UNIVERSIDADE Ð
COIMBRA

Arianna Piscosquito

rhBMP-7 EFFECTS ON WOUND HEALING IN DIABETIC MICE -A PILOT STUDY

Dissertation in Cellular and Molecular Biology,
supervised by Dr Eugénia Maria Lourenço Carvalho and presented to the Department of Life Sciences
of the Faculty of Sciences and Technology of the University of Coimbra

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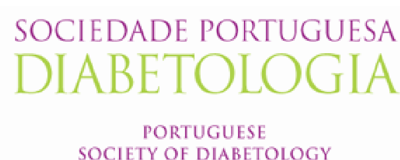
Financial Support by:

We thank for the financial support: European Foundation for the Study of Diabetes, Sociedade Portuguesa Diabetologia, Fundação para a Ciência e a Tecnologia and Programa Operacional Factores de Competitividade – COMPETE. We thank Dr. Yu-Hua Tseng (Joslin, Boston) for providing the recombinant human BMP-7 (rhBMP-7).



FCT Fundação para a Ciência e a Tecnologia

MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR

Acknowledgments

Prima di tutto ci tengo a ringraziare chi mi ha sostenuto nel mio percorso di ricerca. E quindi grazie al mio relatore, Dr Eugénia Carvalho, per avermi permesso di svolgere questo lavoro, fornendomi sempre consigli e indicazioni; grazie al Dr Ermelindo Leal che mi ha aiutato nella pratica e per l'aiuto sempre dimostrato. Un ringraziamento alla professoressa Lorella Canzoniero, che ha avuto fiducia in me e mi ha permesso di poter affrontare questa esperienza. Ai colleghi del Centro di Neuroscienza e Biologia Cellulare (CNC), in particolare a Marija Petkovic che è sempre stata pronta a consolarmi nei momenti di difficoltà, ad ascoltarmi ed avere pazienza per il mio pessimo inglese. Ai miei genitori, che sono il mio punto di riferimento. Grazie perché senza i loro sacrifici non sarei arrivata fino a qui. Grazie per aver creduto in me, per avermi dato fiducia e per avermi sostenuta da lontano e per aver annullato le distanze che ci separavano. A mia sorella Alessia, il mio punto di forza. È stato un anno difficile per entrambe: lontane per la prima volta, i pianti per la distanza che ci separava, ma alla fine abbiamo superato tutte le difficoltà l'una accanto all'altra. Grazie perché sei il mio più grande sostegno e la mia guida. Voglio ringraziare una persona speciale, Cinzia. Grazie perché sei stata tu a stimolarmi in questa esperienza a Coimbra, siamo partite insieme e siamo state complici durante l'intero percorso. Per aver affrontato insieme i momenti difficili, ma oggi voglio ricordare soprattutto i momenti più belli, la palestra Phive, le gite con Erasmuland, le uscite e tutte le risate che ci siamo fatte. Voglio ricordare tutte le amicizie che si sono formate in questo anno: Julia, la brasiliana del mio cuore, che è stata per me coinquilina, amica e sorella. Le amiche di Erasmus Anthea, Giovanna, Claudia, Rosalba, Viviana, Michelle, Rodrigo, Camilla. Un grazie a Giuseppe, amico e fratello per me, che mi è stato vicino in questi anni di Università, anche per noi è stato difficile separarci in questo anno, ma posso dire che ci siamo sempre stati l'uno per l'altro. Un grazie alla mia amica di avventure Viviana, perché sei sempre stata vicina e mi hai sopportata e apprezzata per come sono. Infine voglio ringraziare me stessa, o meglio la mia forza di volontà. Per non aver mollato mai, per aver superato gli ostacoli e per la mia grande determinazione.

Un grazie speciale a tutti.

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List of Abbreviations

AGEs – Advanced glycation end-products

AMP – Antimicrobial peptides

ASCs – Adipose-derived stem cells

bFGF – Basic fibroblast growth factor

BMP-7 – Bone morphogenetic protein 7

BMPRs – Bone morphogenetic protein receptors

BMPs – Bone morphogenetic proteins

BMSCs – Bone marrow-derived mesenchymal stem cells

DFU – Diabetic foot ulcers

DM – Diabetes mellitus

DNA – Deoxyribonucleic acid

ECM – Extracellular matrix

EGF – Epidermal growth factor

H&E – Hematoxylin and eosin

Id-1 – Inhibitor of DNA binding 1

IL-1β – Interleukin-1 β

IL-10 – Interleukin-10

IL-6 – Interleukin-6

KC – Skin keratinocyte-derived cytokine

KGF – Keratinocytes growth factor

MCP-1 – Monocyte chemoattractant protein-1

MMP – Metalloprotease

MMP-9 – Metalloprotease 9

OCT – Optical cutting temperature medium

PBS – Phosphate buffered saline

PDGF – Platelet-derived growth factor

PNS – Peripheral nervous system

rhBMP-7 – Recombinant human BMP-7

RNA – Reactive oxygen species

ROS – Reactive oxygen species

SMAD – Small mother against decapentaplegic

SMAD-4 – Small mother against decapentaplegic 4

STZ – Streptozotocin

TBS – Tris-buffered saline

T-cell – T lymphocyte

TGF-β – Transforming growth factor-β

VEGF – Vascular endothelial growth factor

VEGFR2 – Vascular endothelial growth factor receptor 2

VSMC – Vascular smooth muscle cells

UCP-1 – Uncoupling protein 1

Resumo

A proteína óssea morfogenética 7 (BMP-7) é uma proteína da superfamília fator de transformação de crescimento (TGF-β). Esta proteína foi descoberta pela sua capacidade de induzir células mesenquimais a se diferenciarem em osteoblastos, por promover a proliferação celular e a síntese de proteoglicanos e colágenio tipo II da matriz extracelular. No entanto, os papéis principais da BMP-7 na pele não são bem conhecidos e nunca foram estudados, particularmente na cicatrização de feridas diabéticas.

Objetivo:

Este estudo pretende avaliar o papel fundamental da rhBMP-7 na cicatrização de feridas diabéticas na pele.

Materiais e métodos:

Foram utilizados murganhos adultos C57Bl/6 e a indução de diabetes foi feita com o uso de estreptozotocina (50mg/kg, i.p., por 5 dias consecutivos). Subsequentemente, foram induzidas duas feridas de 6 mm em murganhos diabéticos e as feridas foram tratadas topicamente com proteína óssea morfogénica 7 humana recombinante (rhBMP-7), aplicando 2 ou 10 µg por ferida todos os dias ou solução salina como o tratamento controlo, até 10 dias após a indução de feridas. As células inflamatórias, macrófagos e linfócitos, foram quantificados por imunohistoquímica. A presença de macrófagos foi avaliada com CD68, os macrófagos de fenótipo M1 foram avaliados com dupla marcação de CD68 e TNFα, enquanto os macrófagos de fenótipo M2 foram avaliados com dupla marcação de CD68 e CD206. Além disso, a presença de linfócitos foi avaliada usando CD3, para quantificação de vasos sanguíneos/angiogénese

utilizamos CD31. O factor de crescimento VEGF foi analisado uma vez que esta relacionado com a angiogénese. IL-6 e TNFα, marcadores de inflamação, também foram analisados. Ainda, o KI-67 foi usado para avaliar a proliferação celular. Hematoxilina e Eosina, bem como coloração de Herovici foram usados para avaliar a histologia da pele e a organização da matriz extracelular (ECM), bem como a deposição de colágenio. Além disso, também avaliamos por qRT-PCR a expressão génica de IL-6, KC, TNF-α, IL-β1, MCP-1, MMP-9, VEGF, VEGFR2, TGF-β1, SMAD-4 e BMP- 7.

Resultados:

O tratamento com rhBMP-7 foi induziu significativamente o fecho da ferida em ambos os grupos, 2 µg (**$p<0.01$, $n = 6$ feridas) e 10 µg (*$p<0.05$, $n = 8$ feridas) quando comparados com a solução salina ($n = 6$ feridas). O tratamento com rhBMP-7 reduziu a expressão de moléculas inflamatórias, nos grupos de 2 µg e 10µg de rhBMP-7, tais como IL-6, TNF-α, IL-β1, MCP-1, MMP-9, KC ($n = 3$ em 2 e $n = 4$ em 10 de rhBMP-7, $n = 3$ em solução salina). A redução do fenótipo de macrófagos pró-inflamatórios M1 foi significativamente reduzida pelo tratamento com rhBMP-7 nos grupos de 2 µg e 10 µg de rhBMP-7 (***$p<0.001$, $n = 3$ em 2 µg e $n = 4$ em 10 µg de rhBMP-7 , $n = 3$ em solução salina) quando comparado ao grupo tratado com solução salina. Por outro lado, 2 e 10 µg de rhBMP-7 induziram um aumento da angiogénese, uma vez que o número de vasos aumentou (**$p<0.01$ e ***$p<0.001$). A expressão do VEGF aumentou tanto em 2 µg como em 10 µg de rhBMP-7 (**$p<0.01$ and *$p<0.05$). Além disso, KI-67 aumentou nos tratamentos com rhBMP-7 (***$p<0.001$) quando comparado ao grupo tratado com solução salina, indicando que a rhBMP-7 estimula a fase proliferativa na cicatrização de feridas. A deposição de colágenio também foi estimulada pela rhBMP-7. Não foram observadas diferenças estatisticamente

significantes no número de macrófagos anti-inflamatórios M2, células T, assim como nas expressões dos genes TGF-β1, SMAD-4 e BMP-7, nessas condições.

Conclusões

Em conclusão, o tratamento tópico da ferida com rhBMP-7 foi capaz de melhorar o fecho da ferida devido a uma diminuição significativa na infiltração de células inflamatórias, incluindo macrófagos e particularmente macrófagos M1. Um aumento significativo na neovascularização, juntamente com a estimulação da fase proliferativa, também foi verificado no tratamento com rhBMP7. BMP-7 apresenta várias características benéficas para cicatrização de feridas. No entanto, devido à capacidade da BMP-7 promover a diferenciação de uma matriz óssea na pele, após tratamento tópico, mais estudos são necessários para avaliar se a BMP-7 em concentrações diferentes das que usamos será um bom candidato para tratar feridas na pele diabética.

Palavras-chave: Pele, cicatrização de feridas, diabetes, BMP-7, inflamação, células imunes

Abstract

Bone morphogenetic protein 7 (BMP-7) is a protein from the transforming growth factor (TGF-β) superfamily. It was discovered for its ability to induce mesenchymal cells to differentiate into osteoblasts, for the promotion of cell proliferation and extracellular matrix proteoglycan and collagen type II synthesis. However, the key roles of BMP-7 in the skin are not well known, and have never been studied, particularly in diabetic wound healing.

Objective:

This study evaluates key roles of rhBMP-7 in diabetic wound healing at the skin level.

Materials and methods:

Male C57Bl/6 mice (25-30g) wild type were used for investigation of wound healing kinetics. Diabetes was induced using streptozotocin (50mg/kg), injected intraperitoneally (IP), for 5 consecutive days. Subsequently two 6 mm wounds were induced in diabetic mice and wounds were then topically treated with recombinant human bone morphogenetic protein-7 (rhBMP-7), using either 2 or 10μg per wound every day, or with a saline as the control treatment up to 10 days post wounding. Inflammatory cells, macrophages and lymphocytes, were evaluated by immunohistochemistry. The presence of macrophages was evaluated with CD68 staining, the M1 macrophage phenotype was evaluated with CD68 and

TNF-α co-staining, while the M2 phenotype was evaluated with CD68 and CD206 co-staining. Moreover, the presence of lymphocytes was evaluated using CD3, for quantification of blood vessels/angiogenesis we used CD31. The growth factor VEGF was evaluated since its important for angiogenesis. IL-6 and TNFα, both markers of inflammation, were also analysed. In addition, through KI-67 fluorescent intensity we measured the cell proliferation. Hematoxilyn & Eosin, as well as Herovici staining were used to evaluate skin histology and the organization of the extracellular matrix (ECM), as well as collagen deposition. In addition, we also evaluated the gene expression of IL-6, KC, TNF-α, IL-β1, MCP-1, MMP-9, VEGF, VEGFR2, TGF-β1, SMAD-4 and BMP-7 by qRT-PCR.

Results:

rhBMP-7 treatment was able to induce significant reduction in wound closure in both 2µg (**$p<0.01$, n=6 wounds) and 10µg (*$p<0.05$, n=8 wounds) rhBMP-7 groups when compared to the saline treated group (n=6 wounds). In addition, rhBMP-7 treatment tended to reduce the expression of inflammatory markers, in both 2µg and 10µg rhBMP-7 groups, such as IL-6, TNF-α, IL-β1, MCP-1, MMP-9, KC (n=3 in 2µg and n=4 in 10µg of rhBMP-7, n=3 in saline). The number of the M1 pro-inflammatory macrophages phenotype was significantly reduced by rhBMP-7 treatment in both 2µg and 10µg rhBMP-7 groups (***$p<0.001$, n=3 in 2µg and n=4 in 10µg of rhBMP-7, n=3 in saline) when compared to the saline treated group. On the other hand, 2µg and 10µg of rhBMP-7 induced an increase in the number of blood vessels (**$p<0.01$ and ***$p<0.001$). VEGF expression was increased in both 2µg and 10µg of rhBMP-7 (**$p<0.01$ and *$p<0.05$). In addition, KI-67 expression was also increased with 2µg and 10µg of rhBMP-7 (***$p<0.001$) when compared

to the saline treated group, indicating that rhBMP-7 stimulates the proliferative phase in wound healing. Collagen deposition was also stimulated by rhBMP-7. There were no statistically significant differences observed in the number of M2 anti-inflammatory macrophages, T- cells, as well as for TGF-β1, SMAD-4 and BMP-7 gene expression, under these conditions.

Conclusions:

In conclusion, the rhBMP-7 topical wound treatment was able to improve wound closure due to a significant decrease in inflammatory cell infiltration including, macrophages and particularly M1 macrophage phenotype. A significant increase in neovascularization, along with stimulation of the proliferative phase, was also stimulated by rhBMP-7. BMP-7 has several characteristics that are known to be beneficial in wound healing. However, due to the ability of BMP-7 to promote the differentiation of a bone matrix in the skin, post topical treatment, more studies are needed to evaluate whether BMP-7 at concentrations different from the ones we used will be a good candidate to treat diabetic skin wounds.

Keywords: Skin, wound healing, diabetes, BMP-7, inflammation, immune cells

Chapter 1. Introduction

1.1 Skin

Skin is the largest organ of the human body whose function is to cover the body and ensure its protection against aggression by pathogens, ultraviolet radiations and toxic agent (Fitzgerald, 2018). The skin also has the function of thermoregulation due to the presence of sweat glands, that work to remove large amounts of heat from the body, in addition to the presence of a dense network of capillaries. Skin also produce Vitamin D, necessary for regulation of calcium metabolism and therefore it serves the bone calcification. Moreover, Vitamin D deficiency has different effects on skin diseases including inflammatory skin diseases and skin cancer (Piotrowska et al., 2016). Skin is also important in the absorption of water-soluble molecules and excretion of water and sweat (Moura et al., 2013). Furthermore, the skin also plays an immunological function, in part because keratinocytes produce antimicrobial peptides (AMPs), a large and diverse group of molecules that activate immune response, which is the first line of defense against pathogens (Schröder et al., 2006). The anatomical structure of the skin consists of three overlapping layers: the epidermis, the dermis and the hypodermis (Figure 1). Epidermis is the superficial layer of the skin composed by keratinocytes, dendritic cells, melanocytes, Langerhans and Merkel cells. The dermis provides mechanical support and is composed of fibroblasts, macrophages, mast cells, endothelial cells, vascular smooth muscle cells (VSMC) and lymphocytes. The hypodermis is essentially composed of adipose tissue and collagen and provides support, strength, elasticity, blood flow and oxygen to the skin (Moura et al., 2013).

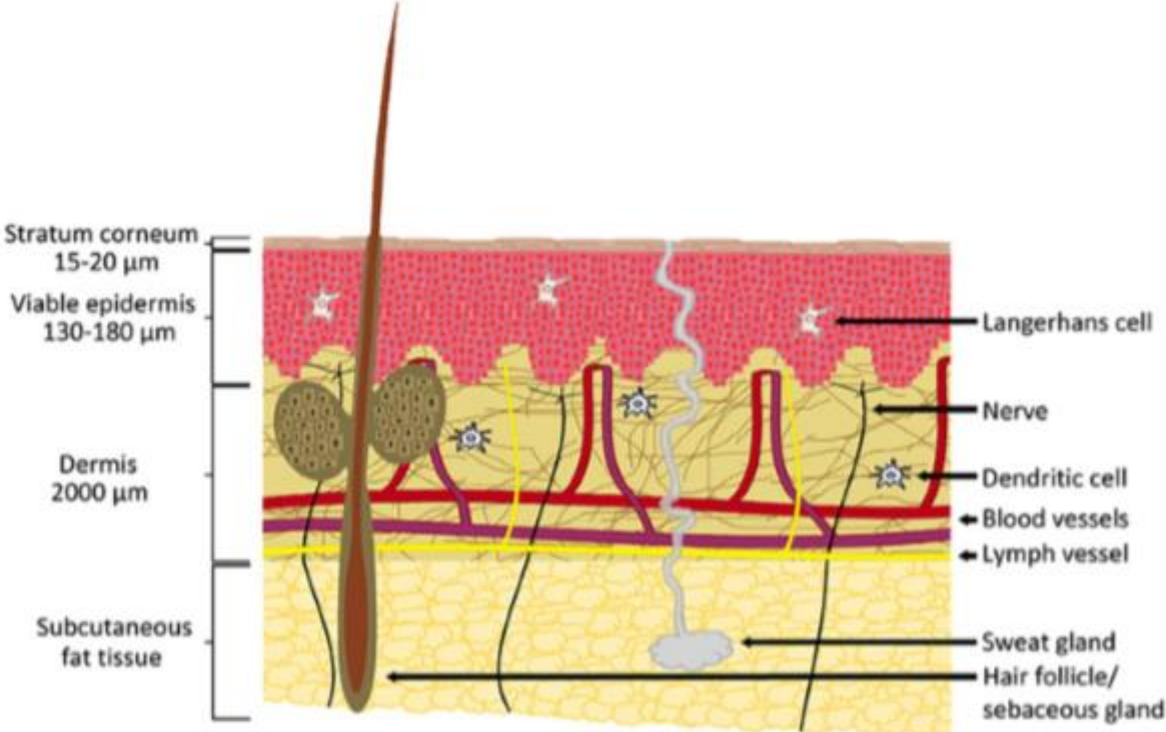

Figure 1 - Anatomy of the skin. Printed with permission (Maaden et al., 2012).

Epidermis

The epidermis is a multi-layered and keratinized paving epithelium and it represents the most superficial part of the skin. It is composed of multiple cell populations: keratinocytes, melanocytes, Langerhans and Merkel cells. Keratinocytes account for over 90% of the epidermal cells; they are epithelial cells that undergo the keratinization process, which is responsible for the regeneration of the epidermis through mitotic divisions. The keratinization process occurs at the level of the epidermis in a series of the different layers:

- Stratum basale
- Stratum spinosum
- Stratum granulosum
- Stratum lucidum
- Stratum corneum

The process of keratinization allows the cells of the basal layer, with germinative activity, to leave the deep portions of the epidermis to reach the more superficial ones in the form of dead cells without a nucleus (Deo et al., 2018). Then there are the melanocytes, responsible for the synthesis of the melanin, that are found mostly in the epidermal germinative layer and in the bulbar portion of the hair follicles (Kanitakis, 2002). Langerhans cells are powerful immune cells stimulators, have the function of capturing antigens, processing and presenting them to the T lymphocytes. Merkel cells play the role of mechanoreceptors, as suggested by their frequent contact with dermal sensory axons with whom they form synaptic junctions (Kanitakis, 2002).

Dermis

The dermis (or corium) is the thickest layer of the skin representing a supportive, compressible and elastic connective tissue protecting the epidermis (Maaden et al., 2012). The most important function of the dermis is to provide nutrients and sebum to the epidermis, supporting it mechanically and metabolically. It contains blood and lymphatic vessels, nerve fibers, sweat glands and the cellular component is composed of fibroblasts, macrophages, lymphocytes and mast cells (Moura et al., 2013).

Fibroblasts are very important in the wound healing process because they have the potential to produce a series of substances for the synthesis of new tissue, in fact they are responsible for collagen and proteoglycan synthesis (Li et al., 2011). They can also stimulate the already secreted cytokines and growth factors, regulate the migration and proliferation of the cells responsible for the neo-formation of tissues and vessels.

Mast cells are mononuclear cells of bone marrow origin that have a villous cell membrane and contain characteristic cytoplasmic granules, in which some inflammatory mediators are stored (Tellechea et al., 2019). Once the mast cells are activated, the degranulation leads to the release of inflammatory molecules from cytoplasmic granules (González-de-Olano et al., 2018).

Hypodermis

The hypodermis is the innermost layer of the skin. It has an energy rich nutrient reserve, it supports and protects the underlying soft tissues, acts as a shock absorber for small traumas and shocks. In addition, it acts as a thermal insulator and does not disperse heat. It is mainly composed of dermal adipocytes, that are found embedded among the collagen fibers and have the function to insulate the body from heat and cold (Kanitakis, 2002). These adipocytes are also important as storehouses for nutrients.

Adipose-derived stem cells (ASCs) are very important in the wound healing process because of their potential to differentiate into endothelial cells that secrete angiogenic and anti-apoptotic factors (Huang et al., 2013). Among the factors secreted by ASCs are vascular endothelial growth factor (VEGF), basic fibroblast growth factor (b-FGF) and platelet-derived growth factor (PDGF)(Kim et al., 2018). At the level of the hypodermis there are also lymphatic vessels, a dense blood vessels and nerves network, the hair follicles, the sweat and sebaceous glands. Sebaceous glands are exocrine glands responsible for the production of sebum, they are found throughout the body except for the palms of the hands and the soles of the feet. The sweat glands are responsible for the secretion of sweat, stimulated by heat, hormonal stimuli and the intake of certain substances (Kanitakis, 2002).

1.2 Biological factors in skin physiology

All cell types in the epidermis and dermis, such as fibroblasts and keratinocytes, produce growth factors, which play an essential role in maintaining the structure and function of the skin. Some of the factors involved in skin regeneration and repair are epidermal growth factor (EGF), vascular endothelium growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblasts growth factor (FGF) and keratinocyte growth factor (KGF)(Werner et al., 2003). FGF, produced by macrophages, stimulates angiogenesis and the synthesis of extracellular matrix proteins. Some FGFs may contribute to the pathogenesis of cancer (Ornitz et al., 2015). PDGF is produced by platelets as well as by other cells, including macrophages, endothelial cells, keratinocytes and muscle cells. It induces the proliferation of fibroblasts and endothelial cells and stimulates the synthesis of extracellular matrix proteins (Werner et al., 2003). EGF, produced by macrophages and other cells, stimulates keratinocyte migration and the formation of granulation tissue (Shen et al., 2017). VEGF determines the development of blood vessels in case of tissue damage stimulating the migration and proliferation of endothelial cells. KGF plays an important role in tissue regeneration because it stimulates cell proliferation and motility. Transforming growth factor (TGF-β) is important for cell proliferation and differentiation, for the production of extracellular matrix and for immune modulation (Penn et al., 2012). Other important factors involved in skin physiology are the family of bone morphogenetic proteins (BMPs) belonging to the superfamily of growth factors (Botchkarev, 2003). They are known to induce bone formation and promote cell proliferation in addition to inducing synthesis of collagen type 2 and proteoglycans (Herrera et al., 2018).

1.3 Wound healing

Wound healing is an important physiological process to maintain the integrity of skin after trauma. A wound alters the epithelial integrity of the skin and can damage other structures such as muscles, vessels, nerves, tendons and bone. It is a dynamic and complex process involving different cell types, immunological and biological systems. It consists of a cascade of events including homeostasis, inflammation, proliferation, and tissue remodelling (Figure 2)(Tellechea et al., 2019). In these phases many types of cells are activated such as neutrophils, monocytes, lymphocytes, dendritic cells, endothelial cells, keratinocytes and fibroblasts. Certain growth factors are required and aid in the regulation in wound healing processes, for example VEGF, FGF, KGF and TGF-β(Werner et al., 2003).

Homeostasis

The first phase of homeostasis begins immediately after wounding (Guo et al., 2010). The bleeding caused by a lesion is characterised by three processes: vascular constriction to reduce blood loss, platelet activation with platelet cap formation and fibrin clot formation (Guo et al., 2010). The result of this phase is the cessation of bleeding. The cytoplasm of platelets contains α-granules in which there are growth factors and cytokines such as TGF-β, PDGF, FGF and EGF, which activate neutrophils, leukocytes, macrophages and fibroblasts. Platelets also contain vasoactive amines that are responsible for vasodilation and increased vascular permeability (Velnar et al., 2009).

Inflammatory phase

The inflammatory phase is characterised by the activation of the cells in innate and adaptive immune system (Agita et al., 2017). The initial event is activation of the innate immunity cells including neutrophils, monocytes and macrophages, followed by adaptive immunity cells composed by the B and T-lymphocytes (Agita e al., 2017). B-lymphocytes are responsible for the antibody production, which are able to recognize pathogens. On the other hand, T-lymphocytes recognize the antigen on the surface of cells expressing these peptide/MHC complexes (Paul, 2011).

Neutrophils arrive at the wound site at the highest concentration 24 hours after injury, and they are the first cells to appear at the injury site. Their roles are to clean debris and bacteria and to provide a good environment for wound healing, being at the peak around days 1-2. Following neutrophils, monocytes migrate towards the injured area and differentiate into macrophages. They migrate into

the wound after 48-96 hours after the injury and release cytokines that promote the inflammatory response by recruiting and activating additional leukocytes (Hesketh et al., 2017). Macrophages are divided into two groups classified as M1 and M2 phenotypes (Tellechea et al., 2019). M1 macrophages are responsible for the initial stages of wound healing as they have pro-inflammatory, bactericidal and phagocytic functions. These cells release cytokines such as TNF-α, IL-6, IL-1β (Hesketh et al., 2017). At 5-7 days post injury M1 macrophages differentiate into M2 macrophages mediating the transition from the inflammatory phase to the proliferative phase, having an anti-inflammatory, matrix producing and pro-angiogenesis functions because they begin secreting IL-10, b-FGF, EGF (Zhang et al., 2008). Therefore, during the initial phase of inflammation there is a high number of M1 phenotype cells (around 85% of macrophages content) while at 5 days post injury the number of M1 decreases and M2 macrophage phenotype increases (Hesketh et al., 2017). Therefore, in wound healing the M1/M2 ratio is critical, since prolonged activation of M1 phenotype may compromise healing and cause chronic inflammation, which inhibits wound closure, as is the case during diabetes (Leal et al., 2015). After the macrophages activation, T-lymphocytes arrive at the wound site and they reach the maximum peak during the proliferative phase of wound healing with the role of killing any cell in which infectious agents have penetrated. T-Lymphocytes can be divided into different classes: Natural Killer, T-Helper CD4, Cytotoxic T-lymphocytes CD8 and Regulatory T-lymphocytes. However the role of T-lymphocytes in wound healing is still not very clear, and this research has been under intensive investigation (Moura et al., 2019).

Proliferative phase

The proliferative phase of wound healing begins around the 4th day after injury and extends approximately until the end of the second week. It is characterised by epithelial proliferation and migration over the provisional matrix within the wound (re-epithelialization). In this phase some of the most important cells are fibroblasts because they can stimulate and secrete cytokines and growth factors. In addition, they regulate the migration and the proliferation of cells responsible for neo-formation of tissue and vessels (Guo et al., 2010). Fibroblasts are responsible for the formation of the granulation tissue and has the function of filling the tissue that is defect, protecting the wound, providing a barrier to infections and containing important myofibroblasts for wound contraction (Guo et al., 2010). Within the wound bed, fibroblasts also produce collagen as well as glycosaminoglycans and proteoglycans, which are major components of the extracellular matrix (ECM) that is important to provide support and to regulate migration, proliferation and differentiation (Guo et al., 2010). Collagen is an important component in this phase because it plays a key role in intracellular matrix formation. The blood vessels formation occurs through different angiogenic factors such as VEGF, PDGF, angiogenin, TGF-α and TGF-β (Nissen et al., 1998). The regulation of angiogenesis is important as high angiogenic activity can cause chronic inflammation or promote the tumor growth, whereas low angiogenic activity can damage tissues resulting in diseases such as ischemia or heart failure (Guo et al., 2010).

Remodelling

Following robust proliferation and ECM synthesis, wound healing enters the final **remodelling** phase, which can last for years. This phase is characterised by the degradation of the immature ECM by metalloproteinases (MMPs) produced by neutrophils. MMPs degrade matrix proteins like collagen, laminin or fibronectin. In this phase, there is also the regression of many of the newly formed capillaries, so that vascular density of the wound returns to normal (Bielefeld et al., 2013). The balance between degradation and synthesis is very important to have normal wound healing. In this phase, in order to the epithelialization process being completed, there is also keratinocyte proliferation (Bielefeld et al., 2013). When the wound heals the number of fibroblasts and macrophages is reduced by apoptosis. This phase also needs a precise balance between apoptosis of existing cells and production of new cells, and any aberration may lead to excessive wound healing or chronic wound formation (Rai et al., 2005).

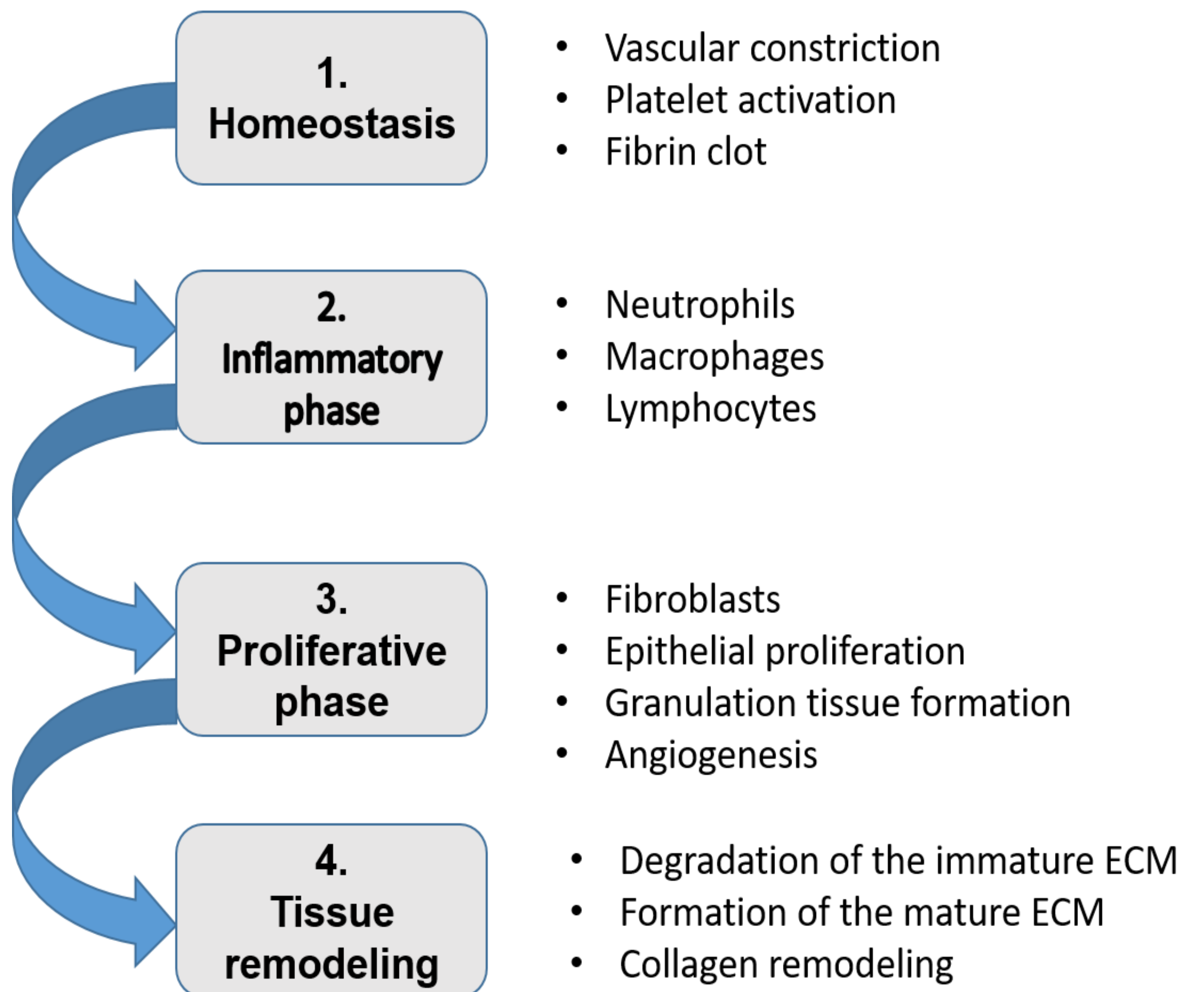

Figure 2 - Phases of wound healing: homeostasis, inflammatory, proliferative and tissue remodelling phases.

1.4 Diabetic wound healing

Multiple factors can lead to impaired wound healing: lack of oxygenation, infection, diseases such as diabetes, obesity and drug induced insulin resistance. Diabetes affects hundreds of millions of people worldwide. According to the World Health Organization (WHO) the number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014. Diabetic wounds typically do not heal and do not progress through the normal stages of healing such as inflammation, proliferation, remodelling. This is a typical case of chronic wound development (Velnar et al., 2009), unlike acute wounds that can heal in a few days or weeks. There are two major types of diabetes: type 1 DM, representing approximately 5-10% of the total cases, characterised by autoimmune destruction of pancreatic β-cells, leading to absolute insulin deficiency (American Diabetes Association), whereas, type 2 DM represents almost 90-95% of the diabetic population and is characterized by insulin resistance (Rubino, 2008). One of the severe and chronic complications of diabetes are diabetic foot ulcers (DFUs). They affect approximately 15% of the diabetic population (Brem et al., 2007) representing the leading cause of lower extremity amputations. In addition to impaired healing, diabetes is also characterized by hypoxia (Ansurudeen et al., 2012), which can increase the early inflammatory response by increasing the levels of oxygen radicals (Ansurudeen et al., 2012). In diabetic wounds we can also find T cell defects (Moura et al., 2019), defects in the chemotaxis of leukocyte and phagocytosis, as well as dysfunctions of fibroblast and epidermal cells (Loots et al., 1998). In addition, diabetic patients are affected by neuropathy that consists of the damage and malfunction of the peripheral nervous system (PNS). Neuropathy alters wound healing because there is a decrease in circulating peptides, such as nerve growth factor, substance P (SP) (Leal et al., 2015), neurotensine (NP) (Moura et al., 2013) and calcitonin gene-related peptide. Some of these peptides are

important in wound healing because they promote cell chemotaxis, induce growth factor production, and stimulate the proliferation of cells (Kanitakis, 2002). In wound healing process the balance between low and high ROS levels is very important. Low levels of ROS are important in the initial wound to maintain homeostasis (Tandara et al., 2004) while high ROS levels results in impaired wound healing with chronic inflammation (Ponugoti et al., 2013). Furthermore, diabetic wound healing is also characterized by high metalloproteinases levels important in extracellular matrix remodelling, and high advanced glycation end-products (AGEs) levels (Guo et al., 2010). AGEs are chemical compounds derived from glycosylation reactions. Normally they are produced in the body in a controlled manner, but under hyperglycaemia they are produced in large quantities becoming toxic to the body (Beisswenger et al., 1993). In summary, diabetic wound healing is accompanied by hypoxia, dysfunction in fibroblasts and epidermal cells, impaired angiogenesis and neovascularization, high levels of metalloproteases, decreased host immune resistance, and neuropathy (Figure 3) (Guo et al., 2010).

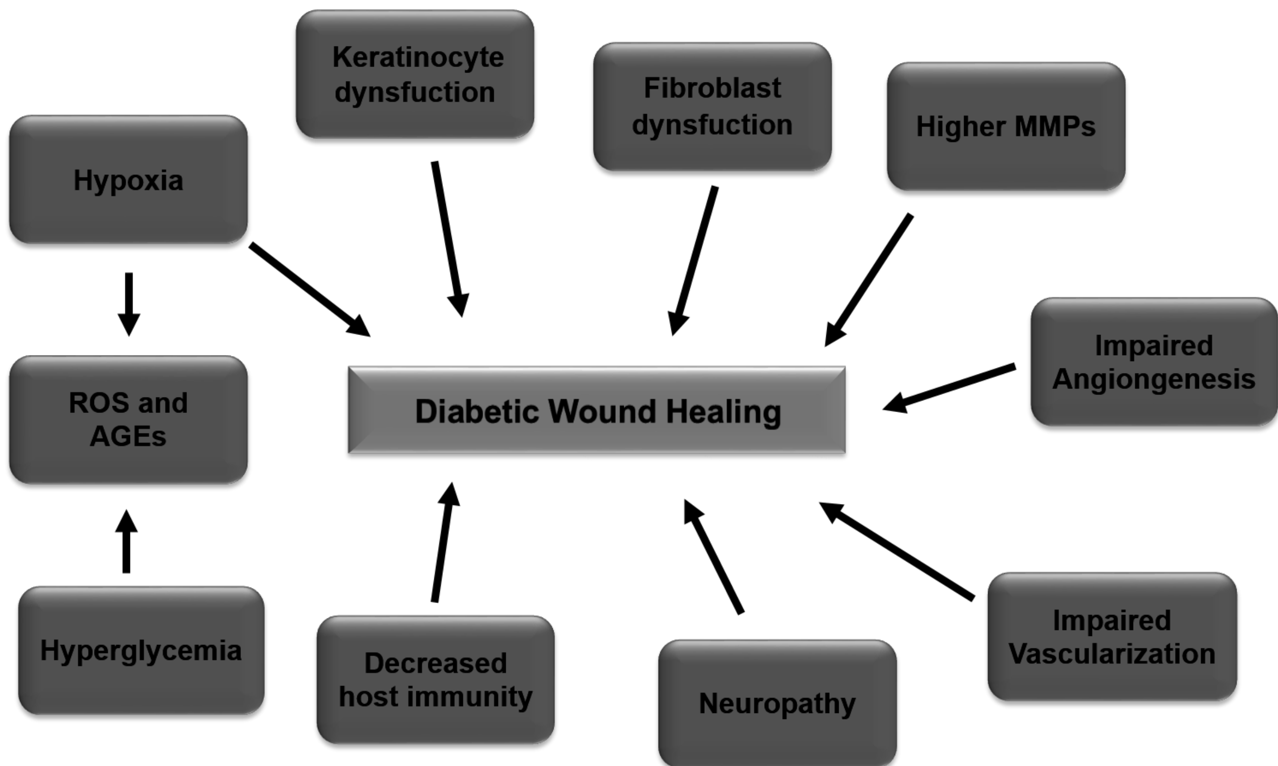

Figure 3 - The potential effects of diabetes on wound healing. MMPs, matrix metalloproteases; ROS, reactive oxygen species; AGEs, advanced glycation end-products.

1.5 BMP pathway

The bone morphogenetic protein (BMP) family belongs to the TGF-β superfamily of growth factors, which are involved in vast cellular processes of fundamental importance including cell-cycle, immunity, development, motility, adhesion, neuronal growth, bone morphogenesis and wound healing (Moura et al., 2013). Up to date, approximately 20 BMP family members are known (Cecchi et al., 2016)(Table 1).

Identification	Description
BMP-2	Bone and cartilage morphogenesis, osteoinduction, osteoblast differentiation, apoptosis.
BMP-3	Negative regulator of bone morphogenesis.
BMP-4	Cartilage, teeth and bone morphogenesis.
BMP-5	Limb development, cartilage and bone morphogenesis.
BMP-6	Osteoblast differentiation, chondrogenesis.
BMP-7	Cartilage and bone morphogenesis.
BMP-8	Bone and cartilage morphogenesis.
BMP-9	Bone morphogenesis.
BMP-11	Axial-skeleton patterning.

Table 1 - Bone morphogenetic proteins. Modified from (Cecchi et al., 2016).

The most important role of BMP proteins is the bone-inductive activity in bone matrix, leading to increased expression of runt-related transcription factor 2 (Runx2) and Osterix (Osx), necessary for osteoblast differentiation (Cecchi et al., 2016). Among the BMP proteins, BMP-7 is one of the important proteins in the bone formation, inducing differentiation of bone marrow-derived mesenchymal stem cells (BMSCs) into osteoblasts (Yan et al., 2018). It is responsible for promoting cell proliferation and extracellular matrix proteoglycans as well as collagen type II synthesis (Abula et al., 2015). The normal concentration of BMP-7 in human blood is 100-300 pg/mL (Aktug et al., 2014). In humans, BMP-7 is expressed in various tissues, such as thymus, bone marrow, spleen, brain, heart, skeletal muscle, kidney, lung, liver, pancreas and prostate (Boon et al., 2011). Several studies have shown that BMP-7 is also involved in brown fat adipogenesis (Tseng et al., 2008) and in proliferation of the human embryonic kidney cells (Saburi et al., 2018). A study conducted by Tseng and collaborators has shown that BMP-7 promotes the differentiation of brown pre-adipocytes by activating key regulators of brown fat, including PRDM16 (PR-domain-containing 16) and Uncoupling protein 1 (UCP-1), in addition to inducing mitochondrial biogenesis via the MAP kinase pathway (Tseng et al., 2008). BMP-7 activates Smad-dependent (canonical) and multiple Smad-independent (non-canonical) signalling pathways to directly affect gene transcription (Moura et al., 2013). BMP-7 binds to its transmembrane type 2 receptor, thus it follows the recruitment of type 1 receptor forming a heterotetrameric complex (Moura et al., 2013). BMP receptors (BMPRs) are serine/threonine kinase receptors, composed of three parts: a short extracellular domain with 10–12 cysteine residues, a single membrane-spanning domain and an intracellular domain with the active serine/threonine kinase region. BMP ligands associate with the extracellular domains of BMP receptors at the cell surface to produce a signalling assembly (Simic et al., 2007). The binding

of BMP-7 to its receptor leads to phosphorylation of SMAD 1/5/8 (Herrera et al., 2018), which then associate with SMAD 4 and translocate to the nucleus to confer gene expression (Herrera et al., 2018). BMP-7 has also been shown to modulate TGF-β1 signalling (Boon et al., 2011), by either inhibiting SMAD 2/3 phosphorylation or by direct competition for SMAD 4, or both (Boon et al., 2011) (Figure 4).

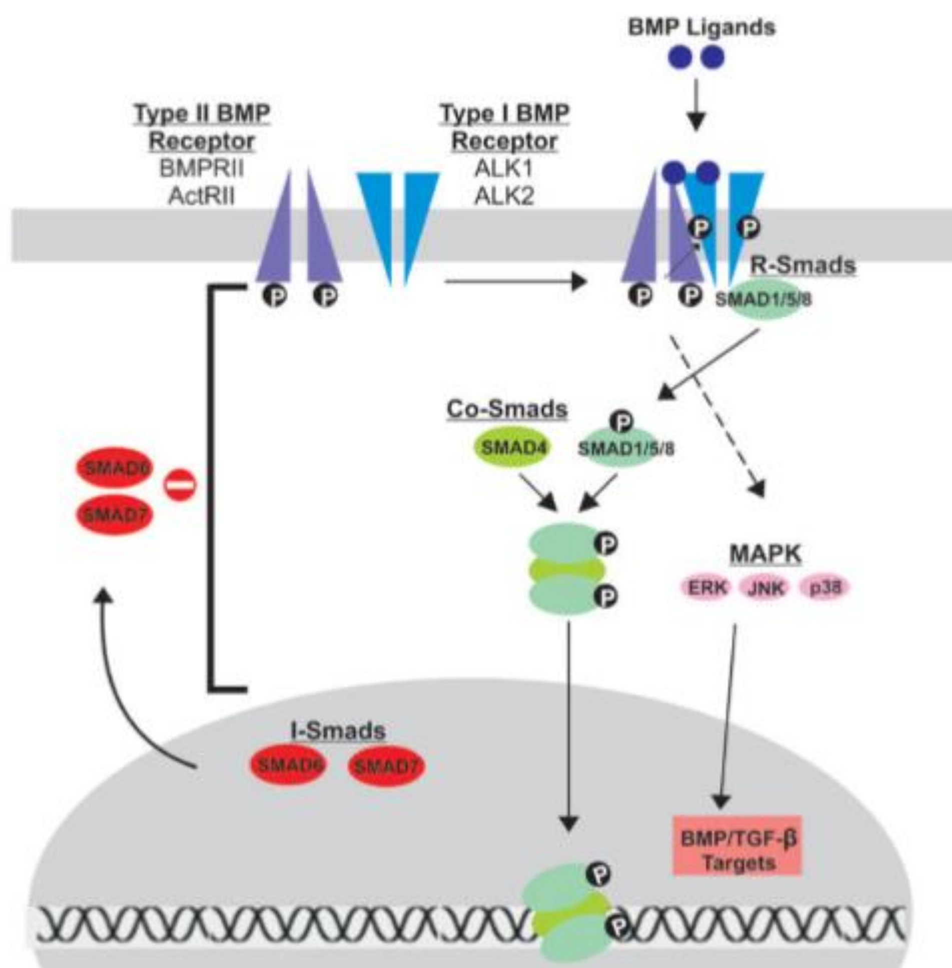

Figure 4 - BMP and TGF-β1 - canonical and non-canonical signalling pathways. BMP = bone morphogenetic protein, TGF-β1 = Transforming growth factor beta 1. Printed with permission (Beederman et al. 2013).

1.6 BMP and tissue regeneration

BMP-7 has been successfully tested in clinical trials and subsequently approved for treating skeletal defects (Simic et al., 2007). BMP-7 was originally discovered for its capacity to induce bone and cartilage formation and bone fracture repair (Boon et al., 2011). In addition, it regulates growth and differentiation of chondroblast and osteoblast cells *in vitro* (Fujii et al., 1999). Regarding bone formation, BMP-7 inhibits myogenesis and promotes the formation of new bone through activating the expression of inhibitor of DNA binding 1 (Id1), an inhibitor for myogenesis. Inhibitor of differentiation (Id) proteins repress transcription by basic helix–loop–helix heterodimers containing myoD/myogenin, a protein that plays important roles in regulating muscle differentiation, which results in the inhibition of myogenesis and leads to the formation of osteoblasts (Figure 5) (Simic et al., 2007).

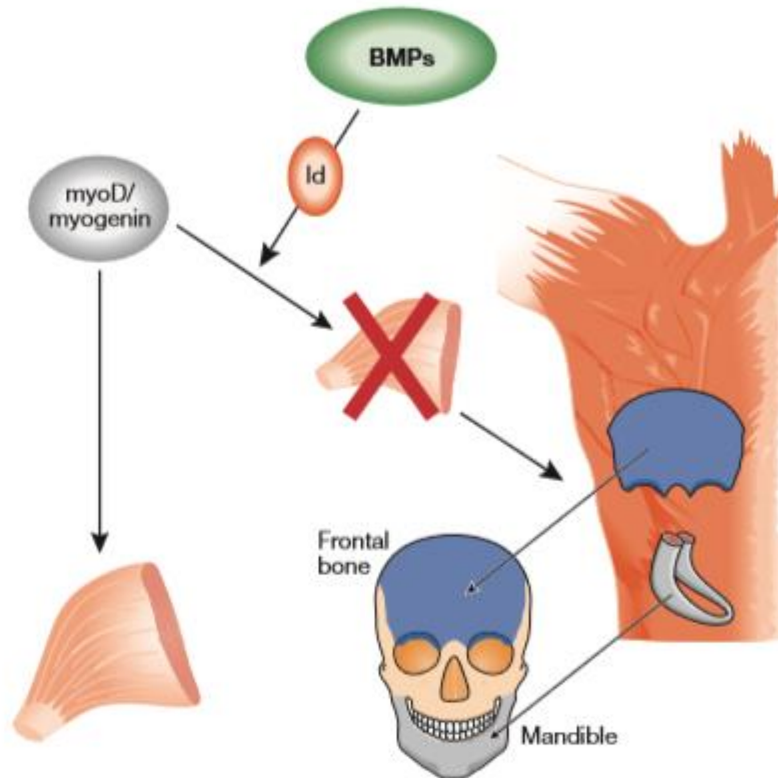

Figure 5 - Bone morphogenetic proteins inhibit myogenesis. BMP-7 inhibit myogenesis and promotes new bone formation through activating the expression of inhibitor of differentiation (Id) genes, in turn preventing the activity of myoD/myogenin. Printed with permission (Simic et al., 2007).

The role of BMP-7 in early development is also well documented and is involved in organogenesis and cell differentiation. Moreover, BMP-7's function in the regeneration of articular cartilage defects has been demonstrated in animal models (Kuo et al., 2006). In addition to bone regeneration, BMP-7 has also been used to increase the volume of the skeleton (Simic et al., 2007), and to regenerate the kidney following acute and chronic failure in rats (Saburi et al., 2018). Other studies have indicated that the liver is also an important target of BMP-7 (Herrera et al., 2018). In addition, BMP-7 is also involved in keratinocyte migration in response to wounding (Ichikawa et al. 2008), and it has also been identified as an instructive factor for human epidermal Langerhans cell differentiation (Yasmin et al., 2013). Thus, BMP-7 improved significantly the recovery rate after acute renal injury in terms of histomorphology as well as function (Boon et al., 2011). Also, chronic injuries including diabetic nephropathy are counteracted. Other studies showed that systemically administered BMP-7 inhibits breast and prostate cancer growth in the bone marrow, and that it is strongly down-regulated in laser micro dissected primary human prostate cancer compared with normal prostate luminal epithelium (Boon et al., 2011). It appears that BMP-7 also controls epithelial homeostasis in the human mammary and prostate gland (Buijs et al., 2007).

Hypothesis

Topical rhBMP-7 treatment improves skin wound healing progression under diabetes conditions.

Aim of the study

a) To evaluate tissue regeneration after topical rhBMP-7 treatment of diabetic wounds in a mouse model of wound healing, measuring its effects on:
    1. Inflammation
    2. Angiogenesis
    3. Proliferation
    4. Collagen deposition

Chapter 2. Materials and methods

2.1 Reagents

The reagents Triton X-100, Isopropanol, Ethanol for molecular biology and Entellan were obtained from Merck, MA, USA. The primary antibody anti-mouse TNF-α was from Bio-Rad AbD Serotec Ltd, CA, USA. The anti-mouse CD206, anti-mouse IL6 were obtained from Santa Cruz Biotechnology, CA, USA. The anti-mouse CD68 was from Abcam Plc, Cambridge, UK and the anti- mouse Ki-67 was from Invitrogen, CA, USA. The anti-mouse CD31 and anti-mouse VEGF were obtained from Millipore, MA, USA. The anti-mouse CD3 was from AbCam. The secondary antibodies Goat Anti-Rat Alexa Fluor 568 and Anti-Rabbit Alexa Fluor 488 were obtained from Molecular Probes, MA, USA. RNase free water, PerfeCTa® SYBR® Green FastMix® were from Quanta Biosciences, Canada. The primers were obtained from Integrated DNA technologies, IA, USA. QIAzol™ Lysis Reagent was from Qiagen, Germany and the normal goat serum was from Life Technologies, CA, EUA. All the remaining reagents were purchased from Sigma-Aldrich, MO, USA or VWR, Portugal.

2.2 Animal experiments

Diabetic male adult mice (C57BL6/J) wild type were used in this work. Diabetes was induced with streptozotocin (STZ) intraperitoneal injections (50 mg/Kg) for 5 consecutive days. The mice with blood glucose over 250 mg/dl were considered diabetic. Streptozotocin destroys the beta cells from pancreas and the mice will not be able to produce insulin. Therefore, insulin signalling and insulin induced pathways are compromised and animals will not be able to regulate the circulating blood glucose levels thus this, is a type 1 diabetes rodent model (Moura et al., 2014).

2.3 Wound procedure and treatment

Mice received analgesia intraperitoneally with buprenorphine (0.05 mg/kg) for 30 minutes before anaesthesia. Anaesthesia was induced with Ketamine/Xylazine (100/10 mg/kg, *i.p.)*. The animals were then maintained on a warm pad during anaesthesia. Then the dorsal hair of the mice was removed with the help of a proper electric shaving machine. The remaining hair was removed using depilatory cream and the skin was cleaned using a sterile swab with an antiseptic solution (Betadine). The mice were divided into 3 groups: saline treatment (n=3), 2µg (n=3) or 10µg (n=4) of rhBMP-7 treatment. Subsequently two 6 mm excision wounds per

mouse were created in the dorsum with sterile surgical punch biopsy tool. The wounds were topically treated, once a day up to 10 days, with either 2 μg or 10 μg of rhBMP-7, or with saline. The wound area was traced daily with acetate paper up to day 10 (Leal et al., 2015; Moura et al., 2014). The wound size was determined with ImageJ (NIH, Bethesda, MD) and the data were presented as a percentage of the original wound (day 0), as previously reported (Leal et al., 2015; Moura et al., 2014; Tellechea et al., 2019).

The animal experiments were not performed during this thesis work but someone else has already performed them before I joined the laboratory. The methods still being described here for better understanding of the experiments that followed from the skin of the previously treated animals. I used the skin samples of these animal experiments to evaluate the role of rhBMP-7 in diabetic wound healing.

2.4 Immunohistochemistry

Skin biopsies were collected at day 10 post - wounding and embedded in optical cutting temperature medium (OCT). Then biopsies were sectioned at 10μm thickness and mounted on the slides. After the frozen slides were removed from the freezer and thawed at room temperature for 10 minutes. Then sections were fixed with ice cold-acetone for 10 minutes and washed in PBS-0.1% / Tween 20. The tissue was surrounded with a hydrophobic barrier using a wax pen. After this step the sections were blocked with 5% goat serum for 1h at room temperature. Subsequently, staining was performed for the following markers: CD31 (a marker of endothelial cells) (1:200), CD68 (a marker of macrophages), CD68 and TNF-α for M1 phenotypes, CD68 and CD206 for M2 phenotypes, CD3 (a marker of

lymphocytes) (1:200), TNF- α, IL-6, KI-67 and VEGF (1:200), at 4ºC overnight in a humid chamber. After 24 incubation with primary antibodies, sections were washed for 5 minutes 3 times in PBS/Tween 20, and then incubated with secondary antibodies, specific for each primary antibody used (anti-rabbit; anti-rat; 1:500 in blocking solution; 1:1000 DAPI) for 45 minutes at room temperature and then washed for 5 minutes (3x) in PBS/ 0.1% Tween 20. Coverslips were mounted in Entellan. Results are expressed as average number/visual field (Leal et al., 2015).

2.5 Histology assays

Skin biopsies were fixed in 4% paraformaldehyde. The samples were sectioned at 3-4µm thickness and routine hematoxylin and eosin (H&E) and Herovici staining were performed. The morphological structure of the skin tissue was determined by H&E staining, and collagen deposition was evaluated by the Herovici staining (Tellechea et al., 2019).

2.6 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA extraction

Total RNA was isolated from skin tissue with the QUIAzol reagent. The samples were homogenized in 1ml of Quiazol and incubated for 5 minutes at room temperature. After the addition of chloroform, the homogenate was separated into an aqueous and an organic phase by centrifugation. RNA partitions to the upper aqueous phase while DNA partitions to the interphase and proteins to the lower, organic phase or the interphase. The aqueous phase was removed, and ethanol was added and transferred to an RNeasy spin column, where the total RNA binds to the membrane. Then Buffer RW1 was added to the RNeasy spin column and the samples were centrifuged, it is used as a stringent washing buffer that efficiently removes biomolecules such as carbohydrates, proteins, fatty acids. Subsequently Buffer RPE was added to the RNeasy spin column, which has the function to remove traces of salts that are still on the column due to buffers used earlier, and centrifuge again the samples. High-quality RNA was then eluted in RNase-free water.

cDNA synthesis

cDNA was prepared using the high capacity cDNA reverse transcription kits. The RT master mix was prepared (2µl of 10✕ RT Buffer, 0,8µl of 25✕ dNTP Mix, 2µl of 10✕ RT Random Primers, 1µl of MultiScribe™ Reverse Transcriptase and 4,2µl of nuclease-free H_2O). Then 1 µg of RNA was added to the Master Mix to perform the reverse transcription in the thermal cycler, according to the following conditions as refered in the cDNA kit instructions (Technologies, A. 2009. RNeasy ® Lipid Tissue Handbook Sample & Assay Technologies QIAGEN Sample and Assay Technologies, February):

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

After the cDNA synthesis, the samples were diluted 20 times in RNase-free water.

Quantitative RT-PCR

After the cDNA synthesis, quantitative RT-PCR was performed in a 20µl volume containing PerfeCTa SYBR Green FastMix for iQ (2X), forward primer, reverse primer, nuclease-free water. Samples were denatured at 95ºC for 3 minutes. Subsequently, 45 cycles were run for 10 sec at 95ºC for denaturation, 30 sec at the appropriate annealing temperature and 30sec at 72ºC for elongation according to SYBR Green FastMix manufacturer instructions (Technologies, A. 2009). The list of primers used, and respective sequences are in Table 2. The results were normalized using the reference gene TATA box binding protein (TBP).

Primer	Mouse primer sequences	
	Forward primer (5’-3’)	Reverse primer (5’-3’)
TBP	ACCCTTCACCAATGACTCCTATG	TGACTGCAGCAAATCGCT TGG
IL-6	TGGCTAAGGACCAAGACCATCCAA	AACGCACTAGGTTTGCCGAGTAGA
KC	ATTAGGGTGAGGACATGTGTGGGA	AATGTCCAAGGGAAGCGTCAACAC
TNF-α	TTCCGAATTCAGTGGAGCCTCGAA	TGCACCTCAGGGAAGAATCTGGAA
IL1-β	ACCTGTCCTGTGTAATGAAAG	GCTTGTGCTCTGCTTGTG
MCP-1	ACTGCATCTGCCCTAAGGTCTTCA	AGAAGTGCTTGAGGTGGTTGTGGA
MMP-9	TCCAACTCACTCACTGTGGTTGCT	AGACTGCCAGGAAGACACTTGGTT
VEGF	CTTGTTCAGAGCGGAGAAAGC	ACATCTGCAAGTACGTTCGTT
VEGFR2	AAACACTCACCATTCCCAGG	CGCAAAGAGACACAT TGAGG
TGFβ-1	TCAATACGTCAGACATTCGGG	CGTGGAGTTTGTTATCTTTGC
SMAD-4	GTGATCTATGCCCGTCTGTG	GGTGTTGGATGGTTTGAATCG
BMP-7	TAAGGGTTCCAGAAACCTGAGCGT	AACGAGGCTTGCGATTACTCCTCA

Table 2 - List of primers and respective sequences used in qRT-PCR. TBP -TATA binding protein; IL-6 - Interleukin 6; KC **-** Keratinocyte-derived chemokine, a murine Interleukin 8 analogue; TNFα - Tumor necrosis factor alpha; IL1-β - Interleukin 1 beta; MCP-1 - Monocyte chemoattractant protein-1; MMP-9 - Metalloprotease 9; VEGF - Vascular endothelial growth factor; VEGFR2 - Vascular endothelial growth factor receptor 2; TGFβ1 - Transforming growth factor beta 1; SMAD-4 - Small mother against decapentaplegic 4; BMP-7 - Bone morphogenetic protein 7.

2.7 Statistical analyses

The results are expressed as mean ± S.E.M (standard error of the mean). Statistical analysis was performed using t-test using Microsoft Excel comparing the saline treated group with 2µg of rhBMP-7, or saline treated group with 10µg of rhBMP-7. P values lower than 0.05 were considered statistically significant.

Chapter 3. Results

3.1 rhBMP-7 improved wound healing in a diabetic mouse model

Wound closure was evaluated from day 0 and until day 10. As shown in Figure 6 A the wound closure in each experimental group was measured every day. In the group where wounds were treated with 10µg of rhBMP-7 there was a significant difference in wound size compared to the saline treated group ($p<0.05$). However, topical treatment with 2µg of rhBMP-7 had an even higher effect in wound closure compared to the saline treated group ($p<0.01$).

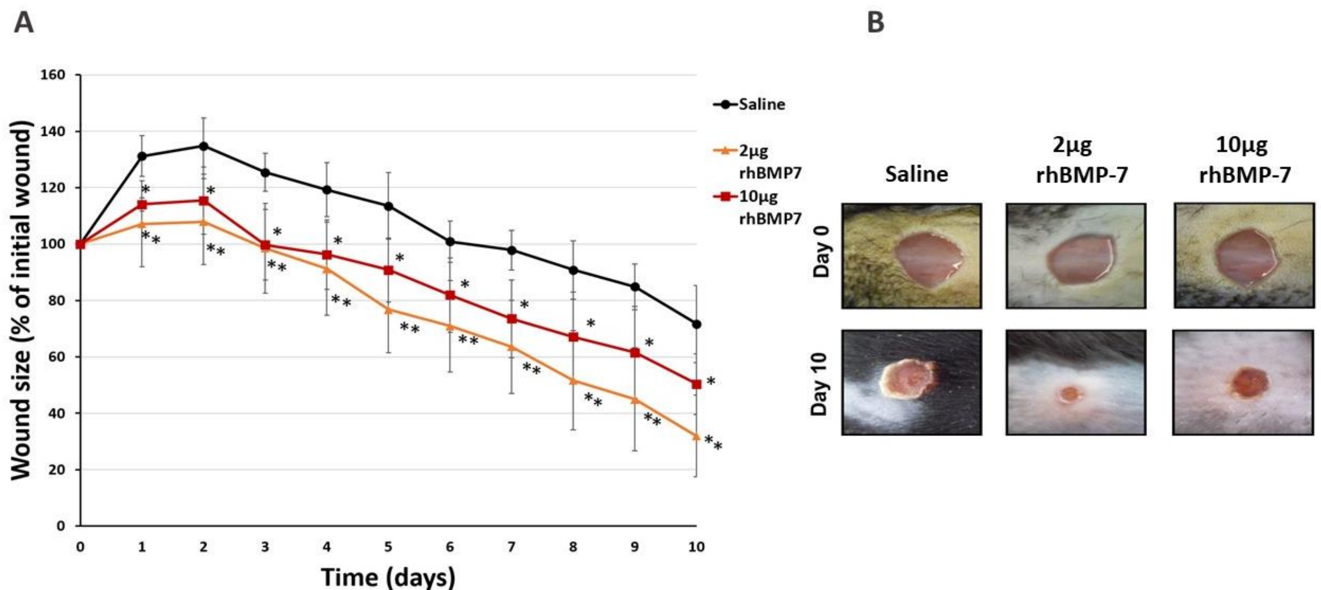

Figure 6 – Skin wound size evaluation. **(A)** Kinetics of wound closure in diabetic mice. Percentage of wound healing progression during the 10 days of experiment in wounds treated with 2µg (n=6 wounds), 10µg (n=8 wounds) of rhBMP-7 and saline (n=6 wounds). **(B)** Representative images for days 0 and 10. The data represent the mean ± SEM. *p<0.05 and ** p<0.01 compared to saline treated group. t-test

3.2 rhBMP-7 decreased pro-inflammatory M1 macrophages

We evaluated the inflammatory cells at the wound site on day 10, by quantifying macrophages and T-cells lymphocytes. As demonstrated in Figure 7, rhBMP-7 reduced significantly the number of macrophages when compared to the saline treatment in both concentrations 2µg and 10µg ($p<0.05$). M1 and M2 phenotypes were also analysed. These results suggest that treatment with both concentrations of rhBMP-7 (2µg or 10µg) significantly decreased the expression of pro-inflammatory M1 macrophages ($p<0.001$) (Figure 7). However, there were no significant effects on the number of M2 anti-inflammatory macrophages (Figure 7).

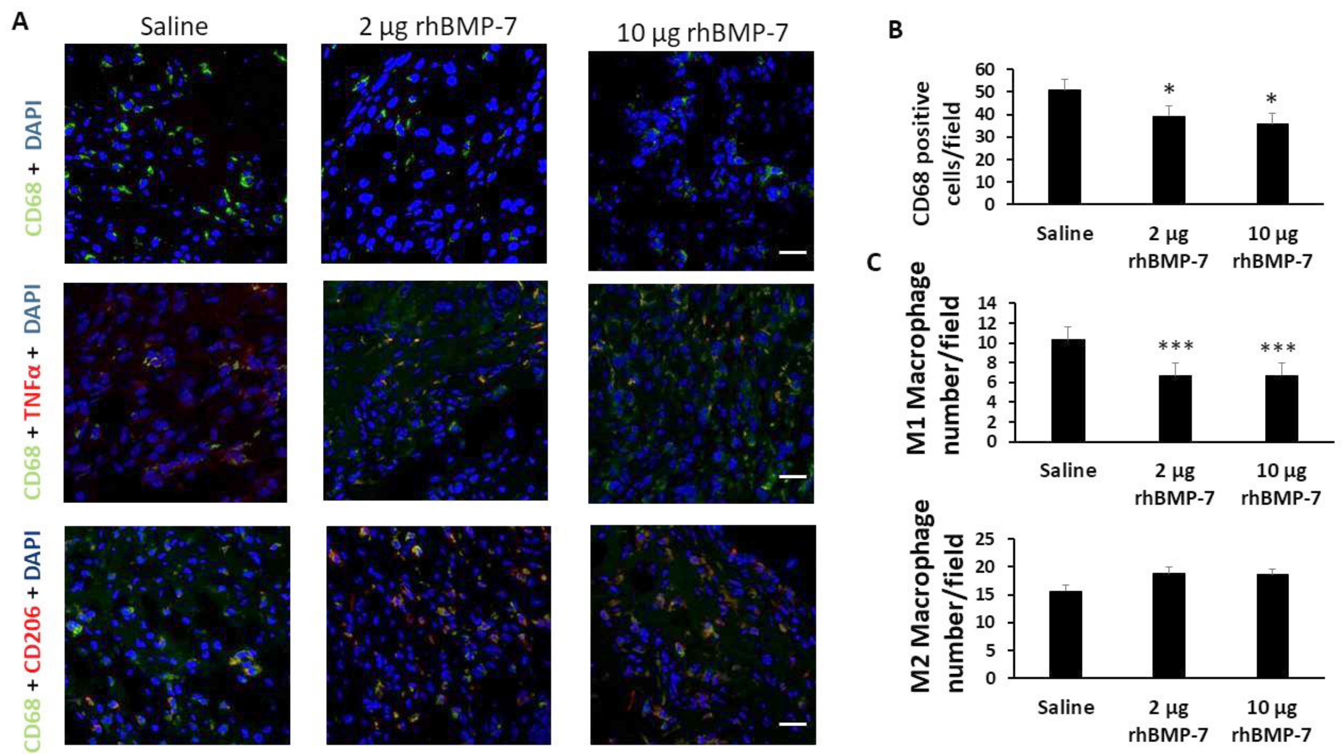

Figure 7 - Effect of rhBMP-7 in the number of macrophages in the skin. **(A)** Representative microscopy pictures of all macrophages, M1 and M2 phenotype in wound skin sections. **(B)** Quantification of the number of macrophages (CD68), **(C)** M1 phenotype and **(D)** M2 phenotype in wounds treated with 2 µg (n=3) and 10 µg (n=4) of rhBMP-7 and saline (n=3). The results are presented as the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001 to saline treated mice; t-test. Scale bar= 50µm. Magnification 200x.

Subsequently, we evaluated the levels of T-cells lymphocytes as demonstrated in Figure 8.

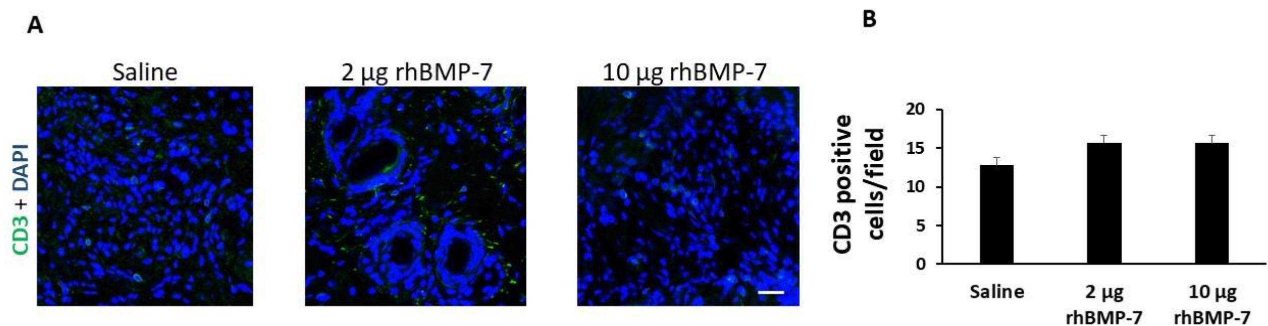

Figure 8 – Effect of rhBMP-7 in T-cells number in diabetic wounds. **(A)** Representative images of CD3 staining and **(B)** quantification of the number of T-cells (CD3) in wounds treated with 2 µg (n=3) and 10 µg (n=4) of rhBMP-7 and saline (n=3). The results are presented as the mean ± SEM. t-test. There is no significant difference between rhBMP-7 treatment and saline treatment groups. Scale bar= 50µm. Magnification 200x.

The results showed that T-cell number did not change between rhBMP-7 treatments compared to the saline group.

3.3 rhBMP-7 promoted a decrease in the inflammatory environment in diabetic wounds

Inflammatory markers were evaluated since we observed a decrease in macrophages, particularly pro-inflammatory M1 macrophages. To evaluate the effect of rhBMP-7 on inflammatory markers, gene expression was measured on skin tissues, at day 10 post-wounding. As shown in Figure 9, the treatment with 2µg and 10µg of rhBMP-7 tended to reduce the gene expression of inflammatory markers when compared to saline group. IL-6 gene expression tended to decrease in both treated groups 2µg and 10µg of rhBMP-7 (Figure 9 A) compared to the saline treated group. Similar results were observed for skin keratinocyte-derived cytokine (KC), the mouse equivalent of human IL-8 (Figure 9 B), TNF-α expression (Figure 9 C) and IL-1β expression (Figure 9 D). In addition, the gene expression of monocyte chemoattractant Protein-1 (MCP-1), which plays an important role in the recruitment of monocytes to the site of injury and then differentiated into macrophages, also tended to be reduced in both 2µg and 10µg of rhBMP-7 group compared to the saline treatment group (Figure 9 E). Moreover, there is also a tendency to decrease in gene expression of MMP-9 in both concentration of rhBMP-7 compared with the saline treatment group (Figure 9 F).

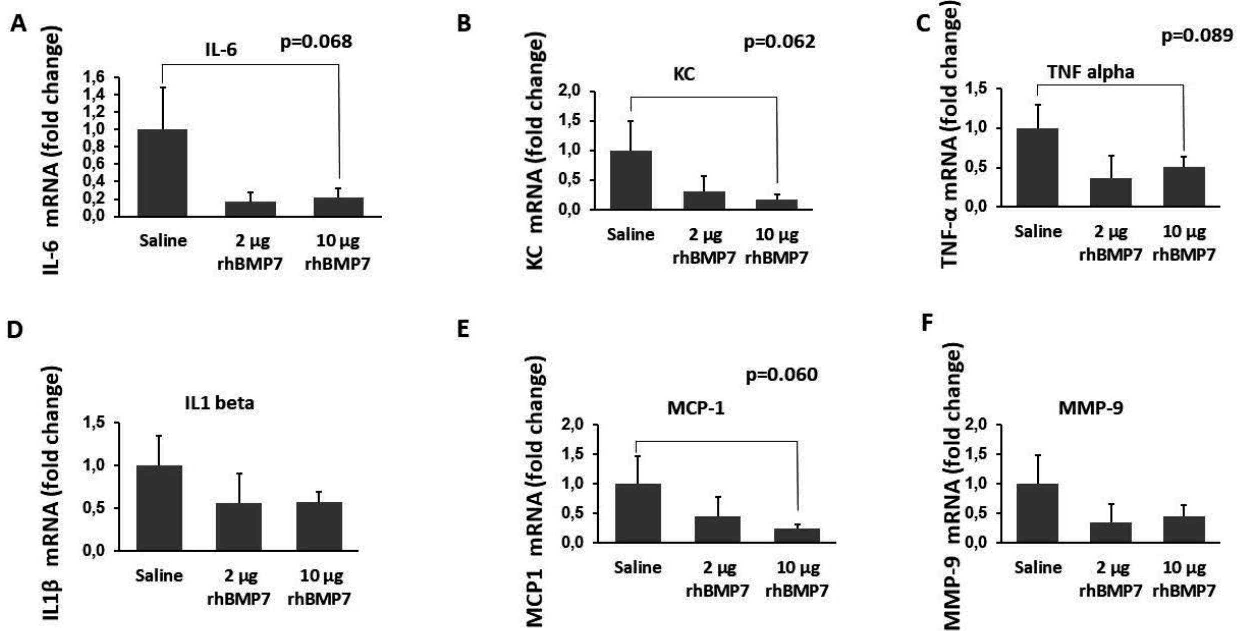

Figure 9 – Effect of rhBMP-7 on molecular markers of inflammation. Gene expression of IL-6 (A), KC (B), TNF-α (C), IL-1β (D), MCP-1 (E) and MMP-9 (F) treated with 2 µg (n=3) and 10 µg (n=4) of rhBMP-7 and saline (n=3). Data are expressed as means ± SEM. No significant differences were found. IL-6= Interleukin 6; KC= keratinocyte-derived cytokine; TNF-α= Tumor necrosis factor alpha; IL-1β= Interleukin 1 beta; MCP-1= monocyte chemoattractant Protein-1; MMP-9= Matrix metalloproteinase 9.

The levels of inflammatory markers, particularly, TNF-α and IL-6, were also evaluated. The treatment with both concentrations of rhBMP-7 reduced significantly the protein expression of TNF-α when compared to saline group ($p<0.01$, in 2µg and $p<0.05$ in 10µg of rhBMP-7) Figure 10. In contrast, there was no significant difference on IL-6 protein expression between treatment and the saline groups (Figure 10).

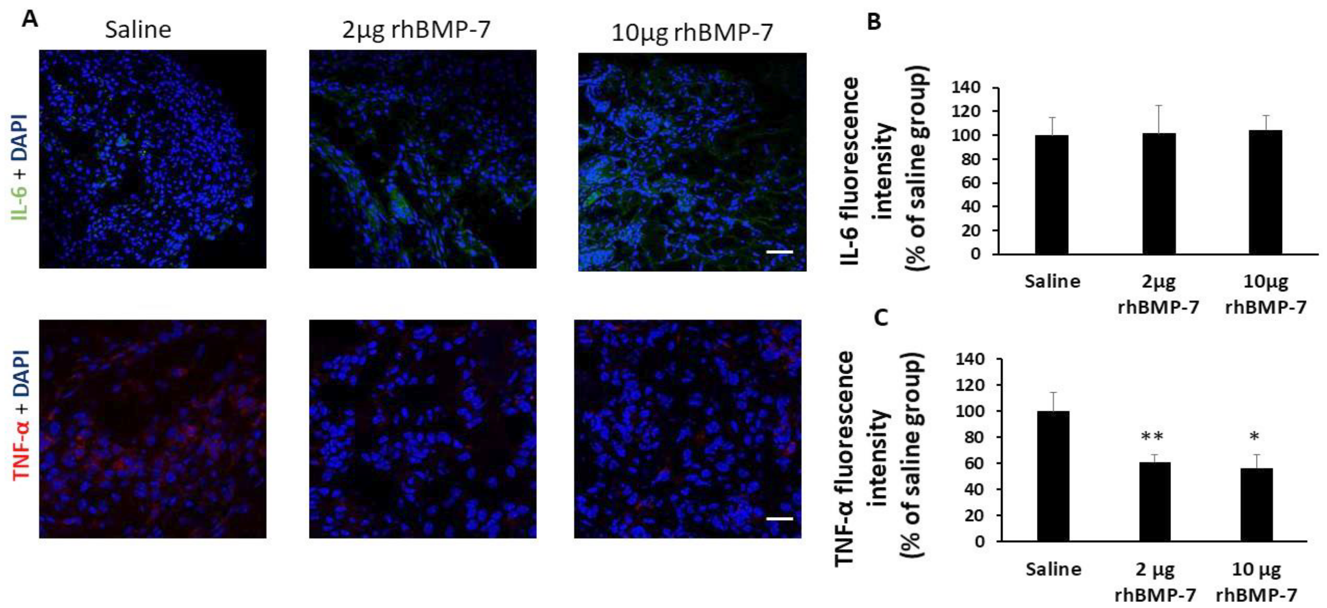

Figure 10 – Effect of rhBMP-7 on IL-6 and TNF-α protein expression in diabetic wounds. **(A)** Representatives images of IL-6 and TNF-α staining. **(B)** Quantification of IL-6 fluorescence intensity in wounds treated with 2 µg (n=3) and 10 µg (n=4) of rhBMP-7 and saline (n=3). **(C)** Quantification of TNF-α fluorescence intensity in wounds treated with 2 µg (n=3) and 10 µg (n=4) of rhBMP-7 and saline (n=3). The results are presented as the percentage of fluorescence intensity as the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001, to saline treated mice; t-test. IL-6= Interleukin 6, TNF- α = Tumor Necrosis Factor α. Scale bar= 50µm. Magnification 200x.

3.4 Angiogenesis increased with rhBMP-7 treatment in diabetic wound healing

VEGF, an important player in wound healing to stimulate angiogenesis, as well as the vascular endothelial growth factor receptor 2 (VEGFR2) mRNA expression were measured but no significant differences were observed (Figure 11 A, B).

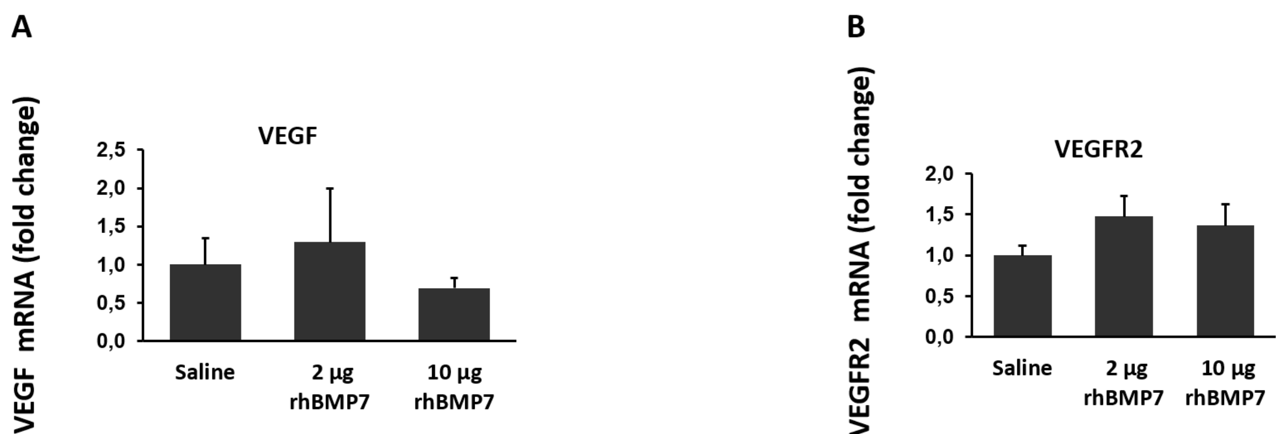

Figure 11 – Effect of rhBMP-7 on molecular markers of Angiogenesis. Gene expression of VEGF **(A)** and VEGFR2 **(B)** treated with 2 µg (n=3) and 10 µg (n=4) of rhBMP-7 and saline (n=3). Data are expressed as means ± SEM; t-test. No significant differences were found. VEGF= Vascular endothelial growth factor; VEGFR-2= Vascular endothelial growth factor receptor 2.

In order to evaluate angiogenesis, we quantify the number of blood vessels with CD31 staining (Figure 12). Our results demonstrated that 2µg of rhBMP-7 increased significantly the amount of vessels at the wound site when compared to the saline treatment ($p<0.01$). Especially the higher concentration of rhBMP-7 had a higher effect on the amount of vessels ($p<0.001$). Moreover, we evaluate VEGF protein by immunohistochemistry, and, contrary to the observed in mRNA levels, VEGF protein expression was significantly increased in both concentration of BMP-7, 2µg of rhBMP-7 ($p<0.01$) and 10µg of rhBMP-7 ($p<0.05$), when compared with saline group (Figure 12).

3.5 rhBMP-7 treatment increased cell proliferation

Moreover, we also evaluated the protein expression of KI-67, which is a marker for cells proliferation. The results suggest that the protein expression of KI-67 increased significantly with both concentration of rhBMP-7 when 2µg and 10µg of rhBMP-7 were compared to the saline treated group ($p<0.001$) (Figure 12).

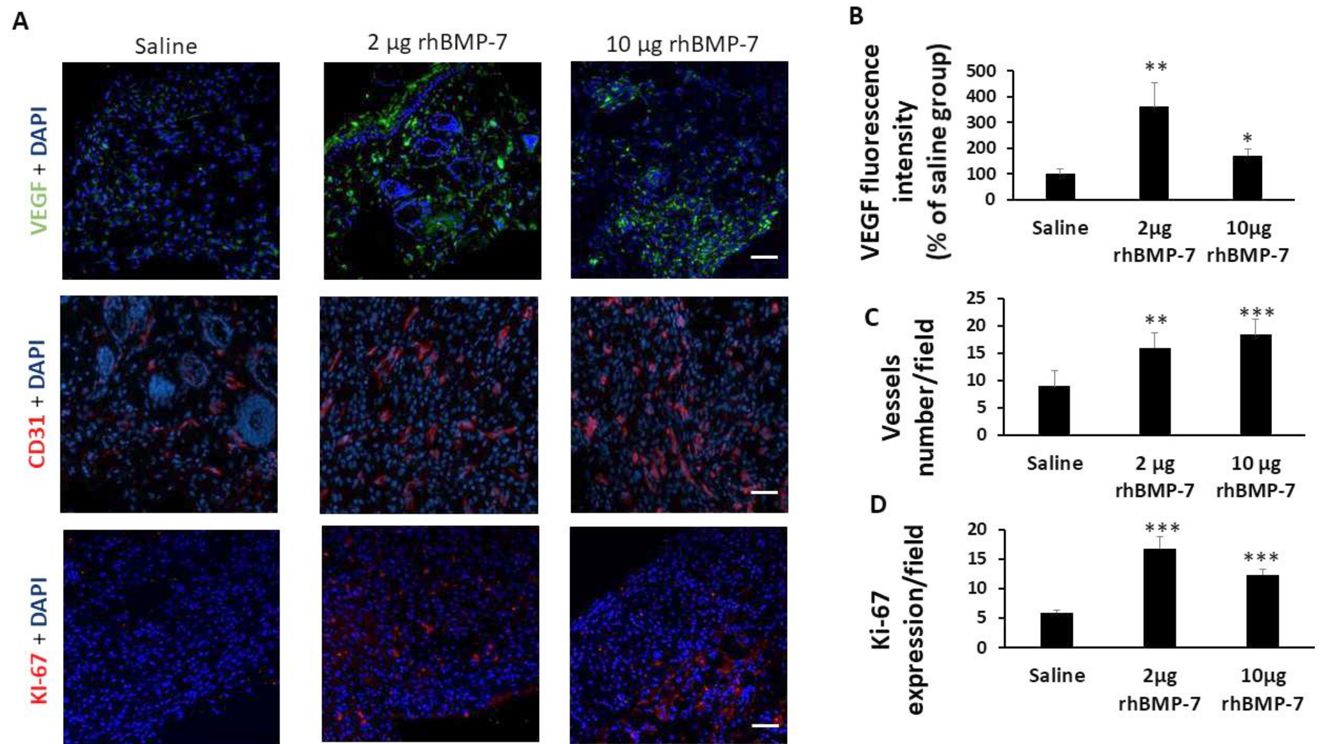

Figure 12 - rhBMP-7 increases angiogenesis and proliferation. **(A)** Representative images of VEGF, CD31 and KI-67 staining. **(B)** Respective quantification of VEGF, **(C)** CD31 and **(D)** KI-67 in wounds treated with 2µg (n=3) and 10µg (n=4) of rhBMP-7 and saline (n=3). The results are presented as the mean ± SEM. *p<0.05, **p<0.01 and ***p<0.001, to saline treated mice; t-test. Scale bar= 50µm. VEGF= vascular endothelial growth factor. Magnification 200x.

3.6 TGF-β1, BMP-7 and SMAD-4 mRNA expression did not alter with rhBMP-7 treatment

In order to study the effect of rhBMP-7 in markers related with BMP-7 signalling we evaluated the gene expression of BMP-7, TGF-β1 and SMAD-4 but no differences were observed (Figure 13 A, B, C).

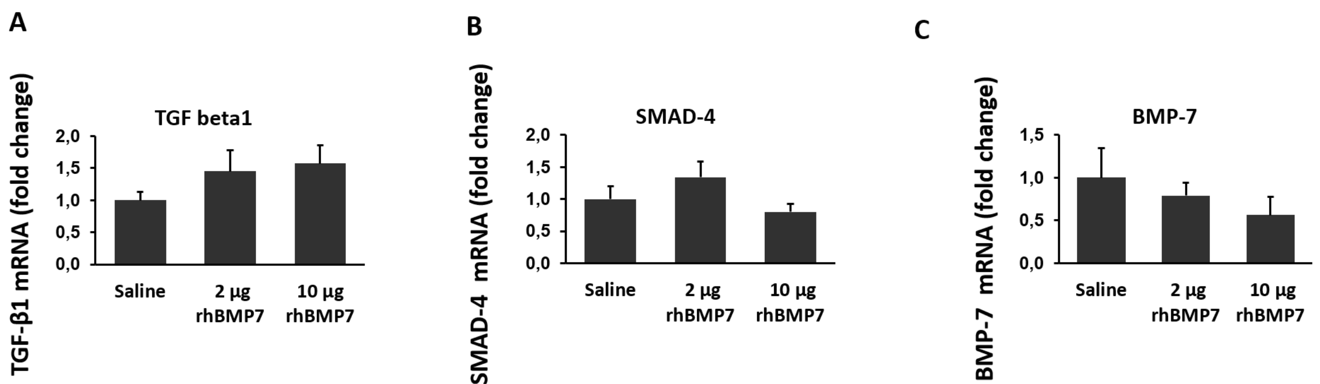

Figure 13 – Gene expression of BMP-7 pathway markers. Gene expression of TGF-β1 **(A)**, SMAD-4 **(B)** and BMP-7 **(C)** treated with 2 µg (n=3) and 10 µg (n=4) of rhBMP-7 and saline (n=3). Data are expressed as means ± SEM. No significant differences were found. TGF-β1= Trasforming growth factor 1 beta; SMAD-4= Small mother against decapentaplegic 4; BMP-7= Bone morphogenetic protein 7.

3.7 rhBMP-7 effect on skin morphology

H&E staining was examined to evaluate the effect of rhBMP-7 on skin histology in diabetic wound healing (Figure 14).

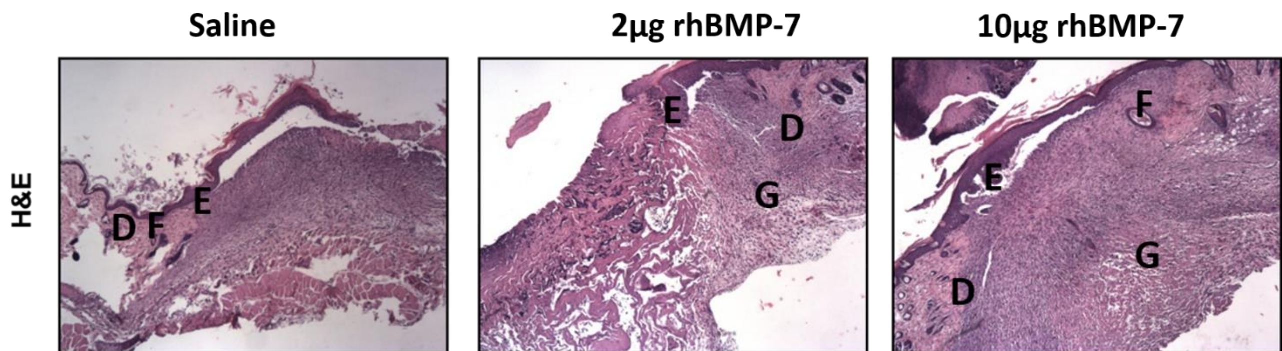

Figure 14 - Skin histology showing the structure of epidermis and dermis. Hematoxilin and Eosin staining in diabetic wounds treated with 2 µg (n=3) and 10 µg (n=4) of rhBMP-7 and saline (n=3). D= dermis, E= epidermis, F= hair follicles, G= granulation tissue. Magnification 40x.

The results demonstrated that both concentration of rhBMP-7 induced granulation tissue formation when compared to the saline treated group, in

addition to an increase in connective tissue cells, as well as the extracellular matrix. Furthermore, the thickness of the epidermis and dermis increased in the treated group with 2µg of rhBMP-7 when compared to the saline treated group. However, it was observed that rhBMP-7 to promote the differentiation of a bone matrix in the skin, so, more studies are needed to evaluate whether different doses of rhBMP-7 will be better candidates to treat diabetic skin wounds.

Collagen deposition in tissues for each experimental group was also determined with Herovici staining. As demonstrated in Figure 15 the collagen stained blue, and rhBMP-7 was associated with an increased collagen deposition when compared to the saline treated group.

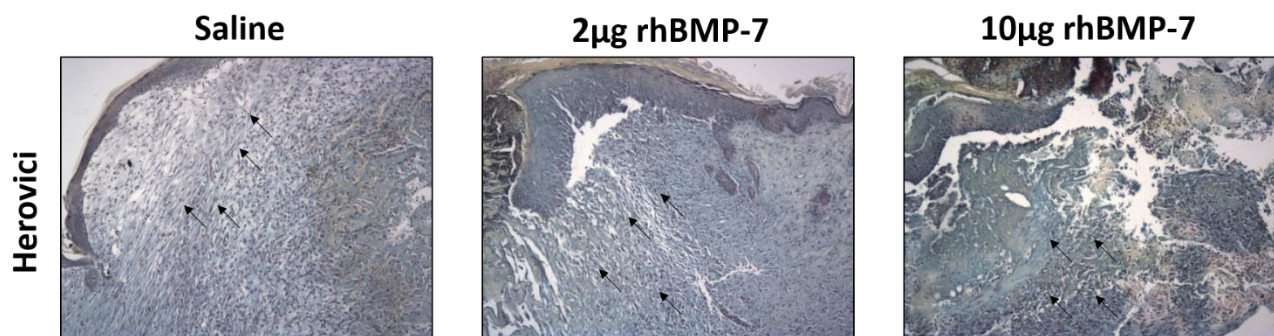

Figure 15 - Skin histology showing collagen deposition. Herovici staining in diabetic wounds treated with 2 µg (n=3) and 10 µg (n=4) of rhBMP-7 and saline (n=3). Blue stain= collagen deposition. Arrows= Collagen fibers. Magnification 100x.

Chapter 4. Discussion

Discussion

Wound healing is a complex process with several overlapped key phases: homeostasis, inflammation, proliferation and remodelling. Diabetic wound healing has a chronic inflammatory phase that typically do not progress through the normal stages of healing. Chronic hyperglycaemia affects wound healing causing a reduction in collagen production, a decrease in new blood vessel formation and a decrease in immune resistance at the wound site. In addition, diabetes alters normal wound healing through increased secretion of MMPs, increased production of ROS, as well as keratinocyte and fibroblast dysfunction.

Our pilot study indicates that rhBMP-7 improves diabetic wound in part via an improved resolution of inflammation. We show an increased expression of inflammatory cytokines and other molecules involved in the inflammatory phase of wound healing in saline treated wounds at a stage of healing when the inflammation should be decreased to allow the regeneration of the tissue. This phenomenon is critical in the healing process thus, impairing wound healing. However, when wounds were treated with rhBMP-7, a reduction in the number of macrophages and M1 phenotype, as well as the reduction of TNF-α protein expression was observed.

TNF-α is one of the most important inflammatory cytokines secreted by macrophages, which plays a key role during this phase because it promotes neutrophil recruitment, induces IL-6 and IL-1 stimulation, as well as the adhesion molecules expression (Selectins and Integrins) (Xia et al., 1998). These results are very significant because they confirm that under diabetic conditions, as in a chronic wounds, the wounds contain high levels of pro-inflammatory molecules. Similar effects of BMP-7 on inflammatory cytokines were observed in another study using mice with type 2 diabetes mellitus (Chattopadhyay et al., 2017). This study showed that injection of recombinant BMP-7 intraperitoneally in db/db and

high-fat diet (HFD) mice was able to reduce serum levels of inflammatory cytokines, including TNF- α, IL-6 and IL-1β compared with untreated db/db and HFD mice (Chattopadhyay et al., 2017). Furthermore, our results showed that there is a tendency for a decrease in the MMP-9 gene expression that is involved in the degradation of the extracellular matrix. Also, we found that rhBMP-7 tended to decrease KC (mouse equivalent of human IL-8) and MCP-1 gene expression (monocyte chemoattractant), responsible for the migration and infiltration of monocytes, in diabetic wounds. Congruent with our results, the study conducted by Rocher and Sigla showed a reduction in concentration of MCP-1, IL-6 and TNF-α, following BMP-7 administration (Rocher et al., 2013). In the same study, it was also observed that BMP-7 treatment induces monocyte polarization into M2 macrophages reducing the expression of M1 macrophage, consistent with our results since rhBMP-7 treatment induce a reduction in the number of M1 macrophages, but we found no difference on M2 macrophage expression. Moreover, M1/M2 ratio is known to be determinant in the inflammatory phase. The dysregulation of M1/M2 ratio can determine the progression of inflammatory disorders as atherosclerosis, cancer and pre-diabetes (Singla et al., 2016). In addition, the reduction of the M2 phenotype in diabetic wounds can lead to a decrease in growth factors levels, which are essential to stimulate the proliferative phase in wound healing. One of these is VEGF, which is analysed in our study. The results showed that rhBMP-7 was able to increase VEGF protein expression in diabetic wounds, suggesting that BMP-7 stimulates production of this angiogenic marker in wound healing process.

Moreover, we found that rhBMP-7 promotes angiogenesis since it increased the number of blood vessels, as showed by CD31 staining. Also, rhBMP-7 showed to increase Ki-67 protein expression, which is a marker of cell proliferation. In a study conducted by Akiyama and collaborators (Akiyama et al., 2014) an effect of BMP-

7 in the promotion of angiogenesis in both granulosa cells (GC) and endothelial cells was observed. The results suggested that BMP-7 stimulates VEGF expression in granulosa cells and VEGF receptor in endothelial cells, suggesting a new role of BMP-7 in folliculogenesis, which is the generation of mature oocytes regulated by cytokines that regulate cell proliferation, differentiation and function (Field et al., 2014).

From our histological results, rhBMP-7 seems to increase collagen deposition and stimulate bone matrix differentiation. Other studies conducted on BMP-7 are in agreement with our results, for example, Xu Yan and collaborators have shown that the expression of BMP-7 enhanced the differentiation capacity of bone marrow mesenchymal stem cells and had a strong ability to induce cell proliferation and extracellular matrix proteoglycan and collagen type II synthesis (Yan et al., 2018). On the other hand, a study conducted to study hypertrophic scar formation showed that BMP-7 inhibits scar formation in a mouse model of thermal injury, suppressing collagen deposition and fibrotic protein expression such as α-SMA, TGF-β1 and CTGF, and it also able to induce the fibroblasts apoptosis (Guo et al., 2017).

Fuguo Chen and collaborators studies have shown that BMP-7 induced the osteogenic differentiation of dermal fibroblast (Chen et al., 2019). In addition, a study conducted by Yu-Hua Tseng showed an additional role of BMP-7 in the differentiation of brown preadipocytes, where BMP-7 induced UCP-1 gene expression and the expression of other brown-fat-selective genes (Tseng et al., 2008). In addition, BMP-7 is able to induce both osteogenic and brown fat cells differentiation from Myf5+ precursor cells, stimulating the expression of PRDM16 that is the key regulator of brown fat differentiation, as well as the expression of myogenin, essential for the development of functional skeletal muscle (Boon et al., 2011).

Chapter 5. Conclusion

Conclusion

BMP-7 was discovered for its ability to induce mesenchymal cells to differentiate into osteoblasts, for the promotion of cell proliferation and extracellular matrix proteoglycan and collagen type II synthesis (Yan et al., 2018). To date no one has evaluated the effects of BMP-7 in tissue regeneration in the presence of diabetes. Due to these features we hypothesized that BMP-7 would play a significant role in diabetic wound healing at the skin level. Our present study is a pilot study and, as such, it is underpowered. Our results indicate that rhBMP-7 was able to induce significant alterations in the wound healing kinetics in a pre-clinical mouse model of diabetes. A decrease in inflammatory cell infiltration was observed, specifically a decrease in the M1 pro-inflammatory macrophages as demonstrated in Figure 7, as well as a decrease in the expression of TNF-α (Figure 10). In addition, IL-6, KC, MMP-9, MCP-1 and IL-1-β tended to decrease (Figure 9 A, B, D, E and F) with rhBMP-7 treatment. All of these markers are involved in inflammation and infection at the wound site. In addition, we also demonstrated that rhBMP-7 treatment increased angiogenesis, since it increases the number of blood vessels and VEGF (Figure 12 A, B), as well as an increased expression of KI-67 (Figure 12 C), which plays an important role in the proliferative phase of wound healing. In H&E (Figure 14) and Herovici stainings (Figure 15) we have shown that rhBMP-7 induced granulation tissue formation, epithelialization and collagen synthesis. Similar results have been found in our previously published results when diabetic wounds were topically treated with Substance P and Neurotensin (Leal et al., 2015; Moura et al., 2013; Moura et al., 2014). In accordance with our results, SP treatment was effective in wound healing in diabetic mice, reducing the markers of the inflammatory phase such as IL-6, KC, MMP-9 and MCP-1 at day 10 post-wounding, and it modulated the macrophage phenotype (Leal et al., 2015). NT treatment showed similar results, where a decreased inflammatory response was

observed (Moura et al., 2013; Moura et al., 2014). Therefore, in accordance with these previous results, our data shows that rhBMP-7 could be a new candidate to treat diabetic wound healing, but more studies are needed to confirm these results.

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