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TESE DE DOUTORAMENTO

DEVELOPMENT OF NOVEL THERAPEUTIC APPROACHES FOR WOUND HEALING IN DIABETES

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Development of novel therapeutic approaches for wound healing in diabetes

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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-epithelializing factors were performed \textit{(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 \textit{in vitro} results, it was concluded that NT impairs macrophage migration under hyperglycemic conditions as well as it decreases their pro-inflammatory cytokines (IL-1\(\beta\) 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-1\(\beta\) 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 \textit{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-\(\alpha\)) 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-\(\alpha\) and IL-1\(\beta\)) 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 hiperiglicémicas 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 biocompatíveis 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:


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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*).
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5.5 Skin inflammatory and granulation tissue histological analysis in the presence of NT, collagen and NT-loaded collagen foams treated control and diabetic wounds at total wound healing day (fd), by H&E and Masson’s Trichrome staining.

5.6 Summary of cytokine expression in wounded control and diabetic skin, at day 3 and after complete healing (fd).
## List of acronyms

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<th>Description</th>
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<tbody>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMSCS</td>
<td>Bone marrow stem cells</td>
</tr>
<tr>
<td>CBC</td>
<td>N-carboxybuthyl chitosan</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin gene-related peptide</td>
</tr>
<tr>
<td>CMC</td>
<td>N-carboxymethylchitosan</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COL1A1</td>
<td>Collagen type I, alpha 1</td>
</tr>
<tr>
<td>COL1A2</td>
<td>Collagen type I, alpha 2</td>
</tr>
<tr>
<td>COL3A1</td>
<td>Collagen type III, alpha 1</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony-stimulating factor 1</td>
</tr>
<tr>
<td>DFU</td>
<td>Diabetic foot ulcer</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DTNB</td>
<td>Ditio-bis(nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>Ditiotreitol</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Endothelial growth factor</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cells</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FSDC</td>
<td>Fetal skin dendritic cell</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
</tr>
<tr>
<td>HPRT-1</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>IDDM</td>
<td>Insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>KC</td>
<td>Interleukin-8 related protein in rodents</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MIP-2</td>
<td>Macrophage inflammatory protein – 2</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Metalloproteinase 9</td>
</tr>
<tr>
<td>MPC</td>
<td>5-methyl pyrrolidinone chitosan</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NEP</td>
<td>Neutral endopeptidase</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non-insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>NK</td>
<td>Tackykinin receptors</td>
</tr>
<tr>
<td>NKA</td>
<td>Neurokinin A</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NT</td>
<td>Neurotensin</td>
</tr>
<tr>
<td>NTR</td>
<td>Neurotensin receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocytes</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>REK</td>
<td>Rat epidermal keratinocytes</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SC</td>
<td>N-succinyl chitosan</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>Stromal cell-derived factor 1 alpha</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TBP</td>
<td>Tata binding protein</td>
</tr>
<tr>
<td>TGF β1</td>
<td>Transforming growth factor β1</td>
</tr>
<tr>
<td>TGF β3</td>
<td>Transforming growth factor β3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor -α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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</table>
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-epithelializing 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, death (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 \(\beta\)-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 \(\beta\)-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 1 diabetes 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
Introduction

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.

<table>
<thead>
<tr>
<th>Diabetes complications</th>
<th>Macrovascular</th>
<th>Microvascular</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Stroke</td>
<td></td>
<td>- Retinopathy</td>
</tr>
<tr>
<td>- Coronary heart disease</td>
<td></td>
<td>- Nephropathy</td>
</tr>
<tr>
<td>(angina, myocardial infarction)</td>
<td>- Peripheral vascular disease</td>
<td>- Neuropathy → diabetic foot ulcer</td>
</tr>
<tr>
<td>- Peripheral vascular disease</td>
<td></td>
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</tr>
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</table>
Chapter 1

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 0.8 mm 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 subcutaneous layer 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.

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.
Introduction

(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.,
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 colleagues (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\textsuperscript{2+} mobilization and also by mitogen-activated protein (MAP) kinase phosphorylation. Furthermore, NTR2 internalization activates the ERK1/2 pathway however no Ca\textsuperscript{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-κB 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 endopeptidase (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, Lithospermum erythrorhizon, 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).

<table>
<thead>
<tr>
<th>Bioactive substances</th>
<th>Models used</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>db/db diabetic mice</td>
<td>Enhanced neovascularization, mobilization of bone marrow-derived cells into the wound site to accelerate wound healing</td>
<td>Galiano et al., 2004</td>
</tr>
<tr>
<td>PDGF</td>
<td>STZ diabetic rats</td>
<td>Enhanced angiogenesis, cell proliferation and epithelialization. Formed thicker and more highly organized collagen fiber deposition in wounds</td>
<td>Li et al., 2008</td>
</tr>
<tr>
<td>bFGF</td>
<td>Human patients with DFUs</td>
<td>Reduction of 75% of wound area in treated patients. Stimulated granulation and epithelialization of tissues</td>
<td>Uchi et al., 2009</td>
</tr>
<tr>
<td>SDF-1α</td>
<td>STZ diabetic mice</td>
<td>Increased EPC mobilization, homing and wound healing</td>
<td>Gallagher et al., 2007</td>
</tr>
<tr>
<td>BMSCs</td>
<td>STZ diabetic rats</td>
<td>Promoted healing and improved the wound breaking strength. In addition, it increased collagen levels and TGF-β, KGF, EGF and VEGF expression</td>
<td>Kwon et al., 2008</td>
</tr>
<tr>
<td>CD133⁺ cells</td>
<td>STZ diabetic rats</td>
<td>Accelerated wound closure and promoted angiogenesis through stimulation of endothelial cell proliferation and migration</td>
<td>Barcelos et al., 2009</td>
</tr>
<tr>
<td>Stem cells</td>
<td>db/db diabetic mice</td>
<td>Increased wound closure and stimulated production of extracellular matrix proteins and secreted soluble factors</td>
<td>Amos et al., 2010</td>
</tr>
<tr>
<td>Human adipose-derived stromal cells</td>
<td>STZ diabetic rats</td>
<td>Reduced significantly wound size and increased expression of EGF and VEGF</td>
<td>Lee et al., 2011</td>
</tr>
<tr>
<td>EPCs</td>
<td>db/db diabetic mice</td>
<td>Promoted wound healing and vascularity and induced expression of VEGF and bFGF</td>
<td>Asai et al., 2012</td>
</tr>
</tbody>
</table>
Table 1.2: Some different and recently proposed approaches to improve DFU treatment (cont).

<table>
<thead>
<tr>
<th>Bioactive substances</th>
<th>Models used</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoviral PDGF-B</td>
<td>db/db diabetic mice</td>
<td>Significantly enhanced wound repair and neovascularization. In addition, adenoviral-PDGF-B stimulated EPC recruitment to the wound site</td>
<td>Keswani et al., 2004</td>
</tr>
<tr>
<td><strong>Gene therapy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lentiviral-containing SDF-1α</td>
<td>STZ diabetic mice obese NOD/Lj mice</td>
<td>Improved diabetic wound healing with completely epithelialization and increased the granulation tissue.</td>
<td>Bodillo et al., 2007</td>
</tr>
<tr>
<td>Adenoviral-Hox B3</td>
<td>db/db diabetic mice</td>
<td>Accelerated wound healing in diabetic mice</td>
<td>Mohebali et al., 2008</td>
</tr>
<tr>
<td>Substance P</td>
<td>db/db diabetic mice</td>
<td>Enhanced wound repair and increased early inflammatory density in the healing wounds</td>
<td>Gibran et al., 2002, Scott et al., 2008</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>STZ diabetic rats</td>
<td>Significantly reduced the time of total wound closure, increased micro vascular density, VEGF, and hydroxyproline contents and reduced extent of apoptosis</td>
<td>Hamed et al., 2010</td>
</tr>
<tr>
<td>Insulin</td>
<td>STZ diabetic rats</td>
<td>Enhanced wound healing and stimulated a faster epithelialization.</td>
<td>Lima et al., 2012</td>
</tr>
<tr>
<td><strong>Natural products</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lithospermum erythrorhizion extract</td>
<td>db/db diabetic mice</td>
<td>Decreased vascular permeability, formation of granulation tissue and accelerated wound healing</td>
<td>Fujita et al., 2003</td>
</tr>
<tr>
<td>Rehmanniae radix</td>
<td>STZ, diabetic rats</td>
<td>Promoted ulcer healing accelerating the processes of tissue regeneration, angiogenesis and inflammation</td>
<td>Lau et al., 2009</td>
</tr>
<tr>
<td>Aqueous extract of <em>Rosmarinus officinalis</em> (Rosemary)</td>
<td>Alloxan diabetic mice</td>
<td>Reduced inflammation and enhancement of wound contraction, re-epithelialization and regeneration of granulation tissue, angiogenesis and collagen deposition in the treated wounds</td>
<td>Abu-Al-Basal, 2010</td>
</tr>
<tr>
<td>Ampucare (polyherbal ingredient)</td>
<td>Alloxan diabetic rats</td>
<td>Significantly reduced the wound size and bacterial count in wound site. Stimulated a well-organized fibrous tissue proliferation, epithelialization and complete scar formation</td>
<td>Dwivedi and Chaudhary, 2012</td>
</tr>
</tbody>
</table>
Table 1.2: Some different and recently proposed approaches to improve DFU treatment.

<table>
<thead>
<tr>
<th>Bioactive substances</th>
<th>Models used</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>db/db diabetic mice</td>
<td>Accelerated healing and increased wound angiogenesis</td>
<td>Jacobi et al., 2002</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>db/db diabetic mice</td>
<td>Increased VEGF mRNA and protein expression. In addition, simvastatin enhanced NO wound content at day 6 impairing the wound healing process</td>
<td>Bitto et al., 2008</td>
</tr>
<tr>
<td>Azelnidipine</td>
<td>STZ diabetic rats</td>
<td>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</td>
<td>Bagheri et al., 2011</td>
</tr>
<tr>
<td>Ciclopirox olamine</td>
<td>db/db diabetic mice</td>
<td>Enhanced wound closure, increased angiogenesis and dermal cellularity</td>
<td>Ko et al., 2011</td>
</tr>
<tr>
<td>Naltrexone</td>
<td>STZ diabetic rats</td>
<td>Reduced wound area in 50% compared with control; stimulated DNA synthesis facilitating wound healing process</td>
<td>McLaughlin et al., 2011</td>
</tr>
</tbody>
</table>
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
Introduction

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 –NH₂ group of chitosan with the carbonyl group of aldehyde- glyoxylic acid followed by hydrogenation with NaBH₄. 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 $–\text{NH}_2$ 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), carboimide (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.

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

*Santyl* is a collagenase gel that possesses the ability to digest the collagen of necrotic tissue proposed to follow the use of *Santyl* 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.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Bioactive substance</th>
<th>Models used</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan-crosslinked collagen</td>
<td>Recombinant human aFGF</td>
<td>STZ diabetic rats</td>
<td>Accelerated wound healing promoting a faster tissue collagen deposition, higher TGF-β1 expression and dermal cell proliferation.</td>
<td>Wang et al., 2008</td>
</tr>
<tr>
<td>Chitosan with different degrees of deacetylation</td>
<td>Acetylg glucosamine oligomers</td>
<td>Human diabetic</td>
<td>Decreased wound size and stimulated angiogenesis and reepithelialization after seven days.</td>
<td>Ben-shalom et al., 2009</td>
</tr>
<tr>
<td>Chitosan and derivatives</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiolated chitosan-oxidized dextran hydrogel</td>
<td>–</td>
<td>STZ diabetic mice</td>
<td>Showed to be non-cytotoxic, resistant to degradation and capable of stimulate tissue regeneration.</td>
<td>Zhang et al., 2011</td>
</tr>
<tr>
<td>Chitosan, alginate, and poly(γ-glutamic acid) hydrogel</td>
<td>–</td>
<td>STZ diabetic rats</td>
<td>Enhanced wound healing. Stimulated collagen deposition, hydroxyproline levels and promoted skin epithelialization. Showed antibacterial properties.</td>
<td>Lee et al., 2012</td>
</tr>
<tr>
<td>Collagen dressing</td>
<td>–</td>
<td>Human patients with DFUs</td>
<td>60% of them healed after two weeks of treatment. A decrease of infection by bacteria and an augment of granulation tissue were also observed.</td>
<td>Singh et al., 2011</td>
</tr>
<tr>
<td>Collagen matrix</td>
<td>Glucose oxidase</td>
<td>STZ diabetic rats</td>
<td>Increased cellular proliferation and stimulated a faster wound contraction.</td>
<td>Arul et al., 2012</td>
</tr>
<tr>
<td>Collagen-gelatin foam</td>
<td>bFGF</td>
<td>db/db mice</td>
<td>Accelerated dermis tissue formation and increased the number of new capillaries.</td>
<td>Kanda et al., 2012</td>
</tr>
</tbody>
</table>
Table 1.5: Commercial collagen based-dressings commonly used for the treatment of DFUs.

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


enable it to accelerate effectively both acute and diabetic wound healing in mice. J Clin Invest 121:4348-4361.


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


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

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 37°C in a humidified incubator containing 5% CO₂. Sub-culturing was performed according to ATCC recommendations. The Raw 264.7 cell line was purchased from ATCC (number TIB-71).

2.3.3 Treatments 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 plus 1 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 plus 1 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, 43 µl of MTT solution (5 mg/ml) was added to each well. The plates were further incubated at 37°C for 1h, in a humidified incubator containing 5 % CO₂. 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 (4×10^5 cells/well) cells were resuspended in 3 mL of DMEM medium in µ-Dish^35mm. (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 95°C, 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 4°C 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.
Neurotensin modulates the migratory and inflammatory response of macrophages under hyperglycemic conditions (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 H₂O 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 H₂O 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: AATGTTTGCAGCTCATAAATAAC Rev: TGCCAACAAGGTCGTCATC |
| NTR1 | For: GGCAATTCTCAGAATCCATCC Rev: ATACAGCGGTCACCAGCAC |
| NTR2 | For: GCCATTACTAACAGTCTAAGC Rev: GCAATTCGTCCTATTTCTACAC |
| NTR3 | For: ATGGCACAAACTTCTTCTTG Rev: AGAGACTTGGAGTAGACAAATG |
| IL-6 | For: TTCCATCCAAGTTGCCTTTC Rev: TTCTCATTCCAGCATTTCC |
| TNF-α | For: CAAGGGACTAGCCAGGAG Rev: TGCCTCTTCTGCGAATTCC |
| IL-10 | For: CCCCCCTGGTGCTCCTTTC Rev: ATGCCCTGTTGTCTCTCCTCCC |
| IL-1β | For: ACCTGTCCTGTGAATGAA Rev: GCCTGCTTCTGCTCTGTG |
| IL-12 | For: CAGAAGCTAACCATCTCCTGGTTTG Rev: TCAGGGATGAATTGTTGCGTTCACAC |
| HPRT1 | For: GTTGAAGATATAATTGACACTG Rev: GGCATATCCAAACAACAAAC |

2.3.8 Statistical analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA
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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
Neurotensin modulates the migratory and inflammatory response of macrophages under hyperglycemic conditions

Figure 2.1: Viability of macrophages, under either 10 or 30 mM 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 7 days. 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 10 mM glucose, NT induced a significant over expression of IL-6 (p<0.05) and IL-1β (p<0.05). On the other hand, under 30 mM 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 10 mM 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 10mM 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 2x10⁶ /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, IκB-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
Neurotensin modulates the migratory and inflammatory response of macrophages under hyperglycemic conditions and 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 (Ser473) and inhibitory protein for NF-κB activation, IκB-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.)
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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 (Ser473) and inhibitory protein for NF-κB activation, IκB-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.
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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
Neurotensin modulates the migratory and inflammatory response of macrophages under hyperglycemic conditions

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 microglia 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

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2.8 References


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 Biosystems (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 37°C in a humidified incubator containing 5% CO₂. 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 (4×10³ 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 7 days. 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 37°C for 1h, in a humidified incubator containing 5 % CO₂. 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 \(5\times10^5\) 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 H₂O 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 H₂O 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 \(5\times10^5\) 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 bicinechonic acid method and the cell lysates were denatured at 95°C, 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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5'-3'Sequence (Forward; Reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>For: GCATACATCAAGATTAGT Rev: TAAAGCAGTAGGAACTT</td>
</tr>
<tr>
<td>NTR1</td>
<td>For: CCATCCACATGCCACCGTCA Rev: TGAATGTGCTGTGCTGCCC</td>
</tr>
<tr>
<td>NTR2</td>
<td>For: TCCAAGTCTTTATCCAGGGT Rev: TACGATGAAGCTGAGGAC</td>
</tr>
<tr>
<td>NTR3</td>
<td>For: TGGTGGAGATAAGCAGTTG Rev: ACGACTTCTCAGACACCT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>For: GCTGTTGAGATGTCTTGTC Rev: GCTGTAAGAAGGTGCAATC</td>
</tr>
<tr>
<td>IL-8</td>
<td>For: TTGGCAGCCCTTCTGATTC Rev: AACTTCTCCAACCCCTG</td>
</tr>
<tr>
<td>EGF</td>
<td>For: AATCATGCTGCTCCTTGGG Rev: CAGGACAGAAACATAAGGAC</td>
</tr>
<tr>
<td>VEGF</td>
<td>For: CAGAATCATCAGAAGTTC Rev: TCTGCATGGTGGATGTTGGA</td>
</tr>
<tr>
<td>PDGF</td>
<td>For: CAGAAGCTAACCATCTCCCTGG Rev: TCGGGAGTAATTTGGTGCTTC</td>
</tr>
<tr>
<td>HPRT1</td>
<td>For: TGACACTGGCAAGCAATG Rev: GGCTTATATCCAACACCT</td>
</tr>
</tbody>
</table>

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 (4x10⁵ cells/well) cells were seeded in 6-well plates and treated with 10nM of NT for 24 h.
Cells were resuspended 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 resuspended 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´st 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 4x10⁴/well and were treated with 10, 50 or 100 nM of NT for 7 days. 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.](image)

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. However, 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 4x10⁵ / 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 with chronic, 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](image-url)
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


3.8 References


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 acts 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 °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 D₂O acidified with acetic acid (10µl of acetic acid in 600µl of D₂O).

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°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°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 spectrophotometrically (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 32°C, 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 predetermined 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 37°C in a humidified incubator containing 5%CO₂. 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 (8×10⁴ cells/well) and HaCaT (4×10⁴ 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 37°C for 1h, in a humidified incubator containing 5%CO₂. 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 (8×10⁴ 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 25mm² 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 iQ5 Optical 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 95°C, 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 °C. 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 120°C for 3 hours. 25μl of the supernatant were transferred to 96-well plate and evaporated in the incubator at 60°C 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 60°C. 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.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’-3’Sequence (Forward; Reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>For: CAAGGGACTAGCCAGGAG Rev: TGCTCTTTCTGCCAGTTC</td>
</tr>
<tr>
<td>IL-6</td>
<td>For: TTCCATCCAGTTGCCTTC Rev: TTCTCATTTCCACAGTTC</td>
</tr>
<tr>
<td>KC</td>
<td>For: ATTAGGGTGAGGACATGTGTTGGA Rev: AATGTTCAAAGGAAGCGTCAACAC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>For: ACCTGTCTCTGTGAATGAAG Rev: GCTTGTCCTGCTGTGTTG</td>
</tr>
<tr>
<td>MMP-9</td>
<td>For: CATAGAGGAAGCCATTACAG Rev: GATCCACCTTCTGAGACTCTCA</td>
</tr>
<tr>
<td>EGF</td>
<td>For: ACGGCAGAGTTTTGTCTTGAATTGC Rev: TGTTGCTACATCCAAATCGCCTTG</td>
</tr>
<tr>
<td>VEGF</td>
<td>For: CTTGTTCAAGAGGAGAAAGC Rev: ACATCTGGAAGTACGTGTTGTTT</td>
</tr>
<tr>
<td>PDGF</td>
<td>For: AGATCTGCCACGCACTCATCCTT Rev: ACGCAGACTGCACCTCTAATCCAT</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>For: TCAATACGTCAACATTCGGG Rev: CGTGGAGTTTGTATCTCTTG</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>For: ACTATGCCAAACTCTGCTCAG Rev: GGTCTGTCGCTTTGGTTT</td>
</tr>
<tr>
<td>COL1A1</td>
<td>For: AGGGCTTCAGTGTTTGGATG Rev: TTCAACCTTACCAACACTG</td>
</tr>
<tr>
<td>COL1A2</td>
<td>For: AAGGATACAGTGGATTCAGG Rev: AACAAAGTCTACGACACCTC</td>
</tr>
<tr>
<td>COL3A1</td>
<td>For: ACCTAAAAATCTGCCACCCC Rev: GCACATCAACGACATCTTCA</td>
</tr>
<tr>
<td>TBP</td>
<td>For: ACCCTTCACCAATGACTCTATG Rev: TGACTGCAGAAATCGCCTTG</td>
</tr>
</tbody>
</table>

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 $^1\text{H}$-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 (32°C). 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 8 hours 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) \( \frac{m_{\text{GSH released}}}{(m_{\text{GSH loaded}} \times m_{\text{polymer}})} \) 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](image)

**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 4.5B 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 4.6A-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 4.6A-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),
Chitosan-based dressings loaded with neurotensin—a efficient strategy to improve early diabetic wound healing

**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 NT 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 were 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 were 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.)
Chitosan-based dressings loaded with neurotensin: an efficient strategy to improve early diabetic wound healing

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.
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 staining (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.
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 when compared 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
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 100x). 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

<table>
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<tbody>
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<td>MPC</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td>Epidermis thickness</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Stratus lucidum</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>- Epithelial layers</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>- Basal layer</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Wound area (mm²)</td>
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</tr>
<tr>
<td>Re-epithelization</td>
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<tr>
<td>- From bottom</td>
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<td>na</td>
</tr>
<tr>
<td>- Top cover</td>
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<td>na</td>
</tr>
</tbody>
</table>

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%

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<td>Control</td>
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<tr>
<td>Epidermis thickness</td>
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</tr>
<tr>
<td>- Stratus lucidum</td>
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<td>- Basal layer</td>
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<tr>
<td>Wound area (mm²)</td>
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<tr>
<td>Re-epithelization</td>
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<tr>
<td>- From bottom</td>
<td>+</td>
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<tr>
<td>- Top cover</td>
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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

<table>
<thead>
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<th>NT</th>
<th>NT-loaded MPC</th>
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<td>Inflammation Status</td>
<td>Control</td>
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<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td>- PMN</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
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<td>- Lymphocytes</td>
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<td>++</td>
<td>+</td>
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<tr>
<td>- Plasma cells</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>- Fibrin</td>
<td>&lt;</td>
<td>&gt;</td>
<td>&lt;</td>
<td>&gt;</td>
</tr>
<tr>
<td>Repair</td>
<td>Fibroblasts</td>
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<td>&gt;</td>
<td>&lt;</td>
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<td>Scar</td>
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<td>+</td>
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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%;

<table>
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<tr>
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<th>NT-loaded MPC</th>
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<td>- Lymphocytes</td>
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<td>- Plasma cells</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>- Fibrin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Repair</td>
<td>Fibroblasts</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Collagen matrix</td>
<td>Loose</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Scar</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>
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

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

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

| 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                  | =            | ↓ MPC, PC+NT | Macrophages, epithelial cells                |
|     | MMP-9                  | ↓ MPC, MPC+NT | = MPC+NT   | Collagenase, monocytes, macrophages           |
|     | EGF                    | =            | =             | Macrophages, platelets                       |
|     | VEGF                   | =            | =             | Fibroblasts, macrophages, neutrophils, endothelial cells |
|     | PDGF                   | =            | =             | macrophages, endothelial cells               |
|     | TGFβ1                  | ↓ MPC, NT, 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                                  |
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 (Blakyn 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).


4.8 References


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


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


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

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 hypochlorite) 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% CO₂. 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 (3×10⁴ cells/well) and HaCaT (1.5×10³ cells/well) cells were seeded individually onto
Neurotensin-loaded collagen dressings reduce inflammation and improve wound healing in diabetic mice

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 (3×10⁴ cells/well) and HaCaT (1.5×10⁵ 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% CO₂. 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 (3×10⁴ 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 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 iQ5 Optical 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 95°C, for 5 min, in sample buffer. 40 μg of total protein were resolved on 12% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline containing 0.1% (v/v) Tween 20 (TBS-T), for 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’-3’ Sequence (Forward; Reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>For: CAAGGGACTAGCCAGGAG Rev: TGCCCTTCTGCCAGTTTC</td>
</tr>
<tr>
<td>IL-6</td>
<td>For: TTCCATCCAGTTGCCTTC Rev: TTCTCATTTCCACAGATTTC</td>
</tr>
<tr>
<td>KC</td>
<td>For: ATTAGGGTGAAGGATGTTGGAG Rev: AATGTTCAAGGGGAGCGTCAACAC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>For: ACCTGCTCTGTAATGAAAG Rev: GCTTGTGCTGTGTTTG</td>
</tr>
<tr>
<td>MMP-9</td>
<td>For: CATAGGAGAAGCCATACAG Rev: GATCCACCTCTGAGACTTCA</td>
</tr>
<tr>
<td>EGF</td>
<td>For: ACGGCACAGTTTGCTTCAATGGC Rev: TGTTGGCTATCCAAATCGCTTG</td>
</tr>
<tr>
<td>VEGF</td>
<td>For: CTTGTCCAGAGGGAGAAAGC Rev: ACATCTGCAAGTACGTTGGT</td>
</tr>
<tr>
<td>PDGF</td>
<td>For: AGATCTGGCCAGCTACATCTTT Rev: ACGCCACAGCCACCTCAATCCAT</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>For: TCAATAGTCAGACATTCGGG Rev: CGTGGAGTTGTATCTTTTC</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>For: ACTATGCCAACCTCTGGCTCAG Rev: GGTCTCTTGTATCTTTTC</td>
</tr>
<tr>
<td>COL1A1</td>
<td>For: AGGCTTCAGTGITTGGGATG Rev: TTCACCTTGGACACGAACAG</td>
</tr>
<tr>
<td>COL1A2</td>
<td>For: AAGGATACAGTGATTGCAGG Rev: AACACAGTCAAGCGACCTTC</td>
</tr>
<tr>
<td>COL3A1</td>
<td>For: ACCTAAATTTGCGCCACCC Rev: GCACATCAACGACATATTTC</td>
</tr>
<tr>
<td>TBP</td>
<td>For: ACCCTTCACCAATGACTCTATG Rev: TGACTGCAAGCAATCGCTTC</td>
</tr>
</tbody>
</table>

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

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

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

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<0.001 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.).
Neurotensin-loaded collagen dressings reduce inflammation and improve wound healing in diabetic mice

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

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.

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.).
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

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<th>Day 3</th>
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<th>Day 3</th>
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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

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### 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

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### 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

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Table 5.6 Summary of cytokine expression in wounded control and diabetic skin, at day 3 post-wounding and after complete healing (fd).

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<tr>
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<th>Cell type that produce cytokine/growth factors</th>
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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,
Neurotensin-loaded collagen dressings reduce inflammation and improve wound healing in diabetic mice

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

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5.8 References


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


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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 \textit{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-1\(\beta\) 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-\(\alpha\) 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-\(\alpha\) 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 (\(\alpha\)-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.).