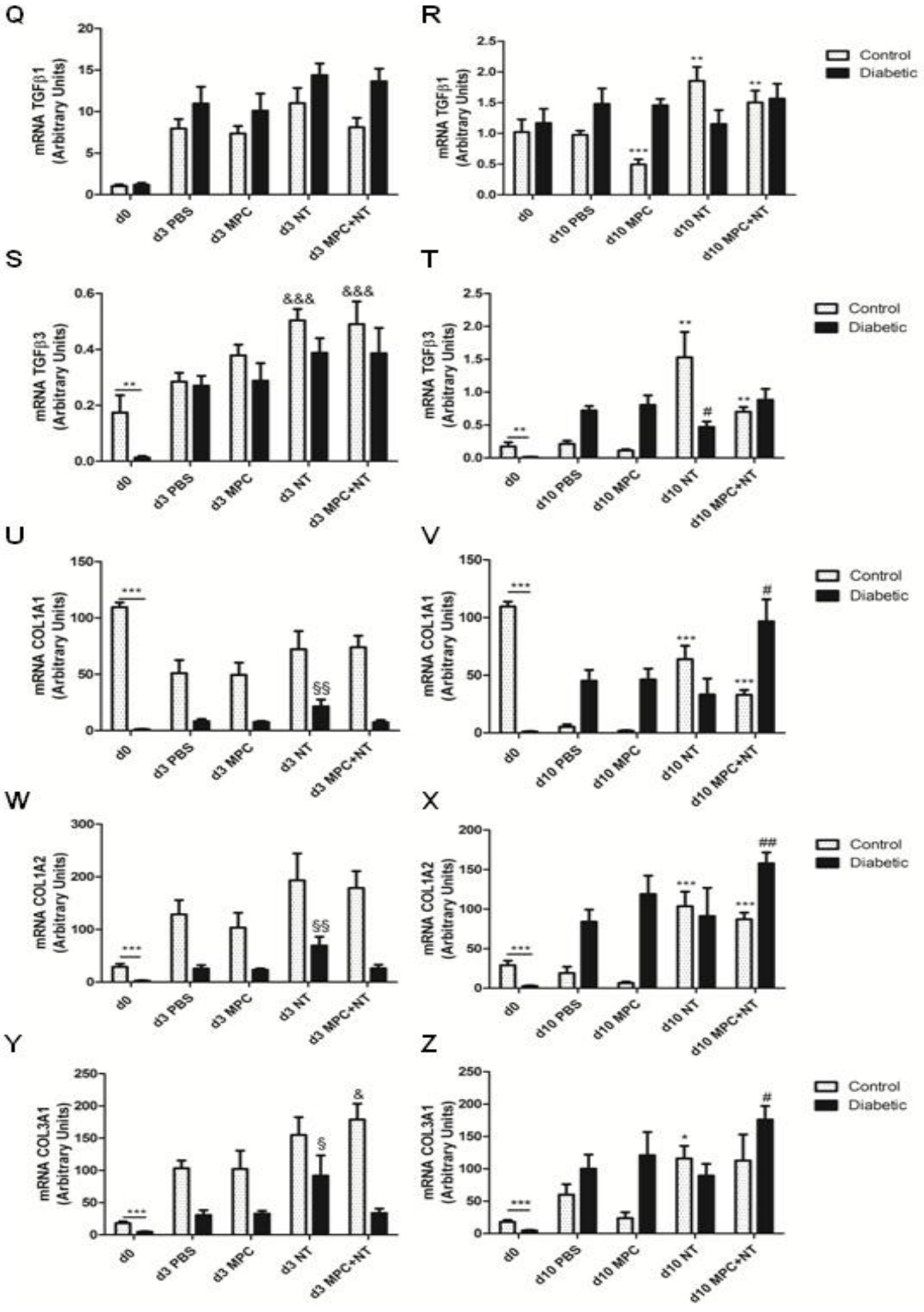

Figure 4.7. The gene expression profile for TNF-α, IL-6, KC, IL-1β, MMP-9, EGF, VEGF ,PDGF, TGFβ1, TGFβ3, COL1A1, COL1A2 and COL3A1 in skin biopsies before and after treatments, at either day 3 (A, C, E, G, I, K, M, O, Q, S, U, W and Y) or 10 (B, D, F, H, J, L, N, P, R, T, V, X and Z) post wounding. Results are presented as mean ± SEM of seven to eighteen independent experiments. & $p < 0.05$ compared with PBS d3, *$p < 0.05$ compared with PBS d10, **$p < 0.01$ compared with PBS d10§ $p < 0.05$ compared with diabetic PBS d3, # $p < 0.05$ compared with diabetic PBS d10, # #$p < 0.01$ compared with diabetic PBS d10.

hydroxyproline levels were significantly decreased ($p<0.01$) in diabetic mice comparing with control skin. At day 3 post-wounding, NT significantly increased ($p<0.05$) hydroxyproline content in diabetic skin, while at day 10, this effect was observed with NT-loaded MPC in control and diabetic skin ($p<0.05$, $p<0.01$), respectively.

4.4.9 Histopathological analysis of the wound

For the histopathological analysis of control and diabetic skin tissue, we used the H&E and Masson´s Trichrome stainning (Figures 4.9A and B, respectively). In unwounded skin the increase in the epidermis skin thickness was evident in diabetic mice when compared with control. At day 3 post wounding, all the treatments stimulated an increase in the epidermis thickness, which was more significant for the non-loaded and NT-loaded MPC treatments in diabetic skin (Table 4.2).

At day 10, the epidermis thickness profile was similar with a stronger effect in diabetic skin (Table 4.3). A specific re-epithelization profile was observed: in control mice, re-epithelization occurred from bottom to top with basal cells in the epidermis covering the scar; in diabetic mice, the re-epithelization occurred over the granulation inflammatory tissue while this was suffering repair, without correlation with the applied treatments, in both groups (Table 4.3 and 4.4). At day 3, neither MPC, NT alone or NT-loaded MPC treatments affected the number of polymorphonuclear leukocytes (PMN) and lymphocytes in control skin, however in diabetic skin, these inflammatory cells were less recruited to the wound site compared with the PBS treatment. In addition, there is a higher production of fibrin in diabetic skin while no plasma cells were observed in either control or diabetic skin (Table 4.4). At day 10, there was no significant recruitment of PMN and lymphocytes observed in control skin, while in diabetic wounds treated with either MPC, NT alone or NT-loaded MPC, PMN cells, lymphocytes and plasma cells were present in higher numbers when compared with PBS treatment. It is important to note that inflammatory cells persisted at day 10

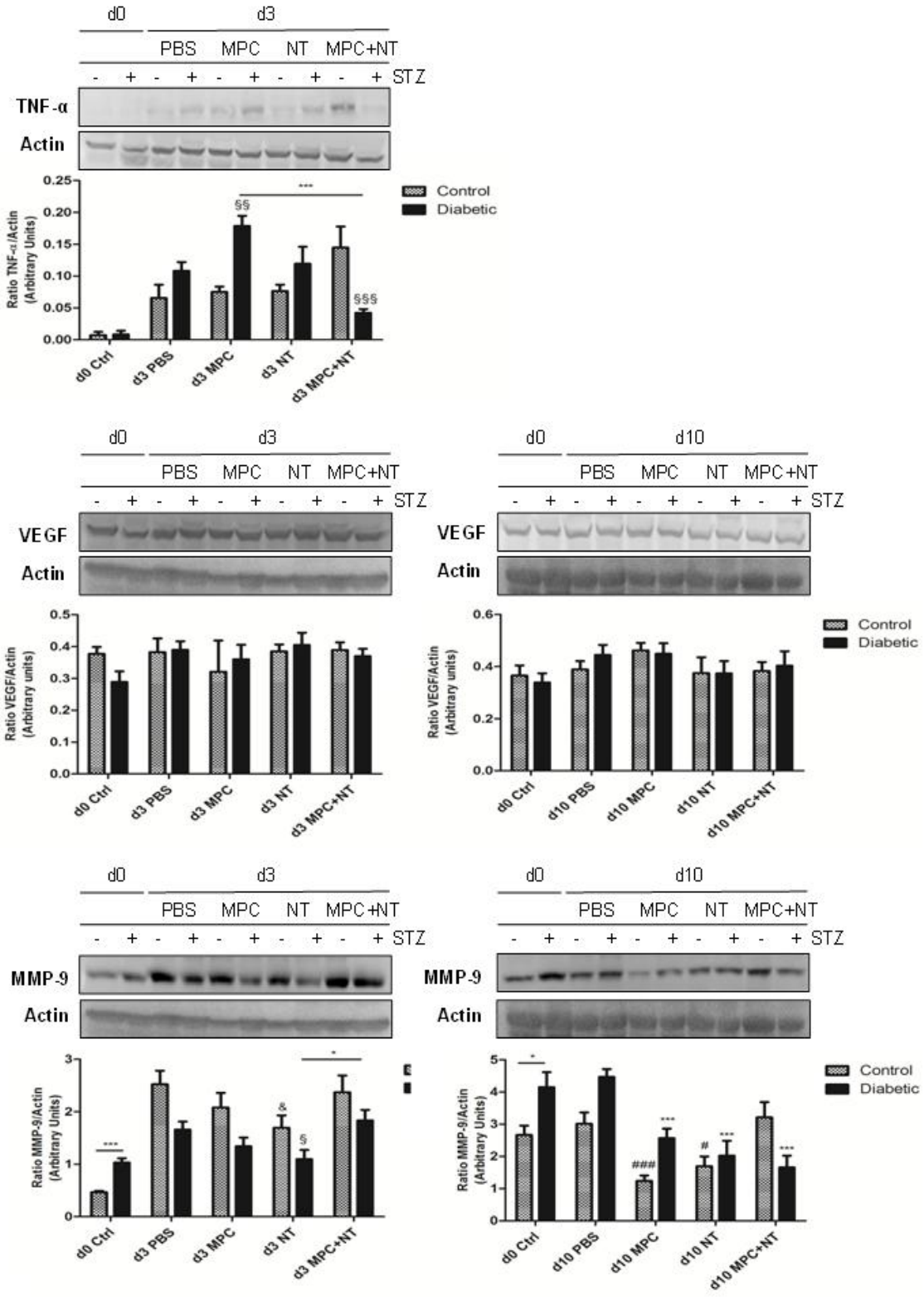

Figure 4.8. Protein expression of TNF-α, VEGF and MMP-9 in unwounded skin (day 0) or after treatments, at either day 3 or 10 post-wounding. Results are presented as mean ± SEM of three to five independent experiments. & $p < 0.05$ compared with PBS d3, *$p < 0.05$ compared with PBS d10, **$p < 0.01$ compared with PBS d10§ $p < 0.05$ compared with diabetic PBS d3, # $p < 0.05$ compared with diabetic PBS d10, # #$p < 0.01$ compared with diabetic PBS d10.

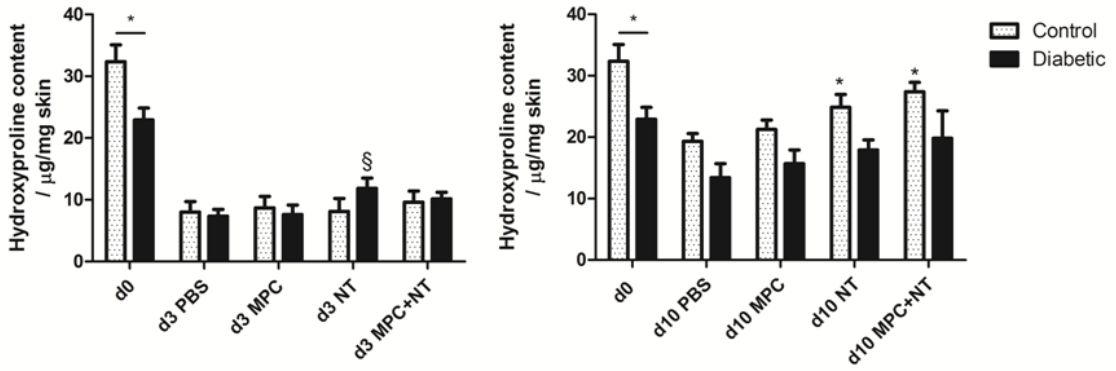

Figure 4.9. Hydroxyproline content levels in unwounded skin (d0) or after treatments, at either day 3 or 10 post-wounding. Results are presented as mean ± SEM of four to six independent experiments. *p < 0.05 compared with PBS d10, § p < 0.05 compared with diabetic PBS d3, # #p < 0.01 compared with diabetic PBS d10.

especially in the diabetic wounded skin. No fibrin was observed either in control or diabetic skin (Table 4.5). Fibroblasts, which are important for tissue repair, were increased in diabetic whencompared to control wounded skin, at day 3. Moreover, collagen matrix production appeared to be more evident in diabetic skin, particularly after the NT or the NT-loaded MPC foam treatment. However, the scar was more pronounced in these same treatments (Table 4.4). Furthermore, at day 10, NT-loaded MPC foam induced the migration of fibroblasts and the production of the collagen matrix. However, the scar obtained after this treatment was more pronounced (Table 4.5). A summary of cytokine expression and corresponding cell type production, in wounded control and diabetic skin, at either day 3 or 10 post-wounding, is represented on table 4.6.

4.5 Discussion

One of the main objectives of this work was to evaluate the capacity of chitosan-based wound dressings to work as biocompatible and biodegradable supports for the sustained delivery of neurotensin, a neuropeptide that has shown to improve wound healing (Brun *et al.*, 2005, da Silva *et al.*, 2011). Three different water soluble chitosan derivatives (CMC, MPC and SC) were synthesized and tested for their water swelling capacities and peptide release profiles in order to infer which of the derivatives would present the best performance (controlled swelling and NT

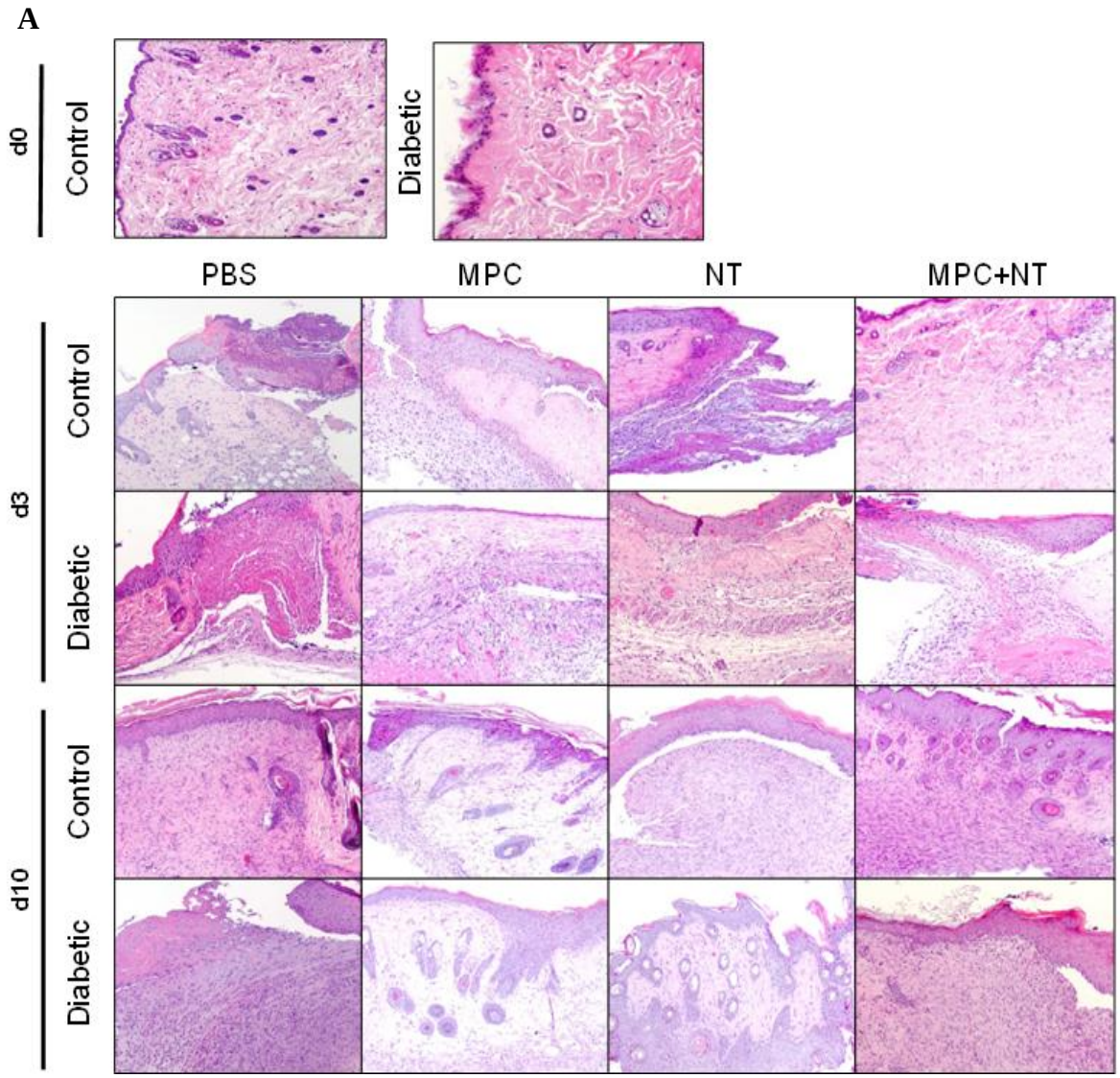

Figure 4.10. Histopathological analysis of Hematoxicilin and Eosin (H&E) (Figure 9A) and Masson´s Trichrome (Figure 9B) staining for control and diabetic mouse skin, untreated or treated with MPC, NT and NT-loaded MPC dressings (magnification 100×). Representative images of three skin stainings analyzed. (cont.).

delivery over time) *in vivo*. At this stage, GSH was used as a model peptide. Although GSH presents lower molecular weight than NT, it has similar functional groups that will permit the simulation of the physical and chemical interactions that may be established between the molecule and the material used as the dressing.

The obtained results showed that the SC foam has the highest water vapor and water swelling capacity probably due to the high number of thin fibers that constitute its matrix, increasing the contact area between the material and the water molecules. SC´s higher affinity for water (higher

B

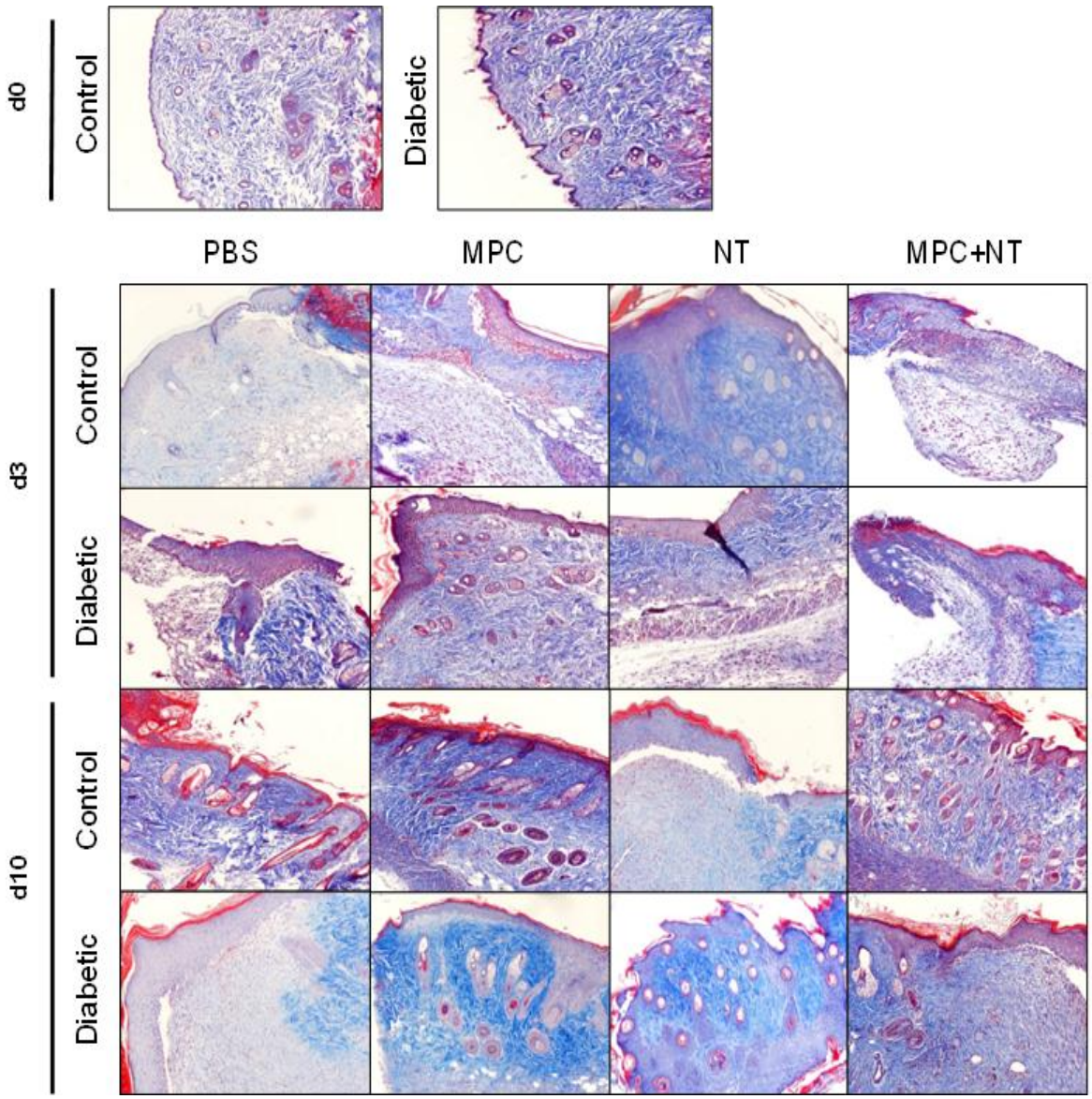

Figure 4.10. Histopathological analysis of Hematoxicilin and Eosin (H&E) (Figure 9A) and Masson´s Trichrome (Figure 9B) staining for control and diabetic mouse skin, untreated or treated with MPC, NT and NT-loaded MPC dressings (magnification 100×). Representative images of three skin stainings analyzed.

hydrophilicity) justifies its faster dissolution in PBS. These results are also in agreement with the ^{1}H-NMR data that showed a higher degree of substitution for SC. This was expected since chitosan substitutions performed in this work aimed to improve the solubility of chitosan in aqueous media. According to the water swelling results, MPC presented an intermediate swelling profile, despite the apparent larger porosity of the CMC derivative observed by SEM analysis.

Table 4.2: Histological analysis of unwounded skin and NT, MPC and NT loaded MPC foams treated wounds at day 3, by H&E staining. - absence or no alterations, + presence <10%, ++ presence 10%,-50%, n.a, not applicable

	Skin (d0)		**Day 3**							
			PBS		**MPC**		**NT**		**NT-loaded MPC**	
	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic
Epidermis thickness										
- Stratus lucidum	-	+	-	+	+	++	-	+	+	++
- Epithelial layers	-	+	-	+	+	++	-	+	+	++
- Basal layer	-	+	-	+	+	++	-	+	+	++
Wound area (mm^2)	26.48 ±4.22	27.71±5.41	30.30±0.17	29.02±0.32	18.68±0.12	22.64±0.22	24.53±0.31	20.95±0.34	17.80±0.18	16.68±0.17
Re-epithelization										
- From bottom	na	na	+	-	+	-	+	-	+	-
- Top cover	na	na	-	+	-	+	-	+	-	+

Table 4.3: Histological analysis of NT, MPC and NT loaded MPC foams treated wounds at day 10, by H&E staining. - absence or no alterations, + presence <10%, ++ presence 10%,-50%, +++ presence >50%

	Day 10							
	PBS		**MPC**		**NT**		**NT-loaded MPC**	
	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic
Epidermis thickness								
- Stratus lucidum	++	+++	+	++	++	+++	+	++
- Epithelial layers	++	+++	+	++	++	+++	+	++
- Basal layer	++	+++	+	++	++	+++	+	++
Wound area (mm^2)	9.02±0.15	13.39±0.31	4.22±0.09	12.11±0.20	7.05±0.30	9.12±0.30	5.88±0.12	9.77±0.29
Re-epithelization								
- From bottom	+	-	+	-	+	-	+	-
- Top cover	-	+	-	+	-	+	-	+

Table 4.4: Inflammatory and granulation tissue histological analysis of NT, MPC and NT loaded MPC foams treated wounds at day 3, by H&E and Masson´s Trichrome staining. - absence or no alterations, + presence <10%, ++ presence 10%,-50%, +++ presence >50%; < not relevant, > predominant

	Day 3							
	PBS		MPC		NT		NT-loaded MPC	
	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic
Inflammation Status								
- PMN	++	+++	++	+	++	+	++	+
- Lymphocytes	+	++	+	-	+	-	+	-
- Plasma cells	-	-	-	-	-	-	-	-
- Fibrin	<	>	>	<	>	<	>	<
Repair								
- Fibroblasts	<	>	<	>	<	>	<	>
Collagen matrix								
- Loose	-	-	-	+	+	+	+	-
- Scar	-	-	-	+	-	+	+	++

Table 4.5: Inflammatory and granulation tissue histological analysis of NT, MPC and NT loaded MPC foams treated wounds at day 10, by H&E and Masson´s Trichrome staining. - absence or no alterations, + presence <10%, ++ presence 10%,-50%, +++ presence >50%;

	Day 10							
	PBS		MPC		NT		NT-loaded MPC	
	Control	Diabetic	Control	Diabetic	Control	Diabetic	Control	Diabetic
Inflammation Status								
- PMN	-	++	-	+	-	+	-	+
- Lymphocytes	+	+++	+	++	+	++	+	++
- Plasma cells	+	+++	+	++	+	++	+	++
- Fibrin	-	-	-	-	-	-	-	-
Repair								
- Fibroblasts	++	+	+	++	+	+	+	+++
Collagen matrix								
- Loose	-	-	-	-	-	-	-	-
- Scar	++	+	+	++	+	+	+	+++

Table 4.6: Summary of cytokine expression in wounded control and diabetic skin, at day 3 and 10 post-wounding.

Day	Cytokine/Growth factor	Control mice	Diabetic mice	Cell type that produce cytokine/growth factors
3	TNF-α	↓ MPC, NT, MPC+NT	↑ NT, MPC+NT	Macrophages, fibroblasts
	IL-6	↓ MPC; ↑ MPC+NT	↓ MPC, NT, MPC+NT	Macrophages, fibroblasts, Keratinocytes, endothelial cells
	KC	↑ MPC+NT	↓ MPC, NT, MPC+NT	Macrophages, fibroblasts
	IL-1β	↓ MPC, NT, MPC+NT	= MPC, NT, MPC+NT	Macrophages, epithelial cells
	MMP-9	=	=	Collagenase, monocytes, macrophages
	EGF	=	↓ NT	Macrophages, platelets
	VEGF	↑ NT, MPC+NT	=	Fibroblasts, macrophages, neutrophils, endothelial cells
	PDGF	=	=	macrophages, endothelial cells
	TGFβ1	=	=	Macrophages, PMN, fibroblasts, keratinocytes, epithelial cells
	TGFβ3	↑ NT, MPC+NT	=	Macrophages, PMN, fibroblasts, keratinocytes, epithelial cells
	COL1A1	=	↑ NT	Fibroblasts
	COL1A2	=	↑ NT	Fibroblasts
	COL3A1	↑ MPC+NT	↑ NT	Fibroblasts
10	TNF-α	↑ NT, MPC+NT	↓ NT, MPC+NT	Macrophages, fibroblasts
	IL-6	=	↓ MPC, NT, MPC+NT	Macrophages, fibroblasts, keratinocytes, endothelial cells
	KC	=	↓ MPC, NT, MPC+NT	Macrophages, fibroblasts
	IL-1β			Macrophages, epithelial cells
	MMP-9	↓ MPC, MPC+NT	=	Collagenase, monocytes, macrophages
	EGF	=	↓MPC+NT	Macrophages, platelets
	VEGF	=	=	Fibroblasts, macrophages, neutrophils, endothelial cells
	PDGF	=	=	macrophages, endothelial cells
	TGFβ1	↓ MPC, ↑NT,MPC+NT	=	Macrophages, PMN, fibroblasts, keratinocytes, epithelial cells
	TGFβ3	↑NT,MPC+NT	↓ NT	Macrophages, PMN, fibroblasts, keratinocytes, epithelial cells
	COL1A1	↑NT,MPC+NT	↑MPC+NT	Fibroblasts
	COL1A2	↑NT,MPC+NT	↑MPC+NT	Fibroblasts
	COL3A1	↑NT	↑MPC+NT	Fibroblasts

Medicated wound dressings have been largely used to deliver healing enhancers and therapeutic substances, such as growth factors or stem cells to stimulate wound healing (Obara *et al.*, 2005, Rossi *et al.*, 2007). Their use allows the protection of the wound against external aggression and avoids the rapid biodegradation of the bioactive healing enhancers that may occur in the enzyme rich wound environment. In this work, the capacity of each dressing to sustain the release of a peptide at different pH conditions was addressed. The measured release kinetics performed was not significantly affected within the pH ranges studied and SC is the material that presented the faster release of GSH, followed by MPC and CMC. The release profiles are in accordance with the water swelling profiles observed for the different chitosan derivatives, indicating that the GSH release is mainly controlled by the water swelling capacity of the material and therefore

GSH is released mainly through a diffusion mechanism. The higher swelling capacity of SC leads to a higher amount of water inside the polymer structure, better dissolving GSH, enhancing its release into the surrounding medium. According to these results (water swelling and GSH release

data), and considering that sustained profiles were envisaged for *in vivo* applications, the use of SC based material was discarded at this stage.

The biocompatibility of CMC and MPC dressings was tested *in vitro*, in Raw 264.7 and HaCaT cell lines and the results showed that both materials were non-toxic against these cell lines, up to 48 h. For the 72 h test period, a slight decrease (not statistically significant) in the viability of the cells was observed probably due to foam dissolution or cell stress in the media conditions. Similar results were observed in L929 cells (fibroblast cell line) by Huang and colleagues(Huang *et al.*, 2009). The production of nitrites by macrophages Raw 264.7 was also quantified since it is known that these cells produce NO when stimulated by inflammatory stimulus. The results presented show that CMC and MPC do not increase nitrite levels *in vitro* suggesting that these compounds do not induce an inflammatory response which is in accordance with data previously reported in the literature (Hwang *et al.*, 2000). The *in vitro* results indicate that both CMC and MPC could be used for wound dressing applications. However, in this work, *in vivo* application and characterization was performed only for MPC, which was the material that presented an intermediate GSH release profile compared to either CMC or SC.

Several studies suggested that chitosan and derivatives accelerate wound healing(Chen *et al.*, 2006, Yang *et al.*, 2010). For instance, MPC freeze-dried dressings were shown to jellify in contact with biological fluids, being progressively absorbed via enzymatic hydrolysis, promoting regeneration of connective tissues (Muzzarelli, 1992). However, no further studies were found in the literature reporting the effect of MPC alone or in combination with NT in diabetic wound healing.

Diabetes mellitus cause important complications, namely at skin level. The healing process involves several overlapping phases: homeostasis/coagulation, inflammation, proliferation (granulation tissue formation), re-epithelialization and remodeling(Enoch and Leaper, 2008). All these processes require the interaction of skin cells, cytokines and growth factors released from inflammatory cells, fibroblasts, keratinocytes and epithelial cells(Tellechea *et al.*, 2010). Wound closure results show that NT induced a faster closure in diabetic mice, even when applied directly over the wound and compared with control mice. This was expected since it has been reported that topical application of neuropeptides, such as Substance P, stimulate diabetic wound healing(Scott

et al., 2008). In addition, previous studies in our group observed that NT modulates inflammatory responses in a skin dendritic cell line (da Silva *et al.*, 2011). Treatments with non-loaded and NT-loaded MPC dressings induced a significant reduction of the wound area, especially in the first 3 days post-wounding and in both control and diabetic mice. Moreover, NT-loaded MPC presented a faster healing profile in diabetic skin wounds. These results suggest a synergistic behavior between the bioactivity of NT alone and the intrinsic healing properties of MPC. Moreover and as intended, a sustained release of NT may also occur which guarantees constant NT levels during the healing process. The adhesive properties of chitosan and its derivatives could explain this enhanced healing profile(Lehr *et al.*, 1992). In addition, wound contraction is necessary for the healing process, probably due to the enhanced proliferation of fibroblasts (Ono *et al.*, 1999). Wound contraction is a biologically important process in wound healing, especially in the healing of chronic wounds such as DFU, although excessive contraction may lead to scar formation(Ishihara *et al.*, 2001). All treatments lead to healing, however, larger scars were developed over diabetic wounds that were treated with MPC dressings, most probably due to the fast initial wound contraction verified in this case.

In unwounded diabetic skin, we observed an overexpression of inflammatory cytokines, growth factors and MMP-9, which is in agreement with the literature (Galkowska *et al.*, 2006). These results suggest a chronic pro-inflammatory state in diabetic skin that can compromise the wound healing. On the other hand, the gene expression of the different types of collagen is downregulated in the diabetic skin suggesting a decreased capacity of the diabetic skin to produce the appropriate matrix essential for wound healing and skin repair. As decreased expression of COL1A1, COL1A2 and COL3A1 is verified, less collagen is deposited as observed by the hydroxyproline assay(Hansen *et al.*, 2003).

In chronic diabetes, the healing process becomes stalled in one or more of the healing phases originating chronic non-healing wounds. One important phase that can become stalled in diabetes is the inflammatory phase (Moura *et al.*, 2013). TNF-α, IL-6, KC 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 attachment of inflammatory cells to vessels, rolling, migration, and eventually chemotaxis into the skin (Bashir *et al.*, 2009). Moreover, IL-6 and IL-1β, produced by macrophages, fibroblasts, keratinocytes and epithelial cells are also important players in the early phase of inflammation and in the wound healing process(Lin *et al.*, 2003). In control mice, the reduction of TNF-α and IL-1β expression with all treatments, at day 3, suggests a decrease in the inflammatory condition which facilitates healing. In diabetic mice treated with MPC, NT or NT-loaded MPC, less infiltrated inflammatory cells was observed at day 3 comparing with control mice, while TNF-α expression is significantly higher, especially for the MPC alone. Moreover, IL-6 and KC expression is significantly reduced. These results may suggest that high expression of TNF-α is produced not only by inflammatory cells present at the wound site, but also by other cells present at day 3, which can stimulate contraction of the wound and consequently have a beneficial effect in the early stages of wound healing. This may further indicate that the granulation tissue fills the wound bed and potentiates re-epithelialization through proliferation of skin fibroblasts, in diabetic mice, treated with NT or/and MPC, in the early phase of wound healing. Similar results were observed with the MPC alone. However, NT-loaded MPC treatment induced a decrease in the TNF-α protein content suggesting that the combination of NT with the MPC foam has an effective anti-inflammatory role in wound healing.

At day 10, the inflammatory status persisted in diabetic mice while in controls it is resolved, as expected (Pradhan *et al.*, 2009). On the other hand, all treatments lead to a reduction in the inflammatory cytokines expression supported by the loose conjunctive tissue observed from the beginning, undergoing different status of collagen deposition in diabetic and control mice. At this time point, fibroblasts have an important role in collagen synthesis and scar formation (Gabbiani, 2003, Diegelmann and Evans, 2004). During the re-epithelization phase, the initial ECM is gradually replaced by a collagenous matrix with the formation of new blood vessels (Singer and Clark, 1999). The expression of angiogenic factors, VEGF and PDGF, did not change with treatments in diabetic mice possibly showing that these treatments do not stimulate the production of growth factors to improve wound healing.

Our results show that the production of the collagen matrix was higher for MPC and NT-loaded MPC treated diabetic skin, which is correlated with increased scar formation. Obara and colleagues (Obara *et al.*, 2005) also observed that application of a chitosan hydrogel in diabetic wounds increased scar formation. Moreover, MMP-9 expression in diabetic skin wound was increased at day 3. Possibly, MMP-9 may affect ECM proteolytic enzymes, allowing the migration of cells into the wound site, resulting in the deposition of new ECM and the development of new tissue. However, it is known that the increased presence of TNF-α in diabetes could reduce the MMP-9/TIMP-2 balance production by fibroblasts, contributing to the elevated proteolytic activity impairing wound healing (Blakytny and Jude, 2009).

Type 1 collagen is the most expressed form of collagen in the skin, serving as the framework for connective tissues such as skin, bone and tendons (Crane *et al.*, 2010) which is also observed in our results. It is known that TGF has an important role in the pathophysiology of tissue repair by the enhancement of type 1 collagen gene expression (Verrecchia and Mauviel, 2004), which is also verified in our results.

In addition, at day 3 we observed an increased expression of all types of analyzed collagen in control compared to diabetic skin at the same time point and the opposite is verified at day 10 suggesting that diabetes impair collagen gene expression and deposition in the skin (Black *et al.*, 2003). Moreover, the NT-loaded MPC foam stimulated COL1A1, COL1A2 and COL3A1 expression at day 10 in diabetic skin, which is also correlated with higher collagen production observed by the hydroxyproline content and the Masson´s Trichrome staining.

4.6 Conclusions

From our results, in control animals both MPC and NT-loaded MPC dressings have great impact on the early phases of the healing process decreasing the inflammatory infiltrate. Furthermore, in diabetic animals the major healing effects were observed with either NT alone or NT-loaded MPC dressings reducing the inflammatory status in the early phase of wound healing and increasing migration of fibroblast and collagen expression and deposition for tissue repair. However, a more

pronounced scar was observed with the MPC application. Table 5 summarizes cytokine expression in wounded control and diabetic skin, at day 3 and 10 post-wounding.

We can suggest that *in vivo* NT combined with the MPC foam application in diabetic wound dressings can promote an inflammatory response and stimulate re-epithelialization which are important phases of the healing process. Human studies are needed to further investigate the potential application of NT-loaded MPC wound dressings as therapy for diabetic foot ulcers.

4.7 Acknowledgments

This work was financially supported by COMPETE , FEDER and Fundação para a Ciência e Tecnologia (FCT-MES) under contract PTDC/SAU-MII/098567/2008, PTDC/SAU FAR/121109/2010 and PEst-C/EQB/UI0102/2011 and PEst-C/SAU/LA0001/2013-2014, in addition to the 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-MES for their fellowships SFRH/BD/60837/2009, SFRH/BPD/40409/2007 and SFRH/BPD/46341/2008, respectively.

4.8 References

Bashir MM, Sharma MR, Werth VP (2009) TNF-alpha production in the skin. Archives of dermatological research 301:87-91.

Berscht PC, Nies B, Liebendorfer A, Kreuter J (1994) Incorporation of basic fibroblast growth factor into methylpyrrolidinone chitosan fleeces and determination of the in vitro release characteristics. Biomaterials 15:593-600.

Black E, Vibe-Petersen J, Jorgensen LN, Madsen SM, Agren MS, Holstein PE, Perrild H, Gottrup F (2003) Decrease of collagen deposition in wound repair in type 1 diabetes independent of glycemic control. Arch Surg 138:34-40.

Blakytny R, Jude EB (2009) Altered molecular mechanisms of diabetic foot ulcers. Int J Low Extrem Wounds 8:95-104.

Brain SD (1997) Sensory neuropeptides: their role in inflammation and wound healing. Immunopharmacology 37:133-152.

Brun P, Mastrotto C, Beggiao E, Stefani A, Barzon L, Sturniolo GC, Palu G, Castagliuolo I (2005) Neuropeptide neurotensin stimulates intestinal wound healing following chronic intestinal inflammation. Am J Physiol Gastrointest Liver Physiol 288:G621-629.

Chen R, Wang G, Chen C, Ho H, Shen M (2006) Development of a new N-O-(Carboxymethyl)/chitosan /collagen matrixes as a wound dressing. Biomacromolecules 7:1058-1064.

Chen X, Wang Z, Liu W, Park H (2002) The effect of carboxymethyl-chitosan on proliferatyion and collagen secretion of normal and keloid skin fibroblasts. 23:4609-4614.

Crane NJ, Brown TS, Evans KN, Hawksworth JS, Hussey S, Tadaki DK, Elster EA (2010) Monitoring the healing of combat wounds using Raman spectroscopic mapping. Wound Repair Regen 18:409-416.

da Silva L, Neves BM, Moura L, Cruz MT, Carvalho E (2011) Neurotensin downregulates the pro-inflammatory properties of skin dendritic cells and increases epidermal growth factor expression. Biochim Biophys Acta 1813:1863-1871.

Dai T, Tanaka M, Huang YY, Hamblin MR (2011) Chitosan preparations for wounds and burns: antimicrobial and wound-healing effects. Expert review of anti-infective therapy 9:857-879.

Dai YN, Li P, Zhang JP, Wang AQ, Wei Q (2008) A novel pH sensitive N-succinyl chitosan/alginate hydrogel bead for nifedipine delivery. Biopharmaceutics & drug disposition 29:173-184.

Diegelmann RF, Evans MC (2004) Wound healing: an overview of acute, fibrotic and delayed healing. Frontiers in bioscience : a journal and virtual library 9:283-289.

Enoch S, Leaper DJ (2008) Basic Science of wound healing. Surgery 26:31-37.

Gabbiani G (2003) The myofibroblast in wound healing and fibrocontractive diseases. The Journal of pathology 200:500-503.

Galkowska H, Wojewodzka U, Olszewski WL (2006) Chemokines, cytokines, and growth factors in keratinocytes and dermal endothelial cells in the margin of chronic diabetic foot ulcers. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 14:558-565.

Hansen SL, Myers CA, Charboneau A, Young DM, Boudreau N (2003) HoxD3 accelerates wound healing in diabetic mice. The American journal of pathology 163:2421-2431.

Huang P, Han B, Liu W, Chang Q, Dong W (2009) Preparation and Biocompatibility of N-carboxymethyl chitosan. Journal of Functional Materials 7:25-33.

Huang S, Fu X (2010) Naturally derived materials-based cell and drug delivery systems in skin regeneration. Journal of controlled release : official journal of the Controlled Release Society 142:149-159.

Hwang SM, Chen CY, Chen SS, Chen JC (2000) Chitinous materials inhibit nitric oxide production by activated RAW 264.7 macrophages. Biochemical and biophysical research communications 271:229-233.

Ishihara M, Ono K, Sato M, Nakanishi K, Saito Y, Yura H, Matsui T, Hattori H, Fujita M, Kikuchi M, Kurita A (2001) Acceleration of wound contraction and healing with a photocrosslinkable chitosan hydrogel. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 9:513-521.

Kalafatakis K, Triantafyllou K (2011) Contribution of neurotensin in the immune and neuroendocrine modulation of normal and abnormal enteric function. Regulatory peptides 170:7-17.

Lazarus LH, Brown MR, Perrin MH (1977) Distribution, localization and characteristics of neurotensin binding sites in the rat brain. Neuropharmacology 16:625-629.

Lehr C, Bouwstra JA, Schacht EH, Junginger HE (1992) In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. International Journal of Pharmaceutics 78:43-48.

Lin Z, Kondo T, Ishida Y, Takayasu T, Mukaida N (2003) Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6 deficient mice. Journal of Leukocyte Biology 73:713-721.

Malafaya PB, Silva GA, Reis RL (2007) Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. Adv Drug Deliv Rev 59:207-233.

Moura LI, Dias AM, Carvalho E, de Sousa HC (2013) Recent advances on the development of wound dressings for diabetic foot ulcer treatment-A review. Acta biomaterialia.

Muzzarelli R (1992) Depolymerization of methyl pyrrolidinone chitosan by lysozyme. Carbohydrate Polymers 19:29-34.

Muzzarelli RAA, Ilari P, Tomasetti M (1993) Preparation and characteristic properties of 5-methyl pyrrolidinone chitosan. Carbohydrate Polymers 20:99-105.

Obara K, Ishihara M, Fujita M, Kanatani Y, Hattori H, Matsui T, Takase B, Ozeki Y, Nakamura S, Ishizuka T, Tominaga S, Hiroi S, Kawai T, Maehara T (2005) Acceleration of wound healing in healing-impaired db/db mice with a photocrosslinkable chitosan hydrogel containing fibroblast growth factor-2. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 13:390-397.

Ono I, Tateshita T, Inoue M (1999) Effect of a collagen matrix containing basic fibroblast growth factor on wound contraction. J Biomed Mater Res (Appl Biomater) 48:621–630.

Park CJ, Clark SG, Lichtensteiger CA, Jamison RD, Johnson AJW (2009) Accelerated wound closure of pressure ulcers in aged mice by chitosan scaffolds with and without bFGF. Acta biomaterialia 5:1926–1936

Prabaharan M (2008) Review Paper: Chitosan Derivatives as Promising Materials for Controlled Drug Delivery. J Biomater Appl 23:5-38.

Pradhan L, Cai X, Wu S, Andersen ND, Martin M, Malek J, Guthrie P, Veves A, Logerfo FW (2011) Gene expression of pro-inflammatory cytokines and neuropeptides in diabetic wound healing. J Surg Res 167:336-342.

Pradhan L, Nabzdyk C, Andersen ND, LoGerfo FW, Veves A (2009) Inflammation and neuropeptides: the connection in diabetic wound healing. Expert Rev Mol Med 11:e2.

Rinaudo M (2006) Chitin and chitosan: properties and applications. Progress in Polymer Science 31:603-632.

Rossi S, Marciello M, Sandri G, Ferrari F, Bonferoni MC, Papetti A, Caramella C, Dacarro C, Grisoli P (2007) Wound Dressings Based on Chitosans and Hyaluronic Acid for the Release of Chlorhexidine Diacetate in Skin Ulcer Therapy. Pharmaceutical Development and Technology 12:415-422.

Santos KSCR, Silva HSRC, Ferreira EI, Bruns RE (2005) 32Factorial design and response surfasse analysis optimization of N-carboxybutylchitosan synthesis. Carbohydrate Polymers 59:37-42.

Scott JR, Tamura RN, Muangman P, Isik FF, Xie C, Gibran NS (2008) Topical substance P increases inflammatory cell density in genetically diabetic murine wounds. Wound Repair and Regeneration 16:529-533

Sell SA, Wolfe PS, Garg K, McCool JM, Rodriguez IA, Bowlin GL (2010) The use of natural polymers in tissue engineering: a focus on electrospun extracellular matrix analogues. Polymers for Advanced Technologies 2:522-553.

Silva L, Carvalho E, Cruz MT (2010) Role of neuropeptides in skin inflammation and its involvement in diabetic wound healing. Expert Opin Biol Ther 10:1427-1439.

Singer AJ, Clark RA (1999) Cutaneous wound healing. N Engl J Med 341:738-746.

Sundler F, Hakanson R, Hammer RA, Alumets J, Carraway R, Leeman SE, Zimmerman EA (1977) Immunohistochemical localization of neurotensin in endocrine cells of the gut. Cell and tissue research 178:313-321.

Sweitzer SM, Fann SA, Borg TK, Baynes JW, Yost MJ (2006) What is the future of diabetic wound care? The Diabetes Educator 32:197-210.

Takei T, Nakahara H, Ijima H, Kawakami K (2012) Synthesis of a chitosan derivative soluble at neutral pH and gellable by freeze-thawing, and its application in wound care. Acta biomaterialia 8:686-693.

Tan Y, Han F, Ma S, Yu W (2011) Carboxymethyl chitosan prevents formation of broad-spectrum biofilm. Carbohydr Polym 84:1365-1370.

Tellechea A, Leal E, Veves A, Carvalho E (2010) Inflammatory and angiogenic abnormalities in diabetic wound healing: role of neuropeptides and therapeutic perspectives The Open Circulation and Vascular Journal 3:43-55.

Verrecchia F, Mauviel A (2004) TGF-beta and TNF-alpha: antagonistic cytokines controlling type I collagen gene expression. Cellular signalling 16:873-880.

Yang C, Zhou Y, Zhang X, Huang X, Wang M, Han Y, Zhai M, Wei S, Li J (2010) A green fabrication approach of gelatin/CM-chitosan hybrid hydrogel for wound healing. Carbohydrate Polymers 82:1297-1305

Chapter 5

In vivo study

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

This Chapter comprises the work submitted in

Biochemical and Biophysical Acta – Molecular Basis of Disease (2013) by

Moura, L. I. F., Dias, A.M.A., Suesca, E., Casadiegos, S., Leal, E. C., Duque, M. R. F., Carvalho, L., de Sousa, H. C., Carvalho, E.

5.1 Abstract

Impaired wound healing is an important clinical problem in diabetes mellitus and results in failure to completely heal diabetic foot ulcers (DFU), 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 STZ diabetic induced mice was evaluated. Results showed that the prepared dressings were not-cytotoxic up to 72h 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, MMP-9 is reduced in diabetic skin ($p<0.05$) which significantly increased fibroblast migration and collagen (COL1A2 and 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.

Keywords: Collagen; wound dressing; diabetic foot ulcer; neurotensin; wound healing

5.2 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 (Shaw *et al.*, 2010). 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 (Silva *et al.*, 2010, Tellechea *et al.*, 2010)

Recently it has been demonstrated that peripheral nerves and cutaneous neurobiology contribute to a correct wound healing process (Pradhan *et al.*, 2009). 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 (Silva *et al.*, 2010). Neurotensin (NT) is a bioactive neuropeptide widely distributed in the brain and in several peripheral tissues (Lazarus *et al.*, 1977, Sundler *et al.*, 1977) 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 (Brain, 1997, Silva *et al.*, 2010, Kalafatakis and Triantafyllou, 2011).

Some neuropeptides namely, substance P and neuropeptide Y have been topically applied to improve diabetic wound healing (Scott *et al.*, 2008, Pradhan *et al.*, 2011). However, the major problem of topical administration of peptides is their short half-life and loss of bioactivity in the peptidase-rich wound environment (Sweitzer *et al.*, 2006). A strategy to overcome this problem is the use of wound dressings made of biocompatible materials for the sustained 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(Malafaya *et al.*, 2007).

After injury, the application of wound dressings based on natural polymers has been proposed to simulate the original cellular environment and extracellular matrix (ECM)(Sell *et al.*, 2010). Collagen is the most abundant protein of ECM being naturally present in human tissues such as

skin, bones, cartilage, tendon and ligaments (Slaughter *et al.*, 2009, Moura *et al.*, 2013a). This biopolymer interacts with cells and regulates cell anchorage, migration, proliferation and survival (Malafaya *et al.*, 2007). Furthermore, collagen-based dressings have shown to promote increase in fibroblast production and stimulate a faster wound healing (Singh *et al.*, 2011).

In addition, collagen is biocompatible, non-toxic, with low antigenicity (Cen *et al.*, 2008) and able to absorb large quantities of wound exudate while simultaneously preserving a moist environment (Antonio *et al.*, 2011). Due to its characteristics, it is usually considered as an ideal biomaterial for tissue engineering and wound dressing applications (Mano *et al.*, 2007). 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 (Adhirajan *et al.*, 2009, Singh *et al.*, 2011, Arul *et al.*, 2012, Manizate *et al.*, 2012). 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 (Kawai *et al.*, 2005, Arul *et al.*, 2012, Kanda *et al.*, 2012).

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, angiogenic 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.

5.3 Material and Methods

5.3.1 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 antibodies against VEGF as well as actin were purchased from the Millipore Corporation (USA).

5.3.2 Preparation of collagen dressings

Collagen isolation and preparation were carried out following procedures from Espinosa et al, 2010. Briefly, collagen dressings were manufactured from type I collagen isolated from mouse-tail tendons. Mouse tails were washed, disinfected (2% sodium hypoclorite) and the tendons were dissected and cut into small pieces. After, they were suspended in 0.5M acetic acid (4ºC during 24 h) and the suspension was centrifuged. The supernatant collagen was dissolved in 0.1M acetic acid and poured in tissue culture dishes, frozen at -20ºC and freeze-dried to yield collagen dressings. The dressings were then rehydrated and cross-linked with glutaraldehyde (0.02%, 4ºC during 24 h). Several washes with water were performed to remove glutaraldehyde residues that not react during the crosslinking. Collagen dressings were cut in circular pieces with 6mm of diameter and then sterilized with ethylene oxide.

5.3.3 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 37ºC 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.

5.3.4 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).

5.3.5 *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 ºC 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.

5.3.6 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 after 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 minutes of incubation in the dark, the absorbance was measured at 550nm in a microplate reader (SLT, Austria). Nitrite concentration was calculated from a previously obtained nitrite standard curve.

5.3.7 *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 of were created with a biopsy punch.

C57BL/6 mice were randomly divided into six groups of treatment for control (non-diabetic) and 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 (5-8 animals), groups II and V with topical application of 50µg/ml NT (7-8 animals) and groups III and VI with 50µg/ml NT-loaded collagen dressings (7-8 animals). For each animal one of the wounds worked as control (PBS application only) and the other received treatment. 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 tissue and skin surrounding the wound were harvested. Complete healing day varies between day 13-16 for control mice and day 14-16 for diabetic mice.

5.3.8 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 in table 5.1. Gene expression changes were analyzed using iQ5Optical system software v2. The results were normalized using a reference gene, TATA box binding protein (TBP).

5.3.9 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 95ºC, 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 1h, at room temperature. After blocking, membranes were incubated with primary antibodies against TNF-α (1:500), VEGF (1:1000) and MMP-9 (1:500), overnight at 4 ºC. After incubation, membranes were washed and incubated for 1h 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.

5.3.10 Hydroxyproline content

This analysis was performed using a Hydroxyproline Assay Kit (Sigma Aldrich). Briefly, 10mg of skin tissue were homogenized in 100µl of water and hydrolyzed with HCl 12M at 120ºC for 3 hours. 25µl of the supernatant were transferred to 96- well plate and evaporated in the incubator at 60ºC 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 minutes at 60ºC.

Table 5.1: Forward and reverse primers sequences used in RT-PCR

Primer	5'-3'Sequence (Forward; Reverse)
TNF-α	For: CAAGGGACTAGCCAGGAG Rev:TGCCTCTTCTGCCAGTTC
IL-6	For: TTCCATCCAGTTGCCTTC Rev:TTCTCATTTCCACGATTTCC
KC	For: ATTAGGGTGAGGACATGTGTGGGA Rev: AATGTCCAAGGGAAGCGTCAACAC
IL-1β	For: ACCTGTCCTGTGTAATGAAAG Rev:GCTTGTGCTCTGCTTGTG
MMP-9	For: CATAGAGGAAGCCCATTACAG Rev: GATCCACCTTCTGAGACTTCA
EGF	For: ACGGCACAGTTTGTCTTCAATGGC Rev: TGTTGGCTATCCAAATCGCCTTGC
VEGF	For: CTTGTTCAGAGCGGAGAAAGC Rev: ACATCTGCAAGTACGTTGGTT
PDGF	For: AGATCTGCCACGCACTCATCCTT Rev: ACGCACACTGCACCTCTAATCCAT
TGFβ1	For: TCAATACGTCAGACATTCGGG Rev: CGTGGAGTTTGTTATCTTTGC
TGFβ3	For: ACTATGCCAACTTCTGCTCAG Rev: GGTCTGTCGCTTTGGTTTTC
COL1A1	For: AGGCTTCAGTGGTTTGGATG Rev: TTCACCCTTAGCACCAACTG
COL1A2	For: AAGGATACAGTGGATTGCAGG Rev: AACCAAAGTCATAGCCACCTC
COL3A1	For: ACCTAAAATTCTGCCACCCC Rev: GCACATCAACGACATCTTCAG
TBP	For: ACCCTTCACCAATGACTCCTATG Rev: TGACTGCAGCAAATCGCTTGG

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.

5.3.11 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.

5.3.12 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* test by GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA) and *p* values lower than 0.05 were considered statistically significant.

5.4 Results

5.4.1 *In vitro* biocompatibility of collagen foam

SEM analysis show that both Raw 264.7 and HaCaT cell lines adhere to the collagen dressing surfaces without apparent change in their morphology (Figure 5.1A).

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 Figure 1B. However, the viability of HaCaT is significantly increased after contact with the collagen dressings for 24 and 48 h (Figure 5.1C). 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 (Figure 5.1D) was not significantly affected when compared to control.

5.4.2 *In vivo* wound healing experiments

Figure 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 B) and diabetic (C and D) mice. PBS was applied in non-treated wounds. For control mice, no major differences where verified among the treatments (Figure 5.2 A). However, in diabetic mice, significant differences were

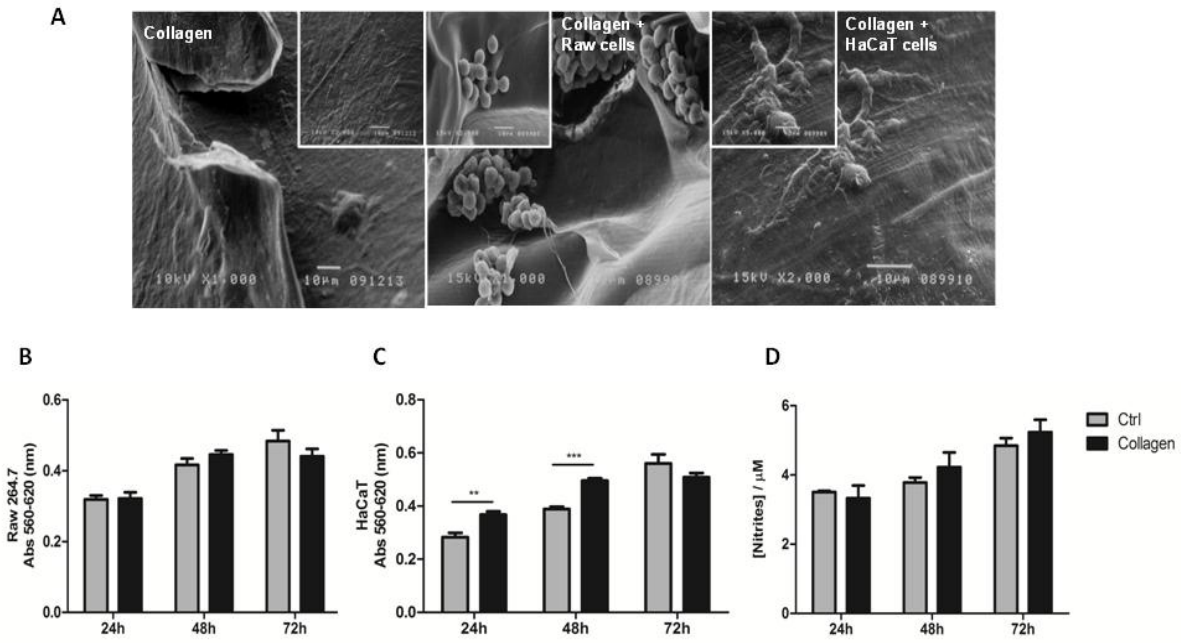

Figure 5.1. SEM micrographs for non-loaded collagen foam structures and/ or after contact with Raw 264.7 and HaCaT cells (Figure 1A). Inner images represent magnifications. Cell viability of Raw (Figure 5.1B) and HaCaT (Figure 5.1C) cells in the presence of collagen foams, during 24, 48 and 72h. NO production in Raw cells (Figure 5.1D). Results are presented as mean ± SEM of three to six independent experiments.

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 (Figure 5.2 C). Collagen treated wounds achieved the significance only at day 5, by 18% compared to control wounds.

Throughout the entire monitored period (12 days), all treatments (collagen, NT and NT-loaded collagen dressings) were effective in decreasing the wound size when compared to PBS, both in control mice ($p<0.001$ for collagen and NT-loaded collagen; $p<0.05$ for NT) and diabetic mice ($p<0.05$, for each treatment) as observed by area under the curve (AUC) analysis.

5.4.3 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, IL-6, KC, IL-1β), MMP-9, growth factors (EGF, VEGF, PDGF), TGFβ1, TGFβ3 and

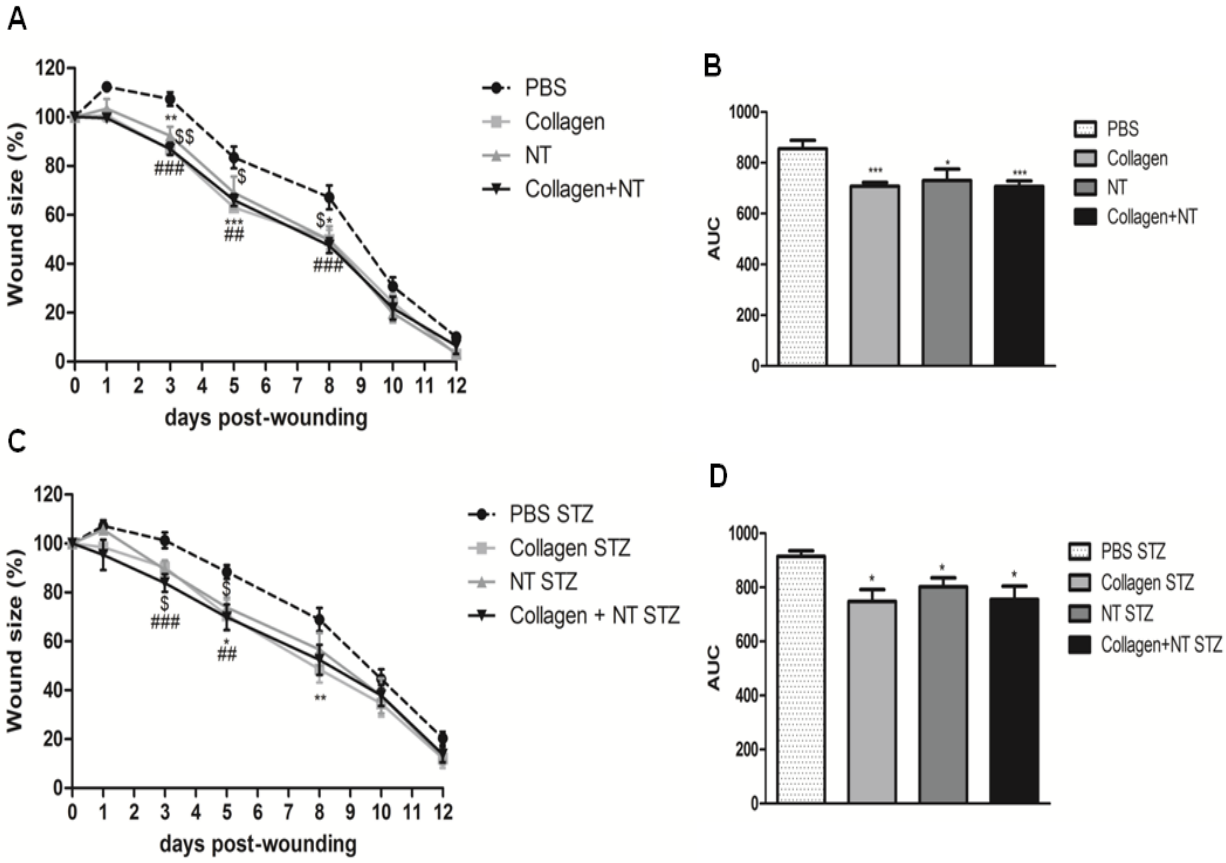

Figure 5.2. Wound size evaluation for collagen, NT and NT-loaded collagen foam treatments in control (A) or diabetic (C) mice. The wound size was determined at days 0, 1, 3, 5, 8, 10 and 12 post-wounding.
Area under the curve (AUC) was obtained from the wound closure with the different treatments in control (B) and diabetic (D) mice. 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.

several types of collagen genes (COL1A1, COL1A2, COL3A1) were measured and the results are presented in Figure 5.3 A-Z.

In unwounded skin (day 0, baseline), and when comparing with control mice, all the mRNA measured for the inflammatory cytokines, MMP-9, and growth factors were significantly increased in the skin of diabetic animals (Figure 5.3 A-P). On the other hand, the expression of TGFβ3 and all types of collagen genes analyzed was significantly reduced (Figure 5.3 S-Z).

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 and TGFβ1 expression are decreased ($p<0.01$) and VEGF increased ($p<0.05$) (Figure 5.3 A, G, M, U, W and Y). For instance, NT-loaded collagen reduced the expression of inflammatory cytokines TNF-α ($p<0.01$),

IL-1β ($p<0.01$) and PDGF ($p<0.05$) (Figure 5.3 A, G and O). COL1A2 was significantly increased ($p<0.01$) in diabetics while in controls all types of collagen are reduced ($p<0.01$) (Figure 5.3 U, W and Y). Collagen alone did not affect inflammatory cytokines 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) (Figure 5.3 I, M, U, W and Y).

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 IL-6 and MMP-9 ($p<0.001$) in diabetic skin (Figure 5.3 B, D and J). Those treatments also reduced significantly EGF and PDGF expression (Figure 5.3 L and P, respectively), with no major differences observed in the expression of the other growth factors. 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 (Figure 5.3 V, X and Z).

5.4.4 Protein expression at the wound site

Western Blots were performed to evaluate the protein expression in the skin at the wound site (Figure 5.4). 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 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.

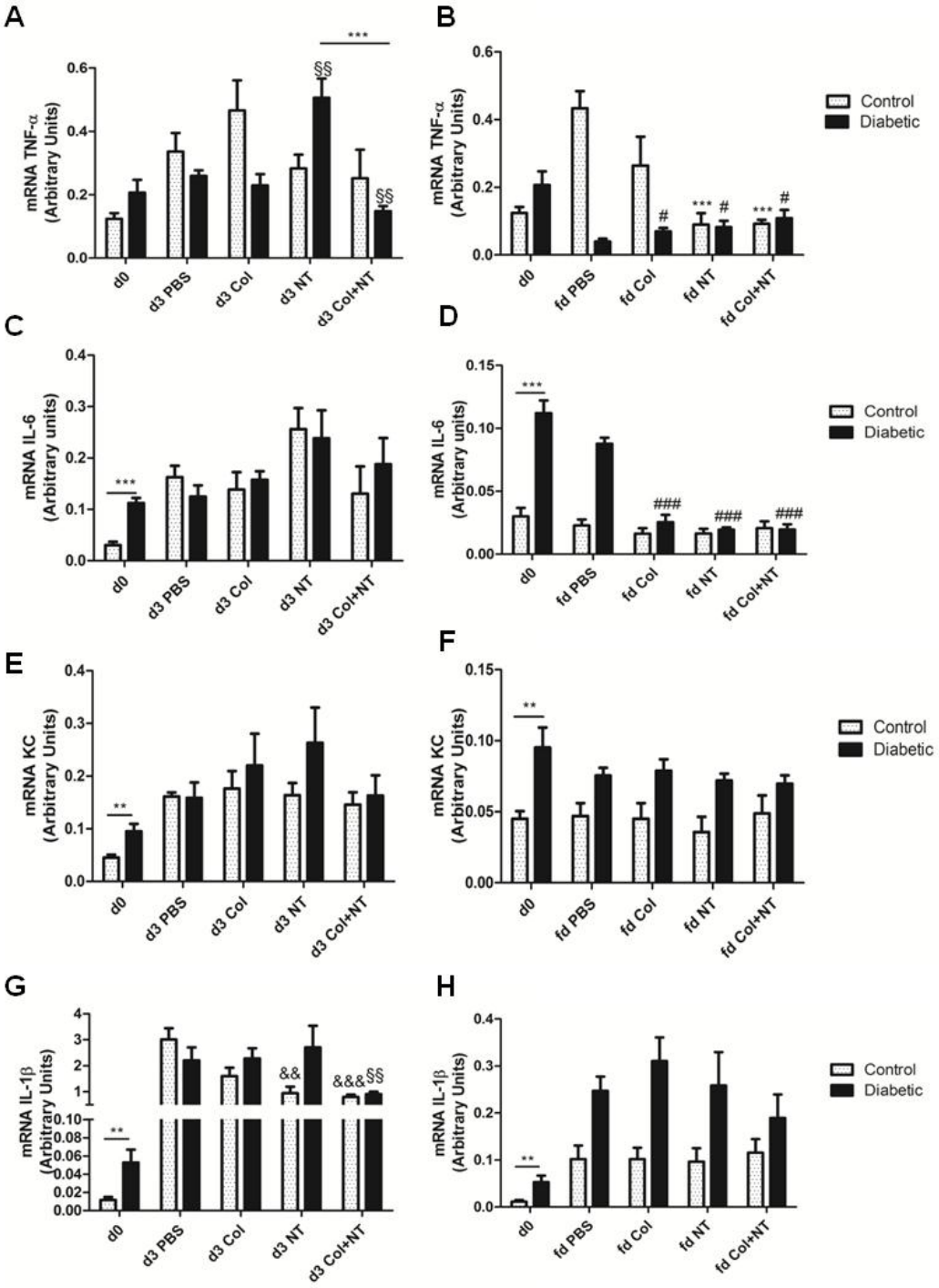

Figure 5.3. The gene expression profile of TNF-α, IL-6, KC, IL-1β, MMP-9, EGF, VEGF, PDGF, TGFβ1, TGFβ3, COL1A1, COL1A2 and COL3A1 in skin biopsies before and after treatments, at either day 3 (A, C, E, G, I, K, M, O, Q, S, U, W and Y) or total wound healing day (fd) (B, D, F, H, J, L, N, P, R, T, V, X and Z) 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 (cont.).

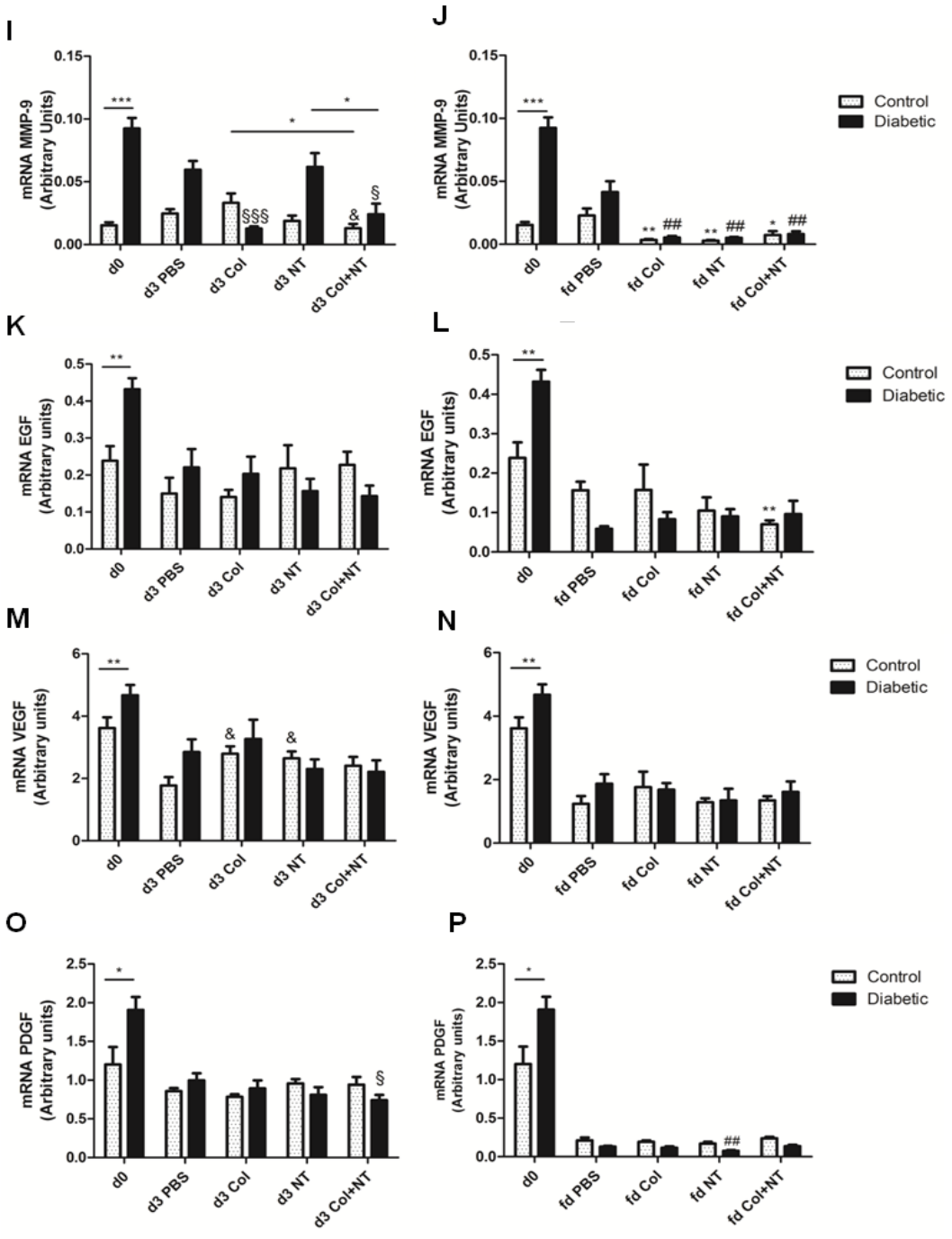

Figure 5.3. The gene expression profile of TNF-α, IL-6, KC, IL-1β, MMP-9, EGF, VEGF, PDGF, TGFβ1, TGFβ3, COL1A1, COL1A2 and COL3A1 in skin biopsies before and after treatments, at either day 3 (A, C, E, G, I, K, M, O, Q, S, U, W and Y) or total wound healing day (fd) (B, D, F, H, J, L, N, P, R, T, V, X and Z) 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 (cont.).

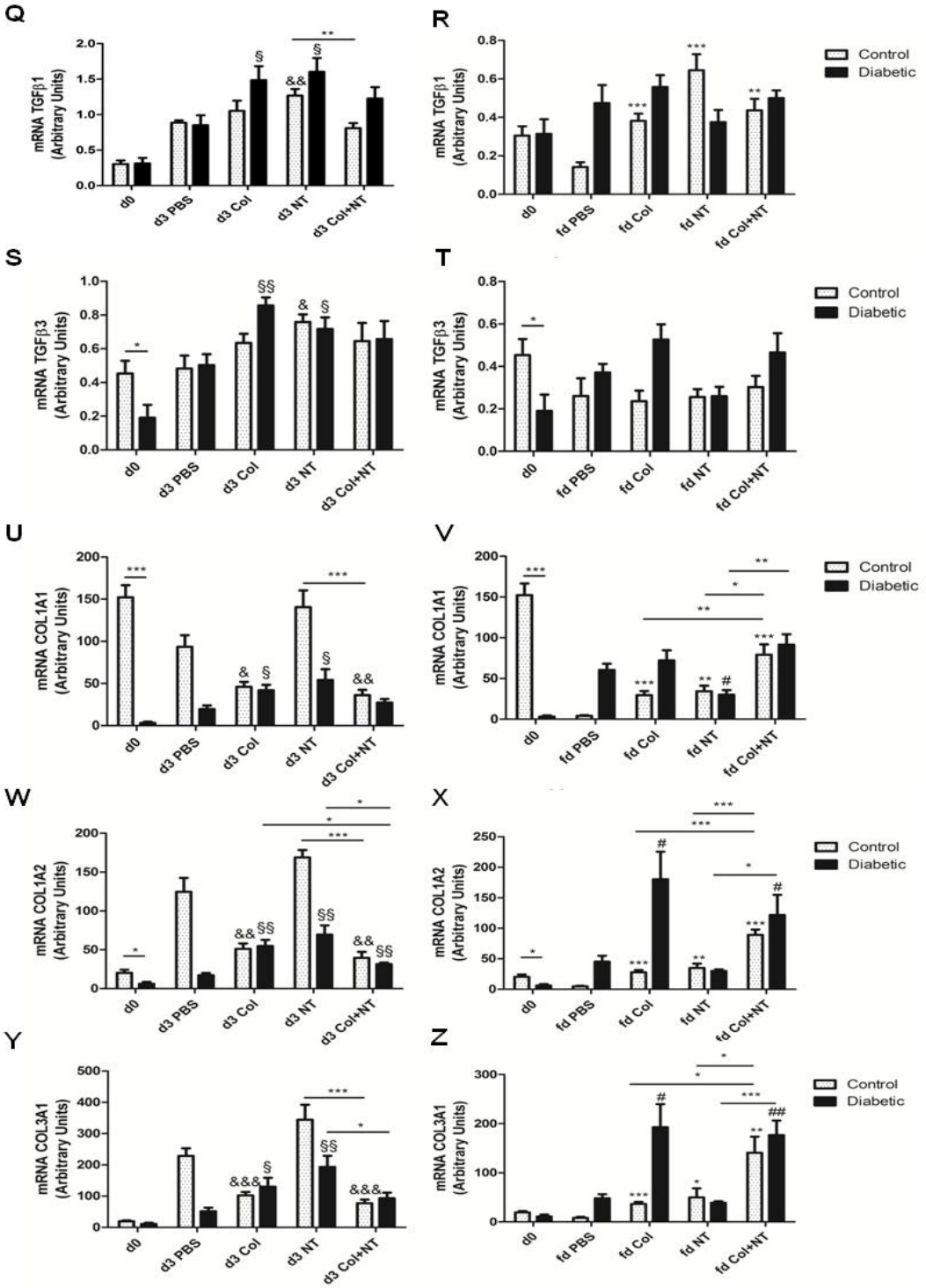

Figure 5.3. The gene expression profile of TNF-α, IL-6, KC, IL-1β, MMP-9, EGF, VEGF, PDGF, TGFβ1, TGFβ3, COL1A1, COL1A2 and COL3A1 in skin biopsies before and after treatments, at either day 3 (A, C, E, G, I, K, M, O, Q, S, U, W and Y) or total wound healing day (fd) (B, D, F, H, J, L, N, P, R, T, V, X and Z) 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.

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).

5.4.5 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 (Figure 5.5). 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 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.

5.4.6 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 (Figures 5.6A 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 5.2).

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 reepithelialization occurred over the granulation inflammatory tissue while it was suffering repair,

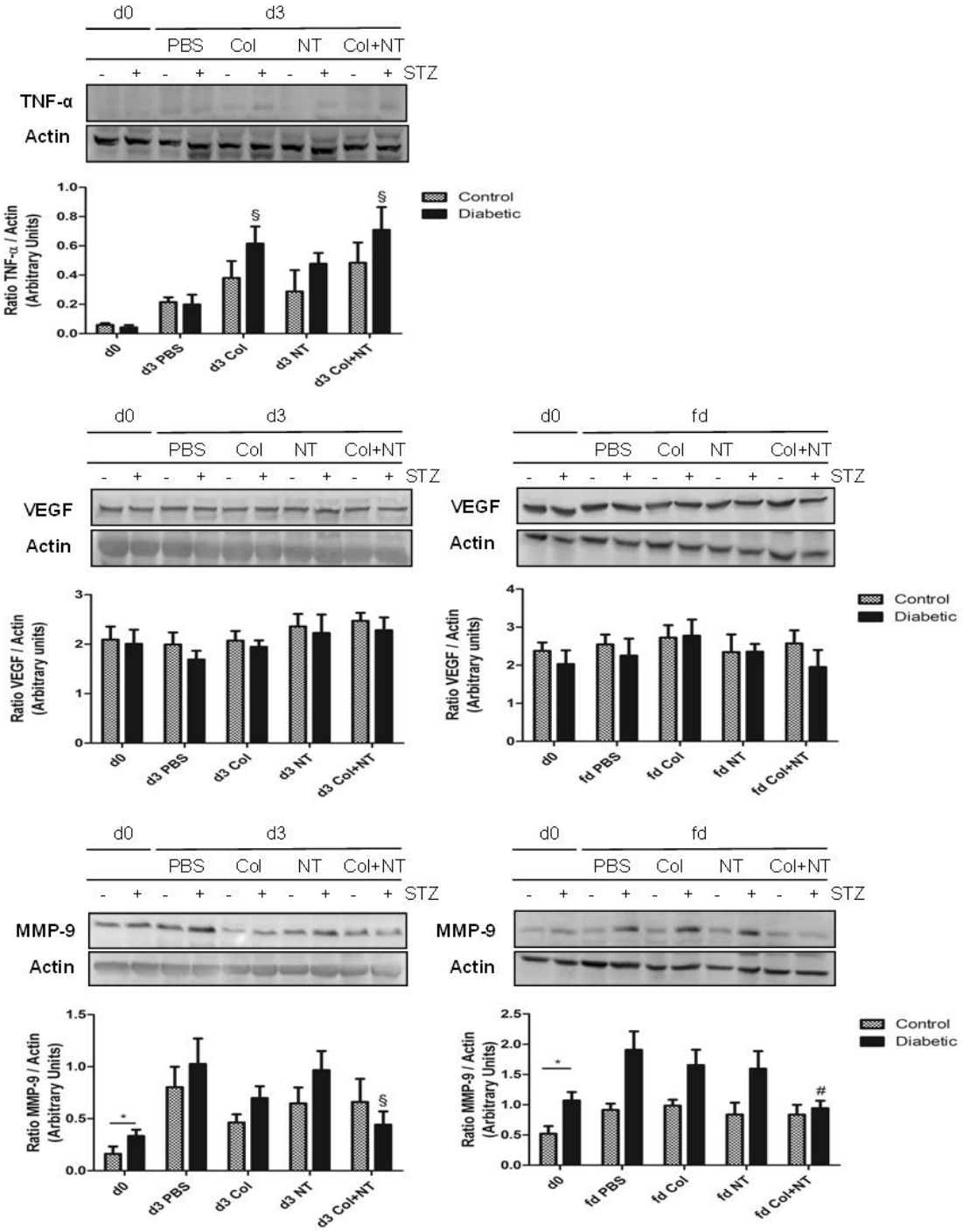

Figure 5.4. Protein expression of TNF-α, VEGF and MMP-9 in unwounded skin (day 0) or after treatments, at either day 3 or total wound healing day (fd). Results are presented as mean ± SEM of three to five independent experiments. § $p < 0.05$ compared with PBS d3, & $p < 0.05$ compared with diabetic PBS d3, #$p < 0.05$ compared with diabetic PBS fd.

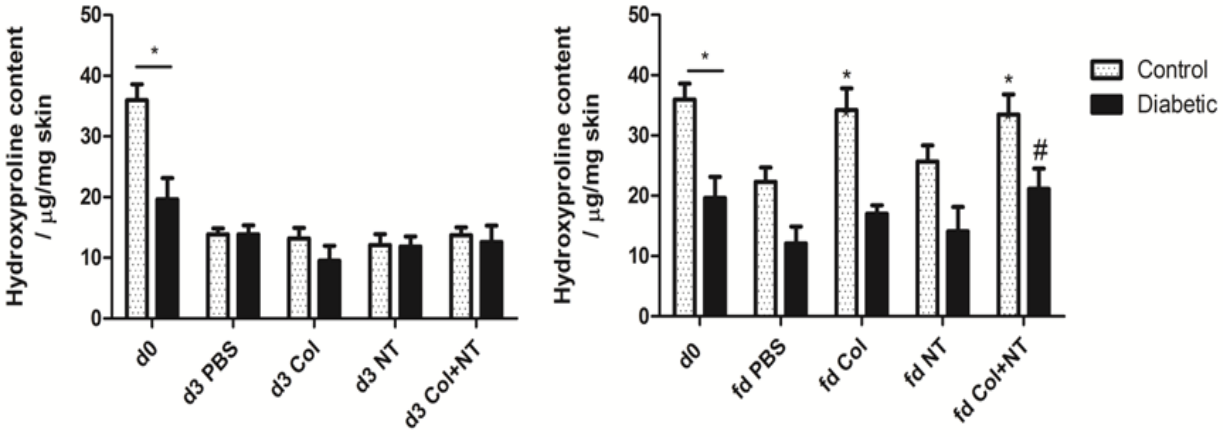

Figure 5.5. Hydroxyproline content levels in unwounded skin (d0) or after treatments, at either day 3 or after total wound healing (fd). Results are presented as mean ± SEM of four to six independent experiments. *p < 0.05 compared with PBS fd.

without correlation with the treatments and in both groups (Table 5.2). 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 5.4). 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 5.5). No fibrin was observed either in control or diabetic skin. There was increased 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 then 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 5.6.

A

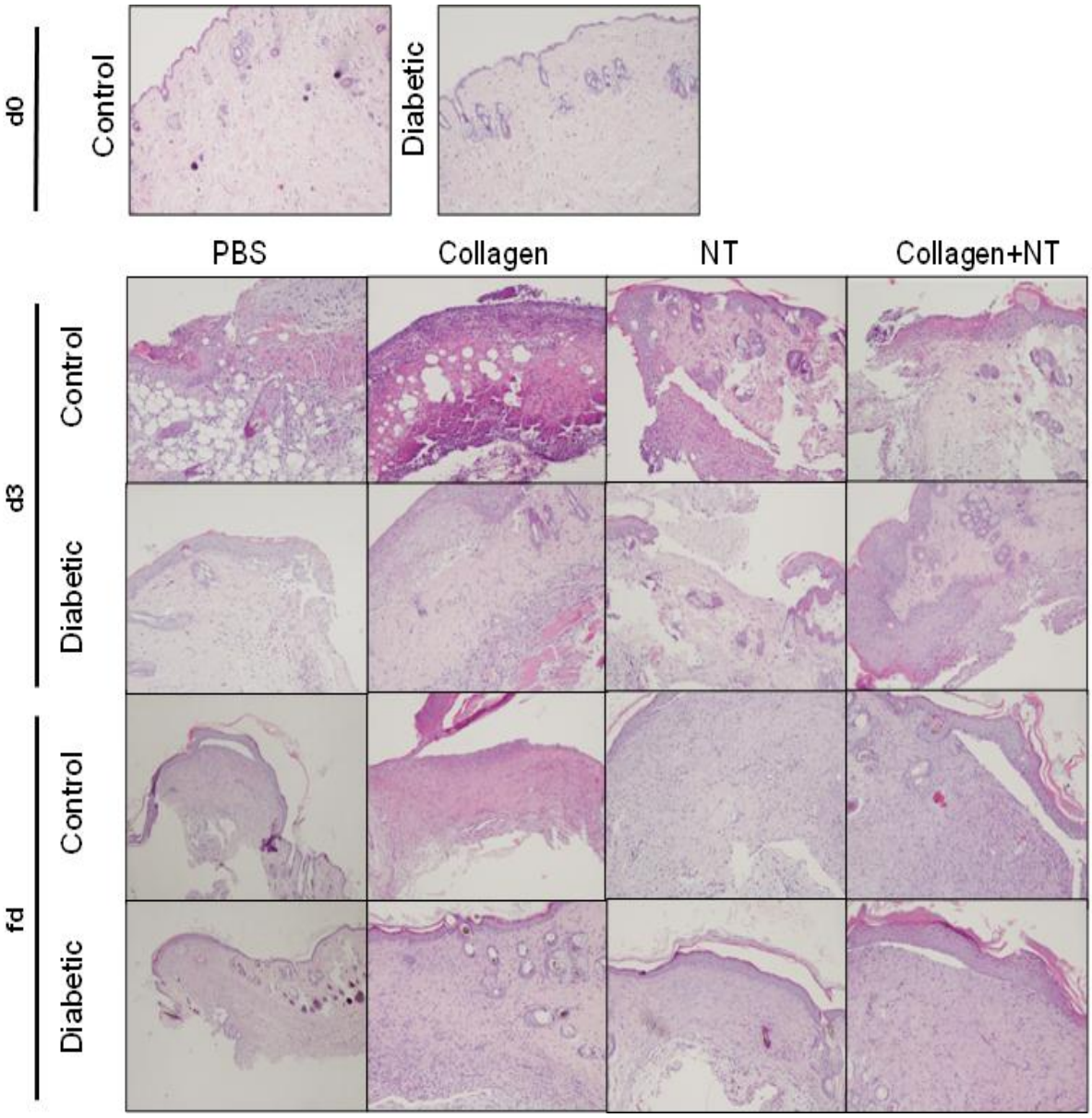

Figure 5.6. Histopathological analysis of Hematoxicilin and Eosin (H&E) (Figure 5.6A) and Masson´s Trichrome (Figure 5.6B) stainings for control and diabetic mouse skin, untreated or treated with collagen, NT and NT-loaded collagen foams (magnification 100×). Representative images of three skin staining were analyzed (cont.).

B

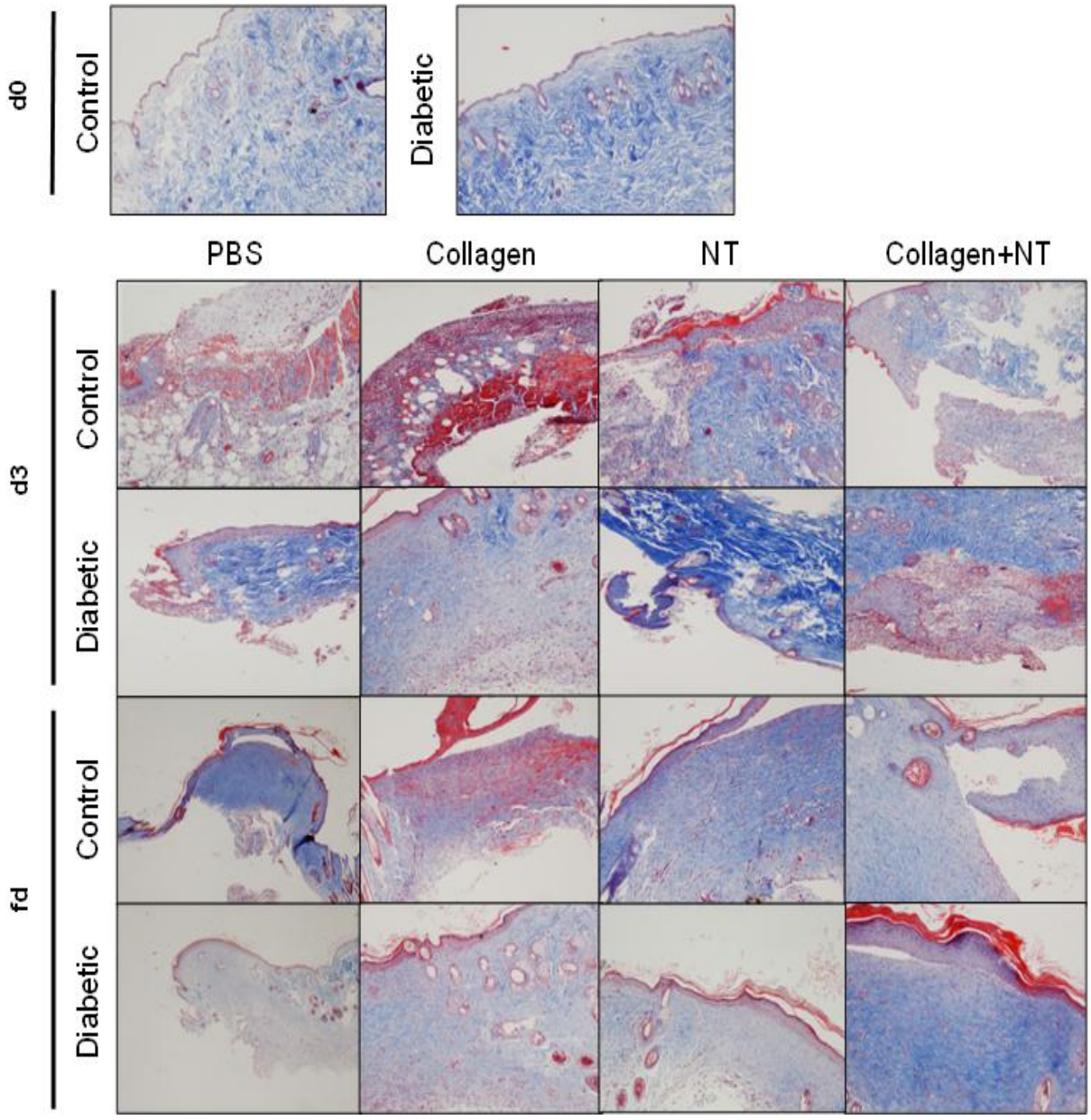

Figure 5.6. Histopathological analysis of Hematoxicilin and Eosin (H&E) (Figure 5.6A) and Masson´s Trichrome (Figure 5.6B) stainings for control and diabetic mouse skin, untreated or treated with collagen, NT and NT-loaded collagen foams (magnification 100×). Representative images of three skin staining were analyzed.

5.5 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

Table 5.2 Histological analysis of unwounded skin and in the presence of NT, collagen and NT-loaded collagen dressings treated wounds at day 3, by H&E staining. - absence or no alterations, + presence <10%, ++ presence 10%-50%, n.a - 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-epithelization										
- From bottom	na	na	+	-	+	-	+	-	+	-
- Top cover	na	na	-	+	-	+	-	+	-	+

Table 5.3: 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

	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	+	++	++	+++	++	+++	++	+++

Table 5.4 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

	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 5.5 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

	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	+	+	+	++	+	+	+	++

Table 5.6 Summary of cytokine 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 cytokine/growth factors
3	TNF-α	=	↑ NT, ↓Col+NT	Macrophages, fibroblasts
	IL-6	=	=	Macrophages, fibroblasts, Keratinocytes, endothelial cells
	KC	=	=	Macrophages, fibroblasts
	IL-1β	↓ NT, Col+NT	=	Macrophages, epithelial cells
	MMP-9	↓ Col+NT	↓ Col, Col+NT	fibroblasts, keratinocytes, macrophages, endothelial cells
	EGF	=	=	Macrophages, platelets
	VEGF	↑ Col, NT	=	Fibroblasts, macrophages, neutrophils, endothelial cells
	PDGF	=	↓ Col+NT	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-6	=	↓ Col, NT, Col+NT	Macrophages, fibroblasts, keratinocytes, endothelial cells
	KC	=	=	Macrophages, fibroblasts
	IL-1β	=	=	Macrophages, epithelial cells
	MMP-9	↓ Col, NT, Col+NT	↓ Col, NT, Col+NT	Fibroblasts, keratinocytes, macrophages, endothelial cells
	EGF	↓ Col+NT	=	Macrophages, platelets
	VEGF	=	=	Fibroblasts, macrophages, neutrophils, endothelial cells
	PDGF	=	↓ NT	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

and co-authors (Nabzdyk *et al.*, 2013) verified that in DFU, neuropeptides such as Substance P and Neuropeptide Y control cytokine release from leukocytes and affect endothelial cell function. Neuropeptides can be produced by skin cells or released by sensory neurons in response to stimuli, promoting different skin cellular responses. In addition, positive NT fibers where identified in the skin suggesting that NT has also important cutaneous functions (da Silva *et al.*, 2011). 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 been frequently used in as wound dressings material to accelerate healing (Wang *et al.*, 2008, Sarkar *et al.*, 2011). Among other advantages it has a prominent role to maintain the biological and structural integrity of the extracellular matrix (ECM) (Parenteau-Bareil *et al.*, 2010).

Results for the *in vitro* biocompatibility of collagen with cells present in skin, namely macrophage

Raw 264.7 and keratinocytes HaCaT cell lines showed that the materials prepared in this work were non-toxic against these cell lines (up to 72h) 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 keratinocytes HaCaT cells adhere perfectly to collagen without apparent change in their morphology. Similar results were observed by Yahyouche and co-authors, 2011 (Yahyouche *et al.*, 2011) 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 keratinocytes cell line NHEK (Rho *et al.*, 2006).

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 (Properzi *et al.*, 1993, Gibran *et al.*, 2002, Scott *et al.*, 2008). 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. During the wound closure experiments, the skin was collected after complete healing (fd) (when the wounds were totally closed) to analyze the effect of each treatment (NT, NT-loaded collagen and also collagen alone) in the formation of the final scar. 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-6, KC and IL-1β), matrix metalloproteinases (MMP-9) and growth factors (EGF, VEGF, PDGF) was observed in unwounded diabetic skin which is in agreement with previously reported data (Galkowska *et al.*, 2006). 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 (Blakytny and Jude, 2006). 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 (Black *et al.*, 2003) 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, IL-6, KC 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 (Galkowska *et al.*, 2006). In addition, IL-6 and IL-1β, produced by macrophages, fibroblasts, keratinocytes and epithelial cells are also important players in the early phase of inflammation and in the wound healing process (Hansen *et al.*, 2003). 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 (Moura *et al.*, 2013b).

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 (Lobmann *et al.*, 2002). 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 (Beldon, 2010).

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 (Volk *et al.*, 2011). 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 amount of inflammatory cytokine IL-6 was significantly reduced by all the treatments in diabetic skin, contributing to the resolution of inflammation. Moreover, 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 (Singer and Clark, 1999). The expression of angiogenic factors, such as, EGF, VEGF and PDGF was not

modulated by any of the studied treatments (NT, NT-loaded collagen neither collagen alone) showing that they may not directly stimulate the production of growth factors for tissue repair.

Fibroblasts promote re-epithelialization by the production of ECM proteins such as collagen (Al-Mulla *et al.*, 2011). Fibroblast accumulation at the wound site was 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 aesthetic 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 (Moura *et al.*, 2013b).

5.6 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 aesthetic 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.

5.7 Acknowledgments

This work was financially supported by COMPETE, FEDER and Fundação para a Ciência e Tecnologia (FCT-MES) 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-MES for their fellowships SFRH/BD/60837/2009, SFRH/BPD/40409/2007 and SFRH/BPD/46341/2008, respectively.

5.8 References

Adhirajan N, Shanmugasundaram N, Shanmuganathan S, Babu M (2009) Collagen-based wound dressing for doxycycline delivery: in-vivo evaluation in an infected excisional wound model in rats. Journal of Pharmacy and Pharmacology 61:1617-1623.

Al-Mulla F, Leibovich SJ, Francis IM, Bitar MS (2011) Impaired TGF-beta signaling and a defect in resolution of inflammation contribute to delayed wound healing in a female rat model of type 2 diabetes. Molecular bioSystems 7:3006-3020.

Antonio F, Guillem R, Sonia T, Clara M, Piergiorgio G, Valeria C, Gianluca C, Tzanov T (2011) Cross-linked collagen sponges loaded with plant polyphenols with inhibitory activity towards chronic wound enzymes. Biotechnol J 6:1208-1218.

Arul V, Masilamoni JG, Jesudason EP, Jaji PJ, Inayathullah M, Dicky John DG, Vignesh S, Jayakumar R (2012) Glucose oxidase incorporated collagen matrices for dermal wound repair in diabetic rat models: a biochemical study. J Biomater Appl 26:917-938.

Beldon P (2010) Basic science of wound healing. Surgery 28:409-412.

Black E, Vibe-Petersen J, Jorgensen LN, Madsen SM, Agren MS, Holstein PE, Perrild H, Gottrup F (2003) Decrease of collagen deposition in wound repair in type 1 diabetes independent of glycemic control. Arch Surg 138:34-40.

Blakytny R, Jude E (2006) The molecular biology of chronic wounds and delayed healing in diabetes. Diabetic medicine : a journal of the British Diabetic Association 23:594-608.

Brain SD (1997) Sensory neuropeptides: their role in inflammation and wound healing. Immunopharmacology 37:133-152.

Cen L, Liu W, Cui L, Zhang W, Cao Y (2008) Collagen tissue engineering: development of novel biomaterials and applications. Pediatr Res 63:492-496.

da Silva L, Neves BM, Moura L, Cruz MT, Carvalho E (2011) Neurotensin downregulates the pro-inflammatory properties of skin dendritic cells and increases epidermal growth factor expression. Biochim Biophys Acta 1813:1863-1871.

Galkowska H, Wojewodzka U, Olszewski WL (2006) Chemokines, cytokines, and growth factors in keratinocytes and dermal endothelial cells in the margin of chronic diabetic foot ulcers. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 14:558-565.

Gibran NS, Jang YC, Isik FF, Greenhalgh DG, Muffley LA, Underwood RA, Usui ML, Larsen J, Smith DG, Bunnett N, Ansel JC, Olerud JE (2002) Diminished neuropeptide levels contribute to the impaired cutaneous healing response associated with diabetes mellitus. J Surg Res 108:122-128.

Hansen SL, Myers CA, Charboneau A, Young DM, Boudreau N (2003) HoxD3 accelerates wound healing in diabetic mice. The American journal of pathology 163:2421-2431.

Kalafatakis K, Triantafyllou K (2011) Contribution of neurotensin in the immune and neuroendocrine modulation of normal and abnormal enteric function. Regulatory peptides 170:7-17.

Kanda N, Morimoto N, Ayvazyan AA, Takemoto S, Kawai K, Nakamura Y, Sakamoto Y, Taira T, Suzuki S (2012) Evaluation of a novel collagen-gelatin scaffold for achieving the sustained release of basic fibroblast growth factor in a diabetic mouse model. J Tissue Eng Regen Med.

Kawai K, Suzuki S, Tabata Y, Nishimura Y (2005) Accelerated wound healing through the incorporation of basic fibroblast growth factor-impregnated gelatin microspheres into artificial dermis using a pressure-induced decubitus ulcer model in genetically diabetic mice. Br J Plast Surg 58:1115-1123.

Lazarus LH, Brown MR, Perrin MH (1977) Distribution, localization and characteristics of neurotensin binding sites in the rat brain. Neuropharmacology 16:625-629.

Lobmann R, Ambrosch A, Schultz G, Waldmann K, Schiweck S, Lehnert H (2002) Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. Diabetologia 45:1011–1016.

Malafaya PB, Silva GA, Reis RL (2007) Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. Adv Drug Deliv Rev 59:207-233.

Manizate F, Fuller A, Gendics C, Lantis J (2012) A Prospective, Single-Center, Nonblinded, Comparative, Postmarket Clinical Evaluation of a Bovine-Derived Collagen With Ionic Silver Dressing versus a Carboxymethylcellulose and Ionic Silver Dressing for the Reduction of Bioburden in Variable-Etiology, Bilateral Lower-Extremity Wounds. Advances in Skin & Wound Care 25:220-225

Mano JF, Silva GA, Azevedo HS, Malafaya PB, Sousa RA, Silva SS, Boesel LF, Oliveira JM, Santos TC, Marques AP, Neves NM, Reis RL (2007) Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends. Journal of the Royal Society Interface 4:999-1030.

Moura LI, Dias AM, Carvalho E, de Sousa HC (2013a) Recent advances on the development of wound dressings for diabetic foot ulcer treatment-A review. Acta biomaterialia 9:7093-7114.

Moura LIF, Dias AMA, Leal EC, Carvalho L, de Sousa HC, Carvalho E (2013b) Chitosan-based dressings loaded with neurotensin-an efficient strategy to improve early diabetic wound healing. Acta biomaterialia Submitted.

Nabzdyk LP, Kuchibhotla S, Guthrie P, Chun M, Auster ME, Nabzdyk C, Deso S, Andersen N, Gnardellis C, LoGerfo FW, Veves A (2013) Expression of neuropeptides and cytokines in a rabbit model of diabetic neuroischemic wound healing. Journal of Vascular Surgery.

Parenteau-Bareil R, Gauvin R, Berthod F (2010) Collagen-Based Biomaterials for Tissue Engineering Applications. Materials 3:1863-1887.

Pradhan L, Cai X, Wu S, Andersen ND, Martin M, Malek J, Guthrie P, Veves A, Logerfo FW (2011) Gene expression of pro-inflammatory cytokines and neuropeptides in diabetic wound healing. J Surg Res 167:336-342.

Pradhan L, Nabzdyk C, Andersen ND, LoGerfo FW, Veves A (2009) Inflammation and neuropeptides: the connection in diabetic wound healing. Expert Rev Mol Med 11:e2.

Properzi G, Villa SF, Poccia G, Aloisi P, Gu X, Terenghi G, Polak JM (1993) Early increase precedes a depletion of VIP and PGP-9.5 in the skin of insulin-dependent diabetics—correlation between quantitative immunohistochemistry and clinical assessment of peripheral neuropathy. The Journal of pathology 169:269–277.

Rho KS, Jeong L, Lee G, Seo BM, Park YJ, Hong SD, Roh S, Cho JJ, Park WH, Min BM (2006) Electrospinning of collagen nanofibers: effects on the behavior of normal human keratinocytes and early-stage wound healing. Biomaterials 27:1452-1461.

Sarkar A, Tatlidede S, Scherer SS, Orgill DP, Berthiaume F (2011) Combination of stromal cell-derived factor-1 and collagen-glycosaminoglycan scaffold delays contraction and accelerates reepithelialization of dermal wounds in wild-type mice. Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society 19:71-79.

Scott JR, Tamura RN, Muangman P, Isik FF, Xie C, Gibran NS (2008) Topical substance P increases inflammatory cell density in genetically diabetic murine wounds. Wound Repair and Regeneration 16:529-533

Sell SA, Wolfe PS, Garg K, McCool JM, Rodriguez IA, Bowlin GL (2010) The use of natural polymers in tissue engineering: a focus on electrospun extracellular matrix analogues. Polymers for Advanced Technologies 2:522-553.

Shaw JE, Sicree RA, Zimmet PZ (2010) Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes research and clinical practice 87:4-14.

Silva L, Carvalho E, Cruz MT (2010) Role of neuropeptides in skin inflammation and its involvement in diabetic wound healing. Expert Opin Biol Ther 10:1427-1439.

Singer AJ, Clark RA (1999) Cutaneous wound healing. N Engl J Med 341:738-746.

Singh O, Gupta SS, Soni M, Moses S, Shukla S, Mathur RK (2011) Collagen Dressing Versus Conventional Dressings in Burn and Chronic Wounds: A Retrospective Study. Journal of Cutaneous and Aesthetic Surgery 4:12-16.

Slaughter BV, Shahana SK, Fisher OZ, Khademhosseini A, Peppas NA (2009) Hydrogels in Regenerative Medicine. Advanced Materials 21:3307–3329.

Sundler F, Hakanson R, Hammer RA, Alumets J, Carraway R, Leeman SE, Zimmerman EA (1977) Immunohistochemical localization of neurotensin in endocrine cells of the gut. Cell and tissue research 178:313-321.

Sweitzer SM, Fann SA, Borg TK, Baynes JW, Yost MJ (2006) What is the future of diabetic wound care? The Diabetes Educator 32:197-210.

Tellechea A, Leal E, Veves A, Carvalho E (2010) Inflammatory and angiogenic abnormalities in diabetic wound healing: role of neuropeptides and therapeutic perspectives The Open Circulation and Vascular Journal 3:43-55.

Volk SW, Wang Y, Mauldin EA, Liechty KW, Adams SL (2011) Diminished type III collagen promotes myofibroblast differentiation and increases scar deposition in cutaneous wound healing. Cells, tissues, organs 194:25-37.

Wang W, Lin S, Xiao Y, Huang Y, Tan Y, Cai L, Li X (2008) Acceleration of diabetic wound healing with chitosan-crosslinked collagen sponge containing recombinant human acidic fibroblast growth factor in healing-impaired STZ diabetic rats. Life Sci 82:190–204.

Yahyouche A, Zhidao X, Czernuszka JT, Clover AJ (2011) Macrophage-mediated degradation of crosslinked collagen scaffolds. Acta biomaterialia 7:278-286.

Chapter 6

Conclusions
Future trends and perspectives

DFU is a frequent complication of diabetes that may lead to severe and persistent infection and in extreme cases, to lower extremity amputation. The therapeutics used to date involve topical application of drugs, antimicrobials, plant extracts, neuropeptides and growth factors. A major problem of topical administration of proteins or neuropeptides is their short half-life and loss of bioactivity in the peptidase-rich wound environment. To overcome this problem the use of biocompatible wound dressings for the sustained delivery of neuropeptides was addressed in this work. In addition, 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. The necessity to develop and improve the efficacy of wound dressings, particularly suitable for DFU treatment, has been a challenge for both researchers and clinicians.

The first main aim of this thesis was to understand the potential therapeutic role of - neurotensin- in compromised wound healing conditions, similar to DFU, which is characterized by pathological hyperglycemia, a pro-inflammatory status and impaired cell migration in different skin cells, namely macrophages and keratinocytes. The results, presented in chapter 2 and 3, showed a reduction in the macrophage migratory profile under hyperglycemic conditions, when compared to normal glycemia. However, NT was able to highly improve the migratory capacity of these cells, either under normal or inflammatory conditions. In addition, under hyperglycemia, NT inhibited the inflammatory response of macrophages, having a pro-inflammatory effect in these cells, however, p38 MAPK, p44/42MAPK and PKB/AKT signaling pathways was not activated by NT in either 10mM or 30mM glucose conditions.

Understanding which of the NT receptors might be involved in the inflammatory response of macrophages induced by NT is important in order to better delineate the mechanisms involved in the effects of NT. The NTR3 was the most expressed receptor in macrophages under both glucose conditions. However, a significant decrease in endogenous NT and NTR expression was observed under hyperglycemic conditions, which may be correlated with the decrease of macrophage migration induced by high glucose. Under 10mM glucose, NT significantly stimulated endogenous NT, NTR1 and NTR2 expression, while no changes was observed for NTR3. On the other hand, in cells under 30mM glucose, NT highly increased NTR3 expression.

Under inflammatory conditions, endogenous NT is highly expressed in either the presence or absence of exogenous NT, while NTR1 and NTR2 was greatly expressed in the presence of LPS but when NT was present their expression returns to control levels. On the other hand, the expression of NTR3 is decreased when compared to non-inflammatory conditions.

In short, NT affected macrophage responses, both under inflammatory and hyperglycemic conditions, through the stimulation of cell migration, inhibition of the pro-inflammatory status and regulation of cytokine expression, contributing to the resolution of inflammation and allowing the progression to the migration-remodeling phases of diabetic wound healing.

Another skin cell line studied was HaCaT - human keratinocytes. The results showed that hyperglycemia reduced significantly NT and all NT receptor expression in human keratinocytes, similar to the results observed for the macrophage cell line. In addition, NT treatment significantly stimulated the expression of NT and NTR2 while NTR1 and NTR3 expression levels were unchanged in hyperglycemia. However, NT did not affect proliferation, migration and expression of inflammatory cytokines. Only hyperglycemia decreased keratinocyte migration after NT treatment.

These results suggest that NT, in keratinocytes under hyperglycemic conditions, could have a paracrine effect on other skin cells, namely on macrophages and dendritic cells.

These results highlight the crucial role of neuropeptides, such as NT, in wound healing and a dysfunctional expression of these molecules under hyperglycemic conditions could be correlated with the physiopathology of diabetic foot ulcers.

The second main objective of the thesis (Chapter 4 and 5) was to develop, characterize and apply wound dressings, prepared from chitosan derivatives (CMC, MPC, SC) or collagen, for a prolonged and efficient NT delivery into diabetic and non-diabetic wounds. The *in vivo* progression of skin wound healing, after topical wound dressing application with or without NT, was monitored over 10 days and evaluated through the analyses of the inflammatory, angiogenic collagen deposition effects of NT using a mouse model of wound healing.

The results showed that MPC had an intermediate profile and the best fluid handling capacities and delivery profile. Even though all treatments reduced the wound area, non-loaded and NT-loaded MPC dressings induced a significant reduction of the wound size, especially in the first 3 days post-wounding in both control and diabetic mice, with the NT-loaded MPC presenting the faster healing profile in diabetic skin wounds. These results suggest a synergistic behavior between the bioactivity of NT alone and the intrinsic healing properties of MPC. However, larger scars are developed over diabetic wounds that were treated with MPC dressings due to the fast initial skin contraction.

In addition, an increase of the inflammatory cytokines, TNF-α and IL-1β, expression, as well as less infiltrated inflammatory cells are observed after NT and NT-loaded MPC application, at day 3 post-wounding in diabetic skin. Shortly, NT-loaded MPC dressings had an effective anti-inflammatory role in wound healing. These results suggest that increased expression of TNF-α is produced not only by inflammatory cells present at the wound site, but also by other cells present at day 3, which can stimulate contraction of the wound and consequently have a beneficial effect in the early stages of wound healing. At day 10, all treatments led to a reduction in the inflammatory cytokine expression supported by the loose conjunctive tissue observed from the beginning, undergoing different status of collagen deposition in diabetic and control mice. At this time point, fibroblasts have an important role in collagen synthesis and scar formation. The production of the collagen matrix was higher for MPC and NT-loaded MPC treated diabetic skin, which is correlated with increased scar formation.

A similar study was performed using collagen, extracted from mouse tails, as a wound dressing.

Treatments with collagen alone or in combination with NT showed to be more effective in reducing the wound area, especially in diabetic mice, already at day 3 post-wounding. All the treatments lead to total healing, however larger scars are developed over diabetic wounds that are treated with collagen dressings, as observed also for MPC dressings. In control mice, a decrease in the expression of IL-1β after treatment with NT and NT-loaded collagen dressings suggest a decreased in the inflammatory status, which promoted the healing process. However, in diabetic mice, NT stimulated an increase of TNF-α gene expression, while collagen alone or combined with NT stimulated protein expression of this inflammatory marker.

In addition, all the treatments studied recruited less inflammatory cells to the wound site when compared to control. This can suggest that the high expression of TNF-α at day 3, is not only produce by the inflammatory cells present at the wound site, but also by other cells, such as fibroblasts and other skin cells. In addition, at day 3, increased expression of all collagen types is observed after treatment with collagen and compared with MPC. After the complete healing day (fd), a more prominent scar was evident when non-loaded and NT-loaded collagen were applied in diabetic skin probably due to higher expression of the different types of collagen by fibroblasts.

From these, *in vivo* results it can be concluded that the application of NT-loaded MPC or collagen dressings in diabetic wound dressings can promote an inflammatory response and stimulate re-epithelialization, which are important phases of the healing process.

In future studies, it would be important to understand the effects of NT in other skin cells, namely in fibroblasts, and in order to evaluate how can NT modulate the proliferation, migration, as well as the inflammatory and angiogenic responses by these cells. In addition, the effects of the combination of NT with other neuropeptides with known healing characteristics, such as SP or NPY, could also be further studied.

The development of alternative dressings can also be another possibility of future work. This could be done by the combination of different biopolymers to create materials presenting improved properties that can guarantee optimal wound environments. Therefore, natural (e.g. chitosan, hyaluronic acid, cellulose, alginate, collagen, fibrin, etc.) or synthetic (e.g. PVA, PEG, PVP, PU, PHEMA, poly (α-esters)) biopolymers combined, modified or cross-linked are potential examples

for these purposes. Other future studies to improve wound healing processes could address the application of wound dressings loaded with NT (and other neuropeptides) in combination with other bioactive substances such as: antimicrobials/antibiotics (to prevent/treat infections); natural extracts with healing properties; fibroblasts and other skin cells; stem cells; or growth factors (e.g. VEGF, PDGF, etc.).