THE UNIVERSITY OF
SYDNEY

ROLE OF NEUTROPHIL GELATINASE -ASSOCIATED LIPOCALIN-2 (NGAL) IN POOR WOUND HEALING IN DIABETES

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A thesis submitted in fulfilment of the requirements for admission to the Degree of Doctor of Philosophy

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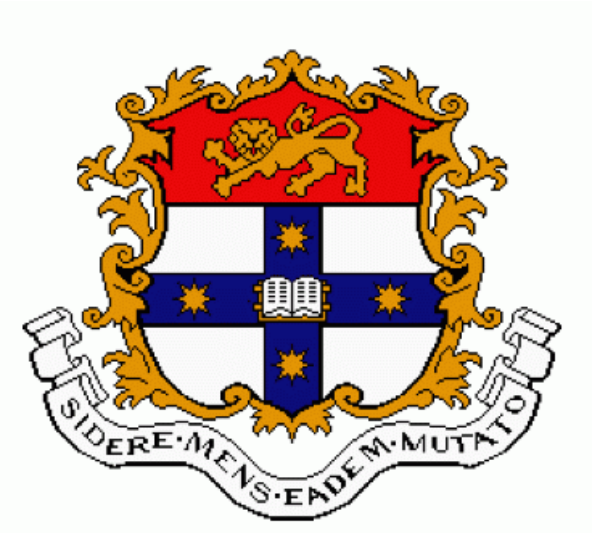

STATEMENT OF DECLARATION

The studies presented in this thesis are the results of original research carried out while the author was enrolled as a candidate for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Sydney. These studies were conducted between September 2011 and December 2014 at the Department of Endocrinology, Discipline of Medicine, The University of Sydney.

All experimental work carried out for this thesis is entirely my own original work except where stated otherwise in the text. No portion of this thesis has been submitted previously for the award of a degree or diploma at any other university.

OVERVIEW

Delayed healing of wounds is a poorly understood condition, which affects 15-30% of all people with diabetes (Hobizal & Wukich, 2012; Mack & Maytin, 2010). This delay in healing is associated with much morbidity and is costly both to the individual and society. The increasing numbers of persons diagnosed with diabetes and the ageing population will further increase the economic and human burden of this complication of diabetes. For this reason understanding the pathophysiology of poor wound healing in diabetes is of critical importance. The wound healing process is complex and is composed of at least three dynamic overlapping stages, which include inflammation, proliferation, and remodelling. During normal wound healing an orderly cellular response to inflammation, cytokines, and inflammatory mediators is required (Falanga, 2005). Impairment in this process such as occurs in diabetic wounds can delay the healing process. The mechanism why wound healing is delayed in diabetes is not fully understood. A number of mechanisms including changes in granulocyte function, alteration in collagen synthesis and accumulation, and altered re-epithelisation and blood vessel formation have been described (Mack & Maytin, 2010; Raja et al., 2007). Persistent inflammation is a characteristic feature of wounds in diabetic subjects (Wetzler et al., 2000), with increased numbers of neutrophils observed in humans and in rodent diabetic wound healing models (McLennan et al., 2008; Rayment et al., 2008; Wetzler et al., 2000). The inflammatory phase is a critical early component of healing and is characterised by an influx of inflammatory cells to the wound site. Neutrophils can be quickly and efficiently mobilised upon inflammation and they constitute the first line of defence against the pathogens. They are essential for the wound healing process; they act as debris scavengers and deliver chemokines to recruit other leukocytes such as

monocytes and lymphocytes to the wound site (Kumar & Sharma, 2010). However due to their toxic contents their persistence in the wound can impair progression from the inflammatory phase to the repair and resolution phases of healing. Neutrophils are also a rich source of matrix metalloproteinases (MMPs), which assist in the migration of monocytes and lymphocytes from the circulation to the wound (Alba-Loureiro et al., 2007; Houreld, 2014; Nishio et al., 2008). On activation the neutrophil degranulates and releases large amounts of pro and active MMPs including Metalloproteinase-9 (MMP-9) (Lin et al., 2005).

MMP-9 is a gelatinase which degrades collagen, elastin and laminin (Chakrabarti & Patel, 2005). It has important roles in wound repair including modulation of growth factor activity, activation of other proteases and it is also implicated in the cleavage of the pro-inflammatory cytokines, such as pro Interleukin-β (IL-1β) to their active forms (Opdenakker et al., 2001). Our previous studies have shown that the wound fluid MMP-9 concentration at presentation can predict future healing (Liu et al., 2009) and we and others have shown that high levels of active MMP-9 correlate with the severity of chronic ulcers (Rayment et al., 2008). Whilst high levels of MMP-9 are associated with delayed healing, MMP-9 is also essential for wound repair as studies in knockout models have also shown delayed healing (Kyriakides et al., 2009). Despite the knowledge that high MMP-9 is associated with delayed healing the mechanism for the increase in MMP-9 is poorly understood.

The expression and activities of MMP-9 are tightly regulated at multiple levels. At the transcriptional level MMP-9 expression is induced by pro-inflammatory cytokines through binding to Activator protein 1(AP-1), Nuclear factor-kappaβ (NF-

κβ) and specificity protein 1(Sp-1) binding sites (McLennan et al., 2007). MMP-9 is synthesised as an inactive pro-zymogen which is activated by other MMPs, plasmin or neutrophil elastase which remove the pro-peptide and cause disruption of the zinc binding site which is essential for its activity. At the post translational level, MMP-9 activities are regulated by Tissue Inhibitor of Metalloproteinase-1(TIMP-1), which binds to the active site and inhibits its degradative activity. In most cell types TIMPs are secreted in 1:1 stoichiometry with the MMPs. The exception to this are neutrophils which produce and secrete MMP-9 in the absence of TIMPs (Opdenakker et al., 2001). The activities of MMP-9 can also be modulated by neutrophil gelatinase associated lipocalin (NGAL) or Lipocalin-2, a 25kda secreted protein that binds covalently with MMP-9 to form an NGAL/MMP-9 complex to stabilise MMP-9 by protecting it from degradation (Chakraborty et al., 2012). NGAL like MMP-9 is stored in the primary granules of neutrophils and is secreted on activation and degranulation. Neutrophils are a major source of NGAL (Bu et al., 2006) and it is also involved in the innate immune response, where it is strongly up-regulated in response to encountering invading bacteria (Grutzner et al., 2004). It has also been reported to play a role in cell homeostasis (Gwira et al., 2005). Whether NGAL expression is altered in wounds and its association with MMP-9 and neutrophils has not been systematically examined in diabetes.

In normal wound healing the highest expression of MMP-9 is seen in the inflammatory phase in association with the influx of neutrophils and monocytes and its expression is decreased as the wound heals (McLennan et al., 2008). Serra et al. have reported increased wound and plasma levels of both NGAL and MMP-9 in persons with chronic ulcers including some diabetic persons (Serra et al., 2013) and

soluble NGAL and MMP-9 levels have been shown to correlate with the clinical course of venous ulcers (Serra et al., 2013). However the effect of diabetes on wound fluid NGAL/MMP-9 complex concentration and its association with overall wound NGAL and MMP-9 levels have received little attention. We hypothesised that the increased activities of MMP-9 in diabetic wounds maybe in part caused by induction of inflammatory cell NGAL and NGAL/MMP-9 concentrations.

Wound tissue and cells obtained from two different wound models were studied. Using a simple excisional model in diabetic rats we showed decreased wound closure and increased expression of MMP-9 and NGAL mainly localised to inflammatory cells (Chapters 3 and 4). To more specifically investigate the effect of diabetes on inflammatory cells we used a sponge implant wound model. In this model PVC sponges ($4x1cm^2$) were subcutaneously implanted. At sacrifice, the sponges were excised and the contained cells and fluids separated for measurement of gene and protein expression. The results of these studies showed, in the absence of change in expression of inflammatory markers of Toll-like receptor 4 (TLR4), Toll-like receptor 2 (TLR2), and Tumour Necrosis Factor alpha (TNFα), diabetes significantly increased NGAL and MMP-8 mRNA and wound fluid NGAL/MMP-9 complex as well as all forms of MMP-9 (all >2 fold, $P<0.05$). Soluble NGAL tended to be lower (Chapters 3 and 4). Interestingly, in wounds in diabetic animals compared to control neutrophil apoptosis as measured by expression of the anti-apoptotic marker MCL-1 is also delayed.

The effects of insulin treatment (Chapter 3) to regulate blood glucose level, or doxycycline (Chapter 4), as a regulator of MMPs, on wound inflammatory cell and

circulating neutrophil NGAL and MMP-9 expression was also investigated. In the excisional wound model insulin treatment prevented the diabetes associated decrease in wound closure. Wound closure was also improved in diabetic animals by treatment with 20mg/kg doxycycline. Insulin treatment also normalised the diabetes related changes in NGAL gene expression and MMP-9 activity. Doxycycline treatment only partially improved NGAL gene expression and had no effect on MMP-9 activity.

Whether diabetes altered circulating neutrophil MMP-9, MMP-8 and NGAL expression was also studied. In the circulation, compared to control, wounding increased the circulating neutrophil number and their activation in diabetic animals. The expression of pro-inflammatory markers of TLR4, TLR2, and TNFα was also increased. Insulin treatment markedly prevented these changes. The same pattern was observed in diabetic group treated with 20 mg/kg doxycycline. In contrast to the pattern seen in the circulating neutrophils obtained from unwounded animals, neutrophil apoptosis was increased in wounded animals.

In vitro studies (Chapter 3) using isolated neutrophils showed that these changes in NGAL and MMP-8 and MMP-9 expression can be induced by glucose treatment suggesting a direct effect of glucose on regulation of MMP-9 activity via NGAL and also on neutrophil apoptosis.

Two small pilot studies in diabetic people were also performed (Chapter 5). The first study showed increased neutrophil activation in those who had a long duration of diabetes but were complications free compared to those of similar durations but had

developed complications. Results from this study showed NGAL was positively associated with neutrophil function. In the second study we examined circulating neutrophil activation and MMP-9 expression in association with wound healing in patients attending the High Risk Foot Service of the Diabetes Centre of RPA Hospital. Results from analysis of samples obtained at the initial visit were compared with those obtained from samples obtained at the last visit. The results from Pilot study 2 showed that decreased MMP-9 expression was related with increased wound closure, indicating the important role of MMP-9 in wound healing. Additionally, the increased expression of NGAL was associated with increased wound healing rate in the first visit. The results of these two small studies suggest increased neutrophil activation in the circulation of diabetic patient in presence or absence of wound. In addition, increased plasma NGAL concentration was associated with increased NGAL/MMP-9 complex in diabetic patients regardless of their complications.

These results from *in vitro* and *in vivo* studies as well as human diabetes suggest that neutrophil activation is altered in diabetes and interestingly is exacerbated by wounding. In addition, in wound tissue and wound inflammatory cells (mainly neutrophils) NGAL levels are increased by diabetes. This increase in NGAL is also associated with an increase in NGAL/MMP-9 complex formation and higher wound fluid MMP-9 activity. Together these observations suggest that in addition to tight blood glucose control from the onset of diabetes therapies targeting NGAL and MMP activities may have utility in diabetic wound healing.

ACKNOWLEDGMENTS

First of all, I would like to express my profound and sincere appreciation to my supervisor, Professor Susan McLennan who opened up a door of opportunities for me to further develop into an independent and self-confident scientist. Her challenging attitude and the great encouragement, support and guidance are of most value for me and made this thesis possible. Many thanks also to her personal advice and friendship whenever I was in dilemma.

I am extremely thankful and indebted to my co-supervisor, Dr. Danqing Min for her valuable comments, generous support and kind encouragement.

I am very also grateful to Professor Stephen Twigg and Associate Prof. Paul Williams for sharing expertise, and insightful comments.

I would also like to express my gratitude and thanks to the all members of the Endocrinology especially, Taria Ng, Surya Sutanto, Auvro Mridha, Sarah Aamidor, Ana Charlton, James Bonner, who have over the years shared knowledge, expertise and friendship and helped me to conduct my research. Thank you to the staff of the study at the Diabetes Centre, Royal Prince Alfred Hospital who assisted with collection of samples for the human studies. I also would like to thank Bosch Institute and histopathology laboratory for the using of the facility and their assistance.

Financial support was provided by International Postgraduate Research Scholarship, Australian Postgraduate Awards and NH&MRC grant.

Finally, I would like to thank my parents Mahmoud and Esmat who lovingly helped me throughout this journey. Special thanks to my husband, Alireza, who patiently assisted and worked beside me, providing me with emotional support. I am very thankful for all the support that I have received throughout my PhD project from my children Kimia and Pouria who steadfastly helped their mother to complete this journey. I would like to thank my sisters Mehri and Soufia, and my brothers Mohammad and Mojtaba for their support and encouragements. These are the moments I will forever remember.

PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

Papers directly related to this work:

Neutrophil Associated Lipocalin-2 (NGAL) is Upregulated and Associates with Increased MMP-9 Activity in an Implant Model of Diabetic Wound Healing

Maryam Abdollahi, Taria Shin Yi Ng, Alireza Rezaeizadeh, Sarah Aamidor, Stephen M. Twigg , Danqing Min, Susan V. McLennan. *Manuscript under review*

Doxycycline Treatment Improves Neutrophil Activation and Wound Healing in Diabetic Animal Models.

Maryam Abdollahi, Taria Shin Yi Ng, Alireza Rezaeizadeh, Sarah Aamidor, Stephen M. Twigg , Danqing Min, Susan V. McLennan. *Manuscript in preparation.*

Diabetes Induced Impairment in Neutrophil Activation: Potential Role in Poor Wound Healing in Diabetes

Maryam Abdollahi, Taria Shin Yi Ng, Alireza Rezaeizadeh, Sarah Aamidor, Stephen M. Twigg , Danqing Min, Susan V. McLennan. *Manuscript in preparation.*

Papers indirectly related to this work:

Connective Tissue Growth Factor, Matrix Regulation, and Diabetic Kidney Disease

McLennan SV, **Abdollahi M**, Twigg SM. Curr Opin Nephrol Hypertens. 2013.

Neutrophil Gelatinase-Associated Lipocalin (NGAL) in Diabetic Kidney Disease

Ernuo Cheng, Alireza Rezaeizadeh, Maryam Abdollahi, Sarah Aamidor, Susan McLennan. *Manuscript under review.*

PRESENTATIONS

Oral presentation at international conferences

Role of Neutrophil Associated Lipocalin-2 (NGAL) in the Increased MMP-9 Activity in Diabetic Wound Healing in Rodents.

Maryam Abdollahi, Taria Ng, Sarah Aamidor, Alireza Rezaeizadeh, Stephen Twigg, Danqing Min, Susan McLennan.

International Diabetes Federation, December 2013, Melbourne, Australia

Oral presentations at national conferences

Increased MMP-9 Activities in A Diabetic Sterile Wound Model are Partially Prevented by Insulin Treatment.

Taria Ng, **Maryam Abdollahi**, Sarah Aamidor, Alireza Rezaeizadeh, Stephen Twigg, Danqing Min, Susan McLennan.

The Annual Scientific Meeting of the Australian Diabetes Society and the Australian Diabetes Educators Association 2014.Australia Diabetes Association (ADS), August 2014. Melbourne, Australia.

Neutrophil Activation is Increased in Diabetes: Possible Role in Poor Wound Healing.

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Inflammation in Disease and Ageing Conference, Sydney 2014. Sydney, Australia.

Association Between Neutrophil Associated Lipocalin-2 (NGAL) and MMP-9 Activity: Possible Role in Poor Wound Healing in Diabetes.

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Doxycycline Treatment Improves Granulation Tissue Quality but Not Wound Closure Rate in Diabetic Animals.

Sarah Aamidor, **Maryam Abdollahi,** , Alireza Rezaeizadeh, Stephen Twigg, Danqing Min, Susan McLennan.

The Annual Scientific Meeting of the Australian Diabetes Society and the Australian Diabetes Educators Association. Australia Diabetes Association (ADS), 2013, Sydney, Australia.

Poster presentations at international conferences

Association between Neutrophil Associated Lipocalin-2 (NGAL) and MMP-9 activity: Possible Role in Poor Wound Healing in Diabetes.

Maryam Abdollahi, Sarah Aamidor, Taria Ng, Alireza Rezaeizadeh, Stephen Twigg ,Danqing Min, Susan McLennan.

American Diabetes Association (ADA), 2014, San Francisco, United States of America.

Poster presentations at national conferences

Neutrophil Activation is Induced by Wounding in Diabetes.

Maryam Abdollahi, Taria Ng, Sarah Aamidor, Alireza Rezaeizadeh, Stephen Twigg ,Danqing Min, Susan McLennan.

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Neutrophil Activation is Induced by Wounding in Diabetes.

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Neutrophil Associated Lipocalin (NGAL) is Increased in Diabetic Rodent Wound Model.

Maryam Abdollahi, Taria Ng, Sarah Aamidor, Alireza Rezaeizadeh, Susan V McLennan.

The Annual Scientific Meeting of the Australian Diabetes Society and the Australian Diabetes Educators Association (ADS), 2012. Gold Coast, Australia.

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AGEs	Advanced glycation end products
AKT	Protein kinase B
AMMP-9	Active-MMP-9
AP-1	Activator protein 1
CON	Control animals with no treatment
CON+HDOX	Control animals treated by oral gavage of doxycycline (100mg/kg)
CON+LDOX	Control animals treated by oral gavage of doxycycline (20mg/kg)
DAG	Diacylglycerol
DM	Diabetic animals with no treatment
DM+INS	Diabetic animals treated with intense dosage of insulin (10 IU daily)
DM+HDOX	Diabetic animals treated by oral gavage of doxycycline (100mg/kg)
DM+LDOX	Diabetic animals treated by oral gavage of doxycycline (20mg/kg)
E	Eosin
ECL	Enhanced Chemiluminescence
ECM	Extra cellular matrix
ELISA	Enzyme-linked immune-Sorbent Assay
ERK1/2	Extracellular signal-regulated kinases
FAK	Focal adhesion kinase
fMLP	N-Formylmethionine-leucyl-phenylalanine
FSC	Forward scatter
G-CSF	Granulocyte colony-stimulating factor
GFAT	Glutamine fructose-6 phosphate amidotransferase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H	Hematoxylin
HBSS	Hanks' balanced salt solution
HMGB1	High mobility group box 1 protein

HRP	Horse radish peroxidase
IGF	Insulin growth factor
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-17A	Interleukin-17A
IL-1β	Interleukin-β
IFN-γ	Interferon gamma
IRS-1	Insulin receptor substrate 1
LPS	Lipopolysaccharide
MCP-1	Macrophage chemoattractant protein-1
MIP-2	Macrophage pro-inflammatory protein-2
MMPs	Matrix metalloproteinases
MMP-2	Metalloproteinases-2
MMP-8	Metalloproteinases-8
MMP-9	Metalloproteinases-9
MAPK	Mitogen-activated protein kinase
NADPH	Nicotinamide adenine dinucleotide phosphate
NAD+	Nicotinamide adenine dinucleotide
NGAL	Neutrophil gelatinase associated lipocalin
NGS	Normal Goat Serum
NF-κB	Nuclear factor-kappaβ
NTC	No template control
PDGF	Platelet-derived growth factor
PKC	Activation of protein kinase
PMMP-9	Pro-MMP-9

pNGAL	Plasma NGAL
PSR	Picro-Sirius Red Stain
RAGE	Advanced glycation end products receptors
RBC	Red blood cell
SDS-PAGE	Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis)
SSC	Side scatter
STZ	Streptozotocin
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween20
TGF-β1	Transforming growth factor beta 1
TIMP	Tissue Inhibitor of Metalloproteinase
TLRs	Toll like receptors
TLR2	Toll like receptor-2
TLR3	Toll like receptor-3
TLR4	Toll like receptor 4
TLR7	Toll like receptor-7
TLR8	Toll like receptor-8
TLR9	Toll like receptor-9
TMMP-9	Total MMP-9
TNFα	Tumor necrosis factor alpha
T1DM	Type 1 diabetes
T2DM	Type 2 diabetes
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

CHAPTER 1: LITERATURE

REVIEW

1.1 **Diabetes Mellitus**

Diabetes is one of the oldest known human diseases. The ancient Egyptians described the clinical symptoms similar to diabetes mellitus 3000 years ago (Rubin, 2007). Diabetes mellitus is a systemic metabolic disorder characterized by elevated blood glucose due to an absolute or relative deficiency of insulin secretion from pancreatic beta cells (Leonardi et al., 2003). It is a disease which is causing serious problems to society health. It is a leading cause of mortality, morbidity, and early disability. The prevalence of diabetes is increasing and has been estimated that from 1995 to 2025 will be increased by 42% among adults living in the developed world and by 170% in adults living in developing countries (Costacou & Mayer-Davis, 2003). This will lead to an increase in the number of people with diabetes worldwide estimated to rise to 366 million by 2030 (Rathmann & Giani, 2004). Diabetes mellitus is also a growing public health concern in Australia. It has been reported that the number of diabetic individuals is rising and there are currently over 1.5 million people with diabetes including those who are undiagnosed (Shaw, 2012). According to estimates two million Australians have pre-diabetes and are at risk of developing diabetes (Health Direct Australia Health Information, 2011). These figures mean that there are approximately eight people with diabetes for every 100 adults. In 2008, a study of Australian children revealed that 5,700 children aged 0-14 years have diabetes. Assuming that new cases of diabetes in 0-14 year old children continues to increase at the rate observed between 2000 and 2008, it is estimated that the prevalence rate will increase by 10% between 2008 and 2013 (Australia's Health 2014).

Diabetes mellitus can be divided into two major types. Type 1 (T1DM) or insulin dependent diabetes mellitus, and Type 2 (T2DM) or non- insulin dependent diabetes mellitus (Mohana et al., 2012). Type 1 diabetes can be diagnosed at any age but frequently is diagnosed from infancy to the late 30s. This type of diabetes is primarily caused by autoimmune pancreatic β cell destruction and is characterized by an absolute insulin deficiency.

Type 2 diabetes mellitus which has been increasing alarmingly worldwide is characterized by reduced insulin secretion in response to glucose and insulin resistance which ultimately leads to the inefficient absorption of glucose into the cell. Insulin resistance is a risk factor for development of diabetes and heart disease. It is present in 90% of the people who are diagnosed with diabetes and affects 18% of the population above 65 years of age (Inzucchi et al., 2015; Mohana et al., 2012).

Despite all treatments for reduction of hyperglycemia such as insulin, metformin, glibenclamide, diabetes is associated with major complications which affect almost all organs including heart, kidney, eyes, liver, and vessels. Another important but less well-known complication of diabetes is impaired wound healing. This complication affects 10-15% of all people with diabetes. Non-healed ulcers often require hospitalisation and in some cases fail to heal and can lead to amputation. This failure to heal precedes 84% of all diabetes-related lower-leg amputation (Brem & Tomic-Canic, 2007) and those who have an amputation are more likely to die within the next 5 years than those with no amputation (Brod, 1998; Nabuurs-Franssen et al., 2005). The prevalence of foot ulcer is greater for diabetic individuals diagnosed at age less than 30 years and it is slightly higher in men than in women.

The prevalence increases with age, especially in diabetic patients diagnosed at age less than 30 years (Palumbo & Melton, 1995; Wu et al., 2007). Despite the prevalence of this condition the pathophysiology of poor wound healing in diabetes is not well understood and an aspect of this is the focus of the research described in this thesis.

1.1.1 **Diagnostic criteria of diabetes mellitus**

Based on the reports from the World Health Organization (WHO) National diabetic group criteria of 2006, in a normal individual, the fasting blood glucose concentration in most mammals is between 4.5-5.5 mmol/l. Irrespective of the type of diabetes, individuals with a fasting blood glucose concentration greater than 7.0 mmol/l (126 mg/dl) or a non-fasting blood glucose concentration of 11.0 mmol/l (200 mg/dl) or higher are considered as having diabetes (Olokoba et al., 2012).

1.1.2 **Metabolic Pathways Altered by Diabetes**

Hyperglycemia causes tissue damage through some main mechanisms including: 1) increased flux of glucose and other sugars through the polyol pathway 2) increased intracellular formation of advanced glycation end products (AGEs) and expression of their receptors 3) activation of protein kinase (PKC) isoforms and 4) increased activity of the hexosamine pathway (Giacco & Brownlee, 2010). Each of these mechanisms will be discussed briefly in the following sections.

1.1.2.1 **Increase polyol pathway flux**

The polyol pathway of glucose metabolism becomes active when intracellular glucose levels are elevated (Lorenzi, 2007). The activity of aldose reductase, a rate

limiting enzyme in the pathway, converts glucose to sorbitol using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Sorbitol is then metabolised to fructose by sorbitol dehydrogenase in a process that uses nicotinamide adenine dinucleotide (NAD+) as a cofactor (Brownlee, 2001; Lorenzi, 2007) shown schematically in Figure 1-1. The polyol pathway activation results in an increase of intracellular sorbitol, and fructose both of which are 10 times more potent glycation agents than glucose. Intracellular accumulation of sorbitol can also result in osmotic stress which damages proteins via oxidation reactions (Forbes & Cooper, 2013). NADPH is required for regeneration of reduced glutathione (Brownlee, 2001). As glutathione functions as a direct free-radical scavenger, as well as a co-substrate for glutathione peroxidase activity, and a cofactor for many other enzymes (Waggiallah & Alzohairy, 2011) these events could induce or impair intracellular oxidative stress (Brownlee, 2001). This increased oxidative stress and production of free radicals results in activation of inflammatory pathways and is thought to be one of the major reasons why diabetes complications including poor wound healing develop and progress (Maritim et al., 2003).

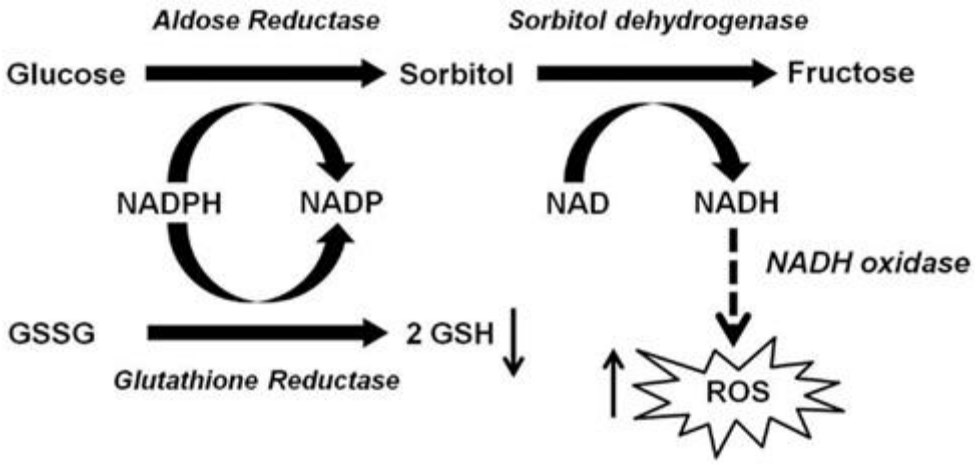

Figure 1-1: Hyperglycemia increases flux through the polyol pathway.
Adapted from (Tang et al., 2012).

1.1.2.2 **Increased formation advanced glycation end-products (AGEs)**

The accumulating metabolites from the polyol pathway result in the formation of AGEs (Lorenzi, 2007). AGEs are formed continuously in the body and the rate of formation are markedly increased in the presence of hyperglycemia and increased oxidant stress (Peppa & Vlassara, 2005). AGEs are formed by the non-enzymatic reaction of glucose and other glycating compounds derived both from glucose and from increased fatty acid oxidation with proteins (Giacco & Brownlee, 2010).

As shown in Figure 1-2, AGEs can modify cells and their matrix. With regard to the matrix, AGE precursors modify matrix to cause them to interact abnormally with other matrix components as well as cell membrane bound molecules such as integrins (Giacco & Brownlee, 2010). Modification of plasma proteins by AGE precursors also creates ligands that bind to AGE receptors (RAGE) and induce changes in gene expression (Brownlee M, 2001). Increased amounts of AGEs are also found in long lived proteins such as collagens which are found in the extracellular matrix. This can accelerate collagen cross-linking which for example contributes to myocardial stiffness in people with diabetes (Candido et al., 2003). AGEs and their receptors negatively affect various aspects of the abnormal inflammatory response in the diabetic wound. AGEs induce the production of oxygen-reactive intermediates from inflammatory and endothelial cells via NADPH activation leading to increased proinflammatory cytokine expression and persistent inflammatory phase in a diabetic wound-healing process (Berlanga et al., 2005). RAGEs are multi-ligand receptors which bind not only to AGEs but bind several ligands to mediate their biologic effects. These ligands include high mobility group box 1 protein (HMGB1), the group of calcium binding cellular factors S100,

amyloid beta peptides and Macrophage-1 antigen (Mac-1) (Mosquera, 2010). RAGE is expressed on several cell types such as monocytes/macrophages (Ohashi et al., 2010) endothelial cells (Pollreisz et al., 2010), fibroblasts (Liu et al., 2010), and keratinocytes (Zhu et al., 2012). Ligation of RAGE by AGEs can directly induce generation of reactive oxygen species (ROS) and the up-regulation of inflammatory pathways and activate the NF-κB, causing multiple pathological changes in gene expression (Giacco & Brownlee, 2010) ultimately leading to inflammation (Mosquera, 2010) and diabetic complications (Bierhaus et al., 2001). RAGEs are highly expressed in a number of diabetic tissues, including blood vessels, atherosclerotic lesions, infected periodontal tissue, and glomeruli (Tanji et al., 2000). Blockade of RAGE restores physiological migration of inflammatory cells (Goova et al., 2001). Induction of type IV collagen by AGEs is mediated by RAGE can be blocked in cells by anti-RAGE ribozyme (Tsuji et al., 1998). Blockage of RAGE has also been shown to suppress levels of TNFα, interleukin-6, and MMPs-2, -3, and-9 (Goova et al., 2001). Many strategies have been tried to either impair AGE formation or to decrease cross-linking of longer-lived proteins. These strategies have not been pursued, as they have been largely ineffective.

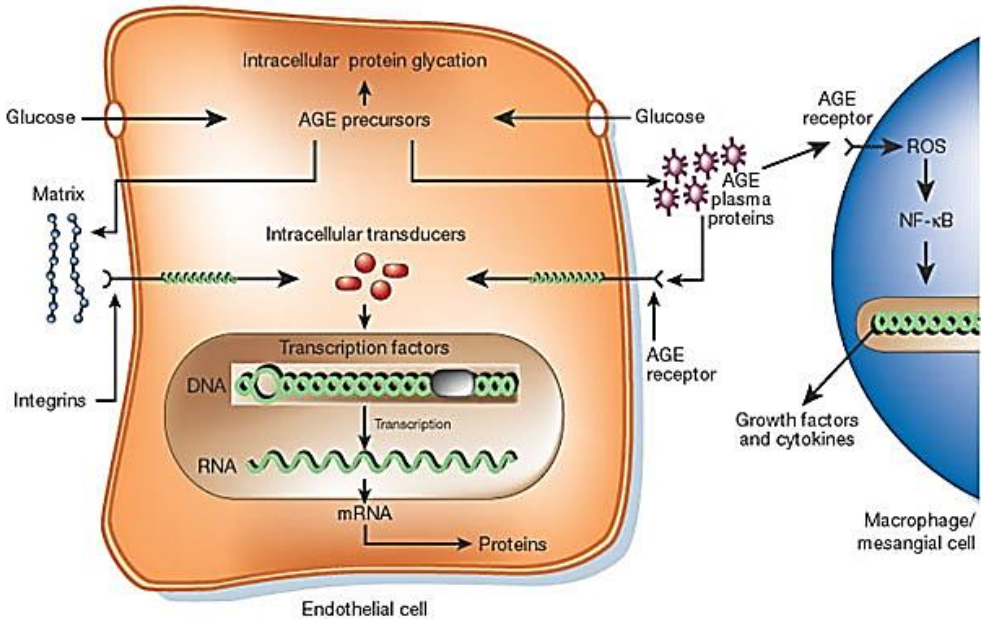

Figure 1-2: Increased production of AGE precursors and its pathologic consequences. *Covalent modification of intracellular proteins by dicarbonyl AGE precursors alters several cellular functions. Modification of extracellular matrix proteins causes abnormal interactions with other matrix proteins and with integrins. Modification of plasma proteins by AGE precursors creates ligands that bind to AGE receptors, inducing changes in gene expression in endothelial cells, mesangial cells and macrophages. Adapted from(Brownlee, 2001)*

1.1.2.3 **Activation of protein kinase C (PKC)**

PKC is a family of protein kinase enzymes which is comprised of a group of related serine/threonine kinases involved in controlling the function of other proteins (Koya, 2014; Mochly-Rosen et al., 2012). PKC is a calcium and phospholipid dependent enzyme which is activated by a variety of signals including increases in the concentration of diacylglycerol (DAG) or calcium ions. Therefore; PKC enzymes play important roles in several signal transduction cascades. With regard to diabetic complications they have an important role in microvascular complications in particular retinopathy, nephropathy, and neuropathy (Mochly-Rosen et al., 2012). As shown in Figure 1-3 activation of PKC contributes to increased microvascular

matrix protein accumulation by induction of expression of Transforming Growth Factor beta 1(TGF-β1), fibronectin and type IV collagen (Brownlee, 2001; Koya et al., 1997). This activation causes a variety of pathologies involved in the development of diabetic complications such as cytokine activation and inhibition, vascular alterations, abnormal angiogenesis, increased risk of insulin resistance, and glucose intolerance (Mochly-Rosen et al., 2012).

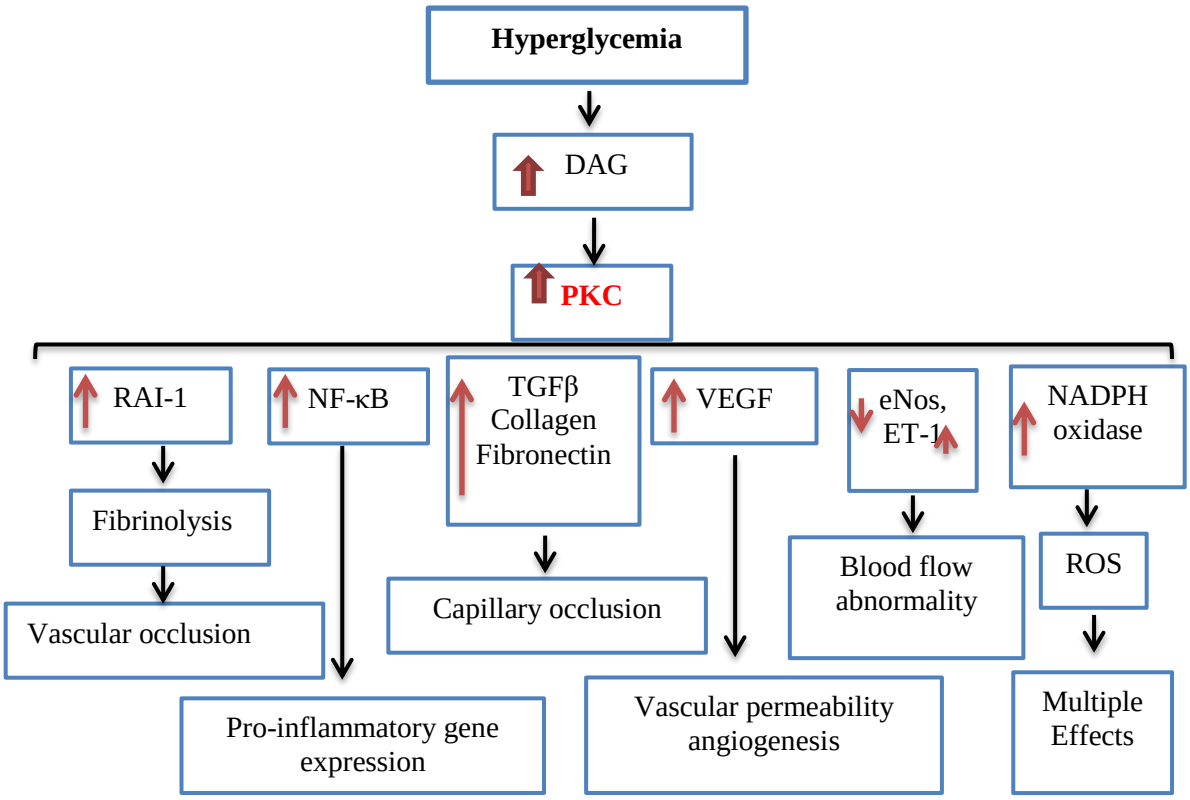

Figure 1-3: Consequences of hyperglycemia-induced activation of PKC.
Hyperglycaemia increases diacylglycerol (DAG) content, which activates PKC, primarily the β- and δ-isoforms. Activation of PKC has a number of pathogenic consequences by affecting expression of endothelial nitric oxide synthetase (eNOS), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and plasminogen activator inhibitor-1 (PAI-1), and by activating NF-κB and NAD(P)H oxidases. Adapted from (Brownlee, 2001).

1.1.2.4 **Increased hexosamine pathway activity**

In the hyperglycemic milieu most of glucose is metabolized through the glycolysis pathway. As shown in Figure 1-4 if glucose is in excess some fructose-6-phosphate is diverted into a signaling pathway and converted to glucosamine-6-phosphate and uridine diphosphate (UDP) N-acetyl glucosamine by glutamine fructose-6 phosphate amidotransferase (GFAT) (Brownlee, 2001). In aortic endothelial cells hyperglycemia can activate the hexosamine pathway via its effect on mitochondrial superoxide (Du et al., 2000). In mesangial cells flux of glucose through this pathway can to increase TGF-β and lead to increased matrix production (Kolm-Litty et al., 1998).

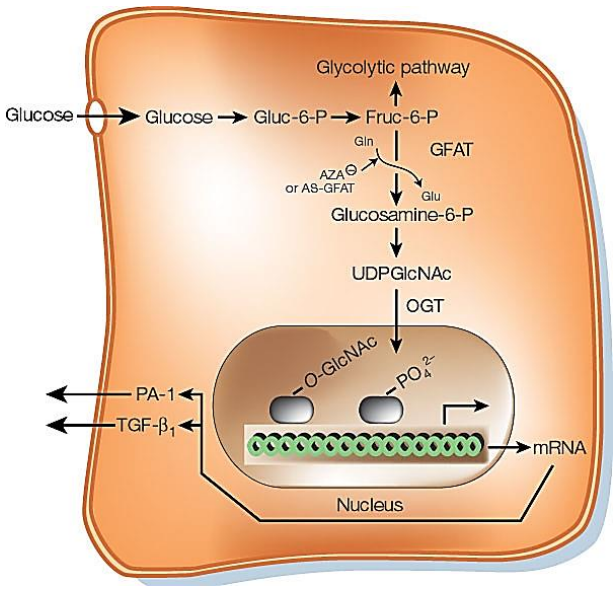

Figure 1-4: Hyperglycemia increases flux through the hexosamine pathway.
The glycolytic intermediate fructose-6-phosphate (Fruc-6-P) is converted to glucosamine-6-phosphate by the enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT). Intracellular glycosylation by the addition of N-acetylglucosamine (GlcNAc) to serine and threonine is catalysed by the enzyme O-GlcNAc transferase (OGT). Increased donation of GlcNAc moieties to serine and threonine residues of transcription factors such as Sp1, often at phosphorylation sites, increases the production of factors as PAI-1 and TGF-beta1. AZA, azaserine; AS-GFAT, antisense to GFAT. Adapted from Brownlee, 2001).

1.1.3 **Complications Associated with Diabetes**

With the frequency and occurrence of diabetes increasing, so are the diabetes related complications. Despite a variety of treatments to improve blood glucose control, chronic hyperglycemia is associated with impaired lipid and lipoprotein metabolism, increased oxidative stress, inflammation, vascular endothelial dysfunction and hypertension (Forbes & Cooper, 2013). As mentioned these metabolic changes can lead to the development of long-term pathogenic conditions such as micro vascular complications, involving damage to small blood vessels, and macro vascular, damage to the arteries (Forbes & Cooper, 2013; Mogensen, 2000). These pathological changes contribute to an increased risk for development of chronic disease such as atherosclerosis, coronary heart disease, stroke, peripheral vascular disease, hypertension (Selvin et al., 2006) eye disease, and kidney failure (Forbes & Cooper, 2013). Diabetic patients also have higher risk of poor wound healing and limb amputation. Neuropathy, vasculopathy and immunopathy are all implicated in the poor healing of diabetic foot ulcers (Hobizal & Wukich, 2012). Other risk factors like infection, smoking and ischemia also contribute to impaired wound healing ability in diabetic foot ulcers (Leung, 2007). As the focus of the work described in this thesis is poor wound healing, factors such as neuropathy and peripheral vascular disease which are associated with delayed wound healing in diabetes will be discussed in more detail in the following sections.

1.1.3.1 **Diabetic neuropathy**

Neuropathy or poor nerve function occurs in both types of diabetes and is characterised by changes to cellular and non-cellular components of the nervous system. Neuropathy affects the central and peripheral nervous system and is more

common with increasing age and longer duration of diabetes (Farmer et al., 2012). Ischemia as a result of poor blood supply has been implicated in the pathophysiology of diabetic neuropathies and it results in axon loss and demyelination of nerves in these subjects. In addition to direct effects on the nerve, ischemia affects the growth of blood vessels which can also impair nerve fibres function (Levin, 2002). Irrespective of the cause, ischemic neuropathy is dominated by pain or sensory loss and weakness in peripheral nerves (Armstrong et al., 1998; Hirsch et al., 2001).

Most diabetic patients have painful neuropathic symptoms and loss of sensation. This loss of sensation can result in a lack of recognition of trauma. This can then contribute to progressive worsening of the injury and by this mechanism contribute to poor wound healing (Clayton & Elasy, 2009). Painful neuropathy occurs in the 16-34% of people with diabetes and the prevalence of this type of neuropathy is greater in those with T2DM (Abbott et al., 2011). Several of the previously listed mechanisms are involved in the development of diabetic neuropathy. Including increased activity of the polyol pathway, formation of AGEs, activation of PKC, enhanced modification of proteins with N-acetylglucosamine via the hexosamine pathway. Other factors such as increased inflammation, and a reduction in neurotrophic factors are also involved (Farmer et al., 2012).

Pro-inflammatory cytokines produced from lymphocytes, macrophage, neurons, and Schwann cells can all cause damage to peripheral nerves (Hur et al., 2011). In diabetic nerves an increased inflammatory response has been reported to lead to excessive injury to the axons and Schwann cell via activation of downstream

pathways such as NF-κB (Wang et al., 2006). Proteases, among them the MMPs, have also emerged as important modulators of non-neuronal pain pathways. Amongst the MMPs cellular expression of MMP-9 and MMP-2 is involved in generation and maintenance of neuropathic pain through degenerative and pro-inflammatory mechanisms (Zhang et al., 2011).The MMPs stimulate the generation of chemokines and cytokines (Cross et al., 1999) such as IL-β and TNF-α, that in turn activate chemokine and cytokine receptors on microglia and astrocytes. Activation of these receptors stimulates MAPK and NF-κB pathways to finally up-regulate the synthesis of IL-β, TNFα, and Interleukin-6 (IL-6) in the microglia (Ji & Suter, 2007). After nerve damage, the Schwann cells release MMP-9, initiating macrophage infiltration and degradation of myelin basic protein (Kobayashi et al., 2008). Inhibition of MMP-9 activity has been shown to reduce neuropathic pain without systemic effects (Kawasaki et al., 2008).

1.1.3.2 **Peripheral vascular disease**

Peripheral vascular disease means blockage of the larger arteries in the thigh and leg causing a reduction in blood circulation and in particular limb ischemia. It can occur in individuals without diabetes but is more common and more severe in people with diabetes (Jude et al., 2010) and is increased by age, duration of diabetes, and presence of peripheral neuropathy. The most common symptom of peripheral vascular disease is cramping, or aching in the thighs, or buttocks that appears reproducibly with walking exercise and is relieved by rest (Sheehan, 2004). As mentioned poor blood supply can exacerbate neuropathy and in addition can decrease flow of important cells to and from the wound site to either commence repair or assist with remodelling.

1.1.4 **The Normal Wound Repair Process**

To understand the mechanisms involved in poor wound healing, it is helpful to first review the normal response to injury. The pathways involved in each of the three distinct but overlapping phases of the normal wound healing response will be discussed in more detail in the following section.

As shown in Figure 1-5 wound healing is a complicated process involving communication and interaction between multiple cell types including fibroblasts, endothelial cells, keratinocytes, inflammatory cells, and the extracellular matrix (Whelan et al., 2005). In normal physiological conditions the wound healing process can be divided into 3 overlapping but distinct phases including: hemostasis and inflammation, proliferation/matrix deposition, and the remodelling phase. Within these three broad phases is a complex and coordinated series of events that includes chemotaxis, phagocytosis, production of new collagen and glycosaminoglycans and proteoglycans, proteolytic degradation of ECM as well as collagen remodelling and re-epithelialization (Masre et al.; Velnar et al., 2009). Disruption of the sequence of these interactions can impair the orderly wound healing process resulting in a delay in healing (Whelan et al., 2005). Many factors can interfere with this process some of these which are particularly relevant to those with diabetes are inflammatory disease, poor blood flow and age (Guo & DiPietro, 2010).

Wounds, irrespective of their cause can be categorised as acute or chronic based on the timeliness of healing. Acute wounds include surgical incisions and traumatic injuries such as lacerations, abrasions, avulsions, penetrations or bites, and burn injuries. Acute wounds normally precede through an orderly and timely reparative

process which results in sustained restoration of anatomic and functional integrity (Nicks et al., 2010). In contrast chronic wounds such as: diabetic foot ulcers, venous leg ulcers, and pressure ulcers do not heal in a predictable time frame (Nicks et al., 2010). The most common causes of chronic wounds are vascular insufficiency, diabetes mellitus, and local-pressure effects (Eming et al., 2014). Compared with acute wounds, chronic wounds contains higher levels of pro-inflammatory cytokines such as IL-1β and TNFα (Tarnuzzer & Schultz, 1996) and lower levels of growth factors including TGF-β, Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF) (Schonfelder et al., 2005). This imbalance between tissue deposition stimulated by growth factors, and tissue destruction mediated by proteases is thought to lead to impaired wound healing (Cullen et al., 2002). Another factor which can impair wound healing is the presence of bacteria (Guo & DiPietro, 2010). Delay caused by bacterial presence is likely due to the release of lipopolysaccharides present in membranes of gram-negative bacteria. Lipopolysaccaharides have been shown to reduce migration of keratinocytes across the wound surface and thus delay wound closure (Thamm et al., 2013).

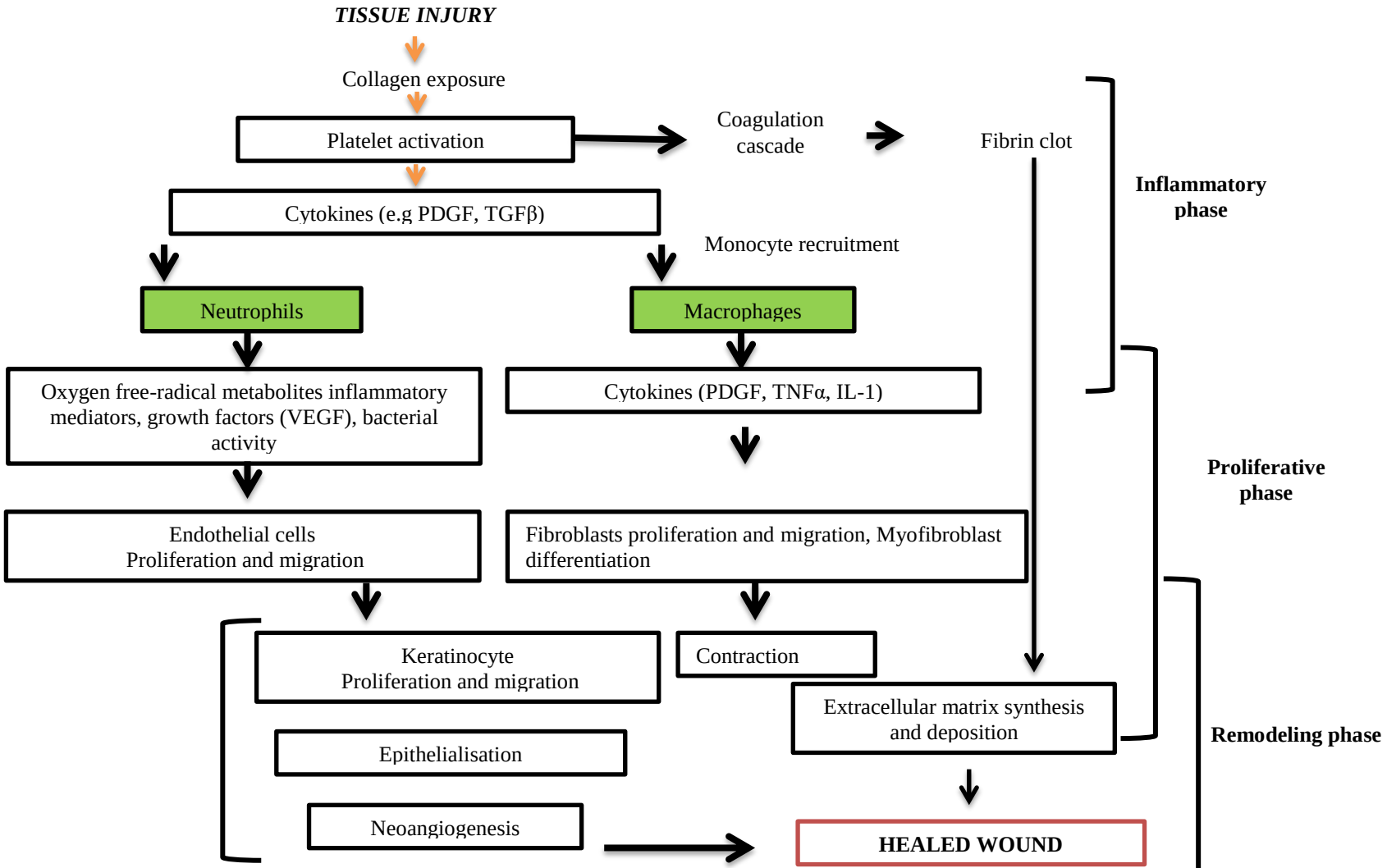

Figure 1-5: A schematic diagram of normal wound healing (Mendes et al. 2012)

1.1.4.1 **The inflammatory phase of healing**

Within minutes after the initial injury, platelets invade the wound area and form a clot by releasing thrombin, which cleaves fibrinogen and forms a cross linked network of fibrin fibres which covers the wound area and protects it from further external injury (Wolberg, 2007). During the beginning of the inflammatory phase i.e. within 24-36 hours of injury, neutrophils which mediate the first line of defence, enter the wound area (Eming et al., 2007). They produce a variety of growth factors including VEGF which can promote re-vascularisation and tissue repair (Dovi et al., 2004). As neutrophils play a key role in inflammation and wound healing and are studied in detail in this work, neutrophil structure and function and their role in wound healing will be discussed in more detail in a later section (section 1.1.6).

The next inflammatory cell type that enters the wound are the macrophages, which produce initially pro-inflammatory and after some delay anti-inflammatory cytokines (Rodero & Khosrotehrani, 2010). They are attracted to the site of inflammation by chemotactic factors such as migration inhibitory factor, lymphocyte-derived chemotactic factor, macrophage activating factor, and macrophage stimulatory protein. Once in the wound, macrophages play a role in host defence, the promotion and resolution of inflammation, the removal of apoptotic cells, and the support of cell proliferation and tissue restoration (Koh & DiPietro, 2011). The phagocytosis of apoptotic neutrophils or other cells induces an anti-inflammatory phenotype in the macrophage. At the same time the macrophages release TGF-β and reduce their production of pro-inflammatory mediators such as TNF-α (Thomson et al., 2010). As a result in healing wounds the macrophages present can be a mixture of M1-like macrophages which are pro-inflammatory and

are characterised by the production of TNF-α, Interleukin-1 (IL-1) and IL-6 or M2-like macrophages, which secrete fewer pro-inflammatory cytokines and assist in tissue repair and are characterised by their expression of IL-10 (Koh & DiPietro, 2011). After the inflammatory cells enter the wound the deposition of granulation tissue commences with the appearance of fibroblasts and endothelial cells and the formation of capillaries in the wounded area (Eming et al., 2007).

1.1.4.2 **The proliferative phase of wound healing**

The proliferative phase starts approximately 3 days after wounding and continues for about 2 weeks. In the proliferative phase, re-epithelialization which involves the proliferation and migration of epithelial cells across the wound surface commences (Gary Sibbald & Woo, 2008). This phase is also marked by the formation of granulation tissue which includes a variety of cell types like inflammatory cells, fibroblasts and endothelial cells. New blood vessel formation commences and a matrix of fibronectin, collagen, glycosaminoglycans, and proteoglycans is laid down. The fibroblasts and myofibroblasts migrate into wound site in response to factors such as TGF-β and PDGF secreted or released by inflammatory cells and platelets (Goldman, 2004). MMPs are secreted by a number of cell types including inflammatory cells, endothelial cells and fibroblasts. The increase in MMPs enables and facilitates cell migration, proliferation and angiogenesis and they can also modify inflammatory reactions (Baum & Arpey, 2005).

As mentioned the growth of new blood vessels occurs at this stage. This process results in greater blood flow to the wound and therefore, increased perfusion of factors necessary to heal the wound. This process is stimulated by macrophage

activity as well as the tissue hypoxia resulting from the disruption of blood flow at the time of injury (Oike et al., 2004). The increase in MMP expression is stimulated by inflammatory cytokines such as TNF-α and interleukins (Siasos et al., 2012). MMPs also induce the release of ECM bound pro-angiogenic factors, including the release of VEGF from endothelial cells (Mazor et al., 2013) a process which also stimulates capillary growth (Mazor et al., 2013; Ucuzian et al., 2010).

1.1.4.3 **The remodelling and maturation phase of wound healing**

Following robust proliferation and ECM synthesis, wound healing enters the final remodelling and maturation phase at about 21 days after injury. This phase can last for many months, or even years in complex wounds (Flanagan, 2000). This is the final phase and it occurs once the wound has closed. It is responsible for the development of new epithelium and final scar tissue formation (Takeo et al., 2015). It involves remodelling of collagen from the less highly cross linked type III collagen to the more highly crosslinked type I collagen (Ramasastry, 2005). Fibronectin gradually disappears and hyaluronic acid and glycosaminoglycans are replaced by proteoglycans. Cellular activity reduces and the numbers of blood vessels in the wounded area regress and decrease (Ramasastry, 2005). One critical feature of the this phase is the remodelling of the ECM to an architecture that approaches that of the normal tissue (Campos et al., 2008).

As mentioned previously, chronic wounds are caused by multiple factors in particular pressure sores, diabetic ulcers, and venous ulcers which are responsible for 70% to 90% of all leg ulcer cases (Mustoe, 2004). The focus of the work described in this thesis is impaired wound healing in diabetes and for this reason

factors known to be involved in poor wound healing in diabetes will be discussed in more detail in the following section.

1.1.5 **Diabetes and Poor Wound Healing**

Insufficient angiogenesis (Lim et al., 2015) disturbances in collagen metabolism and deposition (Lee et al., 2015) and decreased immune function (Takeo et al., 2015) as well as increased bacterial load all contribute to the delay in healing in diabetes (Xu et al., 2007). In addition to these factors which affect cellular function, diabetes specific changes in nerve function, peripheral blood supply, callus formation and excessive pressure to the site can all exacerbate the problem (Falanga, 2005).

As mentioned (Section 1.1.2) the diabetic milieu affects multiple cellular pathways with downstream effects on cellular function. Of importance to this work is the effect of diabetes on inflammatory cell function in particular neutrophils. Diabetes is known to cause defective T-cell immunity, defects in leukocyte chemotaxis, phagocytosis, and bactericidal capacity (Eming et al., 2014). With regard to wound healing, wounds in diabetic patients show decreased early inflammatory cell infiltration but increased numbers of neutrophils and macrophages in the later stages (Wetzler et al., 2000). The initial inflammatory phase is associated with immediate and rapid infiltration of neutrophils into the wound. Their main function at the wound site involves protection against micro-organisms and destruction of contaminating bacteria by phagocytosis (Flanagan, 2000). Clinical investigations of circulating neutrophil from diabetic patients and experimental studies using diabetic rats and mice have clearly demonstrated a deficiency in neutrophil activation and function including neutrophil phagocytosis (Lin et al., 2006), adhesion to the

endothelium and migration to the inflammatory sites (Chanchamroen et al., 2009), production of pro-inflammatory cytokines (Alba-Loureiro et al., 2007) and bactericidal activity (Kewcharoenwong et al., 2013).

In addition to alterations in neutrophil function diabetes can also impair macrophage function. In particular, in normal wounds the M1 macrophage phase is shorter than the phase with M2 macrophages. This is important as the switch from the M1 to M2 phenotype, characterised by increased delivery of TGF-β, promotes granulation tissue formation angiogenesis, and re-epithelialization. In diabetic wounds there is prolonged M1 macrophage presence, which delays influx of M2 macrophages and ultimately healing (Khanna et al., 2010). For example, in non-healing diabetic wound tissues, increased pro-inflammatory cytokines such as TNFα and Interleukin-6 (IL-6) and decreased anti-inflammatory cytokines such as IL-10 are observed (Khanna et al., 2010; Wetzler et al., 2000). Whether this delay in progression of macrophage phenotype from a pro-inflammatory to an anti-inflammatory phenotype is due to altered neutrophil function in diabetic wounds is not known.

The increased level of pro-inflammatory cytokines has downstream effects to further exacerbate the pro-inflammatory environment by phosphorylation of inhibitor κB to cause translocation of NF-κB to the nucleus. Intracellular NF-κB can then induce the transcription of pro-inflammatory cytokines, adhesion molecules, chemokines and enzymes generating ROS (Dandona et al., 2002). These changes lead to sustained expression of chemokines such as Chemokine Ligand 2 (CCL2) that can lead to prolonged infiltration of leukocytes during impaired healing in diabetes (Wetzler et al., 2000).

Increased activation of the Toll like receptor (TLR) pathway is another factor which can contribute to poor wound healing. Persistent activation of TLRs is associated with increased levels of cytokines such as IL-1, IL-8 which can contribute to the non-healing of venous leg ulcers (Dunne et al., 2011).TLRs are present on immune cells such as macrophages, neutrophils, and dendritic cells. TLRs-1–6 recognise bacterial and fungal components on the cell surface whereas TLR3 recognises intracellular viral double-stranded RNA, viral single-stranded RNA are recognised by TLR7 and TLR8, and microbial DNA by TLR9 (Dasu & Isseroff, 2012). Increased expression of TLR2 mRNA and protein and TLR4 has been described in diabetic wounds and are thought to contribute to the prolonged inflammation. TLR2 absence is associated with reduced inflammation and improved wound healing (Dasu et al., 2010) and increased TLR4 has been shown to alter microvascular leakage and leukocyte adhesion (Breslin et al., 2008).

1.1.6 **Neutrophils and Wound Healing**

1.1.6.1 **Neutrophil structure and function**

Neutrophils are short-lived leukocytes which undergo rapid programmed cell death both *in vitro* and *in vivo*. The number of neutrophils varies across the species and is affected by age. For example in humans approximately 50-70% of blood cells are neutrophils whereas in mice this number is 10-25% (Mestas & Hughes, 2004). In addition to being different in number the circulatory half-life of neutrophils also varies between species. It is difficult to exactly measure the half-life of neutrophils and as a result it is controversial with some reports suggesting 1.5 hours in mice and 8 hours in human (Galli et al., 2011) and others 12.5 hours for mice and up to 5.4

days for humans (Pillay et al., 2010). Nevertheless, the half-life for mice neutrophil is less than that observed for human neutrophils.

Neutrophils are produced within the hematopoietic cords interspersed within the venous sinuses of the bone marrow at a rate of roughly 1×10^9 cells per kilogram body weight per day (Borregaard, 2010). Their proliferation is driven by multiple factors including Granulocyte Colony Stimulating factor (G-CSF), which is produced in response to Interleukin-17A synthesized by T cells (Ley et al., 2006).

Shown in Figure 1-6 neutrophil precursors originate in the bone marrow and undergo differentiation in the blood. During maturation, neutrophils progress through several stages including; promyelocyte, neutrophilic myelocyte, neutrophilic metamyelocyte and finally to a segmented or polymorphonuclear neutrophil (Kolaczkowska & Kubes, 2013; Nathan, 2006).

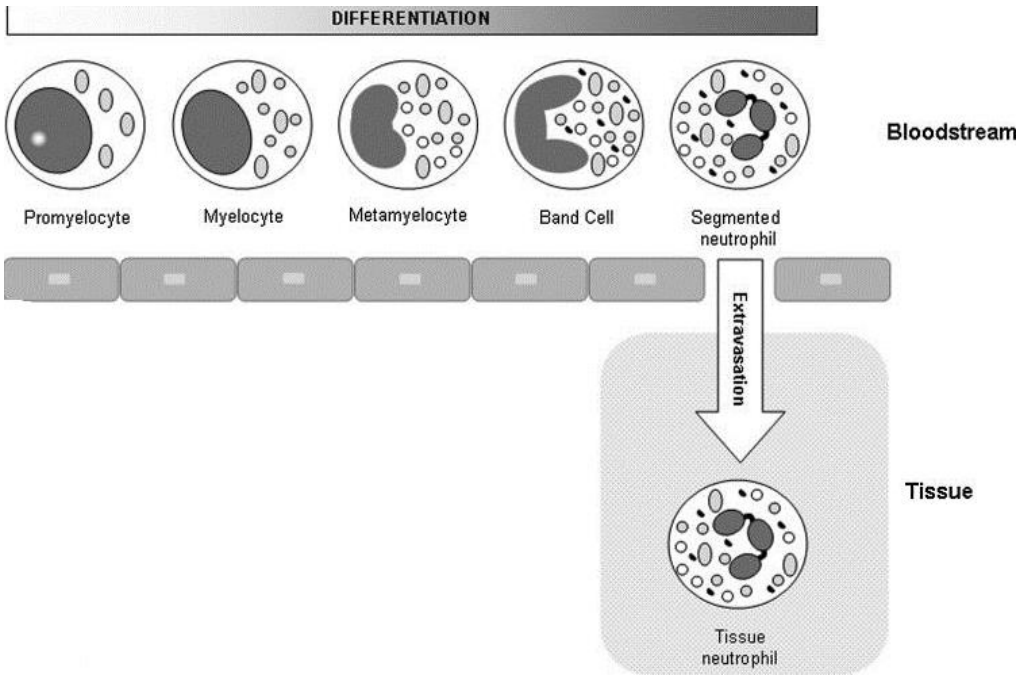

Figure 1-6: Neutrophil maturation and granule formation.
Adapted from (Wilgus et al., 2013)

Neutrophils are markedly different to other inflammatory cells as they contain a number of granules. These granules, in addition to contributing to their distinctive morphologic appearance, are critical for the neutrophil's inflammatory functions. The neutrophil granules can be classified on the basis of their size, morphology, electron density or presence of characteristic granule proteins. According to a review by Borregaard & Cowland, 1997 there are three types of granules and one secretory vesicle which are formed sequentially during the neutrophil granulocytic differentiation process (Kolaczkowska & Kubes, 2013). All granule subsets share common structural features such as a phospholipid bilayer membrane and an intragranular matrix which contains proteins destined for exocytosis or delivery to the phagosome (Faurschou & Borregaard, 2003).

The peroxidase-positive or primary granules azurophilic granules are large, round, and electron dense. They contain toxic mediators such as Myeloperoxidase (MPO), elastase, lysozyme, cathepsin G and acid hydrolases. This type of granule is formed in the promyelocytes (Hager et al., 2010). They are a source of enzymes for digestive and bactericidal functions and supply MPO to the MPO-halide-hydrogen peroxide bactericidal system. These enzymes also regulate inflammation by degrading inflammatory products (Faurschou & Borregaard, 2003).

Peroxidase-negative or secondary/specific granules are smaller than primary granules and are electron lucent. They contain collagenase, gelatinase, NGAL, lactoferin and lysosomes. The specific granules also appear to be necessary for neutrophil recruitment to sites of inflammation, for upregulation of receptors,

important in the control of chemotaxis and the respiratory burst, for bactericidal activity, and for chemoattractant generation (Falloon & Gallin, 1986).

Gelatinases are also contained in gelatinase or tertiary granules that also considered being peroxidase-negative granules. These granules are defined by their high concentration of gelatinase.

Secretory vesicles constitute a reservoir of membrane-associated receptors needed at the earliest phases of the neutrophil-mediated inflammatory response. Their membranes are rich in the β2-integrin CD11b/CD18 which was discovered in a systematic search for organelles that could explain the extensive and rapid upregulation of CD11b ⁄ CD18 in response to stimulation with fMLP or other chemoattractants (Hager et al., 2010). Shown in Table 1-1 are the contents of the different types of neutrophil granules (Borregaard & Cowland, 1997). Of particular relevance to the work described in this thesis, NGAL is located in the specific granules, whilst MMP-9 is located in both specific and gelatinase granules.

Table 1-1: the content of the different types of neutrophil granules

Azurophic granules	Specific granules	Gelatinase granules	Secretory vesicles
Membrane components:	**Membrane components:**	**Membrane components:**	**Membrane components**
CD63	CD11b	CD11b	Alkaline phosphatase
CD68	CD66	Cytochromeb	CD11b
V-typeH-ATPase	CD67	fMLP− R	CD14
	fMLP− R	VAMP-2	CD16
Matrix components:	Fibronectin-R		fMLP-R
	G-protein α-subunit	**Matrix components:**	CD10, CD13, CD45
MPO			
Lysosome	**Matrix components:**	Gelatinase (MMP-9)	**Matrix components:**
Heparin binding protein		Lysosome	
	Collagenase	Plasminogen activator	Plasma proteins including tetranectin
Low molecular weight cationic proteins	Gelatinase (MMP-9)		
	NGAL		
Acid hydroease	Histaminase		
Neutral protease	Heparanase		
Elastase	Lysosome		
Myeloperoxidase	Lactoferrin		
Proteinase			

Adapted from (Borregaard & Cowland, 1997)

As mentioned previously neutrophil apoptosis is not only important for neutrophil production but also increasingly recognised to play a critical role in neutrophil function (Scannell et al., 2007). During neutrophil apoptosis, increased expression of apoptosis markers such as annexin mark the cell for phagocytosis by macrophages, an action which prevents dispersal of their toxic contents (Li et al., 2003). Additionally, the apoptosis of neutrophils is essential to wound repair as it stimulates macrophages to become more anti-inflammatory and to secrete TGF-β1 which is important for myofibroblast differentiation and formation of ECM (Eming et al., 2007). Additionally, neutrophil apoptosis can inhibit the activities of activated neutrophils (Koedel et al., 2009).

1.1.6.2 **Neutrophils and inflammation**

Neutrophils play an important role in the innate immune response and are the first leukocyte to arrive at the inflammatory site. They have a special role in host defence, immune regulation, and in the regulation of inflammation (Houreld, 2014). The migration of neutrophils from the circulation to the site of inflammation is controlled by their interactions with the vascular endothelium. During rolling under inflammatory conditions, neutrophils are activated by ligation of G-protein coupled receptors with chemokines. They also receive signals via the engagement of adhesion molecules including the selectins, a family of cell adhesion molecules, and β2 integrin (Ley, 2002). MMPs such as MMP-9 and MMP-8 are also induced in response to inflammation (Nissinen & Kahari, 2014).They assist neutrophil migration from the blood stream across basement membrane to the site of inflammation (Lin et al., 2008) by degradation of ECM. MMP driven proteolysis can also affect the biological functions of chemokines and cytokines. Shedding and

release of TNFα and activation of pro-IL-β are examples of how MMPs might influence an inflammatory reaction by modulating cytokines (Lin et al., 2008). Other proteases such as elastase also contribute to this process by cleaving the pro-gelatinase to its biologically active form (Delclaux et al., 1996).

As shown in Figure 1-7 activation of neutrophils in response to inflammation increases their longevity by several fold (Colotta et al., 1992; Summers et al., 2010). Increased levels of ROS, acute phase proteins, pro-inflammatory cytokines, and fatty acids can all prime neutrophils (Hatanaka et al., 2003). This priming process ensures the presence of neutrophils at the site of inflammation and contributes on the one hand to the regulation of host resistance and resolution of inflammation. On the other hand as discussed earlier persistence of neutrophil in the tissue may lead to cell injury by activation of neutrophil and increased secretion of cytokines such as interleukins (IL-1, IL-6, IL-8, IL-12, IL-10), TNFα and interferon gamma (IFN-γ) (Cowburn et al., 2005). Cytokines such as IL-1β, TNFα, IFN-γ, G-CSF, Granulocyte-macrophage colony-stimulating factor (GM-CSF), and bacterial products have been shown to prolong neutrophil survival by inhibition of apoptosis (Colotta et al., 1992; Kolaczkowska & Kubes, 2013).

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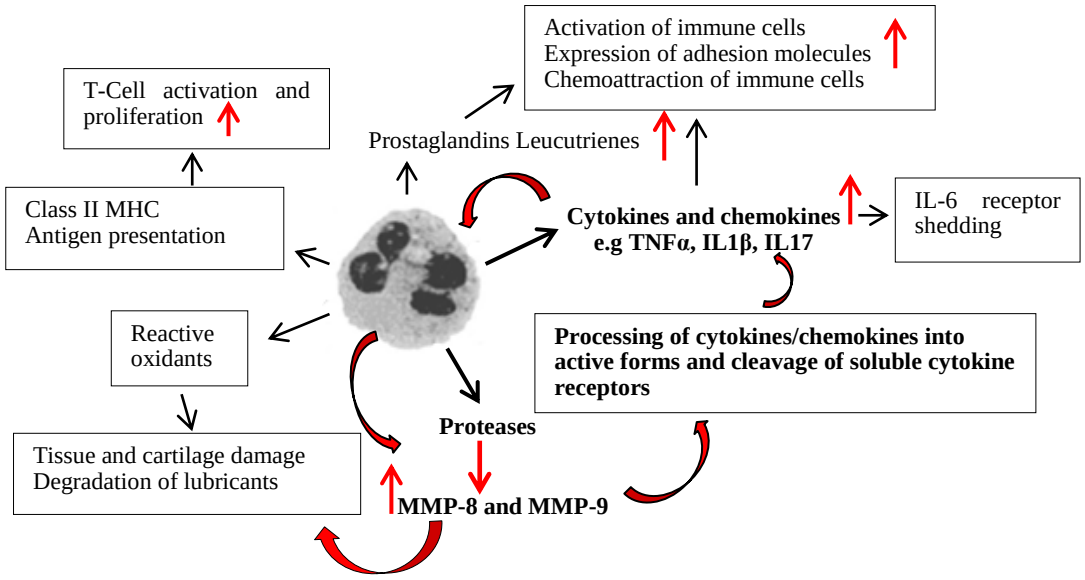

Figure 1-7: Neutrophils and inflammation
Resting neutrophils can become primed by agents that include bacterial products and cytokines or chemokines, e.g. TNF-a and primed neutrophils are then mobilized to the site of infection or inflammation. Activated neutrophils produce MMP-8 and MMP-9 and consequently increase destructive functions of MMPs.

Whether these processes occur in neutrophils in diabetes is not certain. Some studies have shown that a high glucose environment can increase neutrophil apoptosis (Peleg et al., 2007). Whilst other studies investigating the effect of high glucose concentration in combination with exposure to Lipopolysaccharide (LPS) have shown a reduction in the number of apoptotic neutrophils (Tennenberg et al., 1999). In these later studies of neutrophils obtained from diabetic patients, LPS induced the production of the anti-apoptotic cytokines IL-8, IL-10 (Glowacka et al., 2002). Another study has shown that decreased glutamine utilisation can contribute to an increased rate of apoptosis in neutrophils from diabetic patients (Alba-Loureiro et al., 2007). Interestingly, and of relevance to this work the neutrophils used in these studies are circulating neutrophils obtained from people with diabetes but who do

not have a wound. Whether wound presence affects circulating neutrophil profile and additionally whether neutrophils obtained from a wound environment are altered in diabetes has not as yet been studied.

1.1.6.3 **Neutrophils and delayed wound healing in diabetes**

Neutrophils are essential for the wound healing process because they act as a wound site scavenger but as described above excessive neutrophils or persistence of neutrophils can contribute to a delay in wound healing (Dovi et al., 2003; Falanga, 2005). Neutrophil persistence has been shown to delay wound closure by inhibiting re-epithelialisation (Dovi et al., 2003). In that study wound closure was shown to progress at a significantly faster rate in neutrophil-depleted diabetic mice than animals that were not neutrophil depleted (Dovi et al., 2003). Persistent inflammatory cells particularly neutrophils is one the feature of diabetic wounds (Casqueiro et al., 2012; McLennan et al., 2008). The mechanism of this effect is uncertain but sustained expression of Macrophage Pro-Inflammatory Proten-2 (MIP-2) and Macrophage Chemoattractant Protein-1 (MCP-1) during the late phase of wound healing in the diabetes is involved in the persistence of both neutrophils and macrophages (Wetzler et al., 2000). In the diabetic condition persistence of neutrophils may lead to increased wound pro-inflammatory cytokines, for example IL-8, IL-1β, and TNFα (Hatanaka et al., 2006). The excessive production of these cytokines leads to reduced neutrophil clearance (Hanses et al., 2011), inappropriate activation and consequently tissue damage (Hatanaka et al., 2006).

As mentioned in section 1.1.6.1 neutrophil granules contain a variety of proteases. Neutrophil proteases are responsible for the modulatory effects of neutrophils

(Gresnigt et al., 2012). For instance, elastase contributes to the regulation of inflammation and the breakdown of ECM proteins including collagen-IV and elastin fibronectin, laminin, and vitronectin (Dovi et al., 2003; Weinrauch et al., 2002). Neutrophils also contain MPO, a peroxidase enzyme, which can oxidatively activate MMP-8 and MMP-9 and consequently increase the destructive function of the MMPs (Nizam et al., 2014; Sorsa et al., 2006). A common feature of all poorly healing wounds is increased MMP content. This increase can contribute to impaired keratinocyte migration (Dovi et al., 2003). Previous studies in my laboratory have shown increased initial MMPs in particular MMP-9 which can predict future poor healing of wounds in persons with diabetes (Liu et al., 2009). The mechanism of this effect is not fully understood and will be investigated in the work described in this thesis.

1.1.7 **Matrix Metalloproteinases Structure and Function**

As discussed in preceding sections the wound healing process involves high levels of extracellular proteolytic activity which is mediated primarily by the MMPs, serine proteinases and cysteine proteinases. There is considerable evidence that shows that increased MMP activity is associated with poor healing of chronic wounds and future delayed healing in people with diabetes. Additionally, the expression of the TIMPs is decreased in chronic wounds and as MMP activity is regulated by TIMPs this altered balance between MMPs and TIMPs also likely contributes to poor healing. In addition to regulation by TIMPs the expression and activity of MMPs can be regulated by other factors. Interestingly, neutrophils are different to other cell types and they do not express TIMPs, suggesting that MMP activities in this cell type are regulated in a different manner. The next section will discuss MMPs and

their role in wound healing including in poorly healing wounds in those with diabetes.

The MMPs also referred to as matrixins, are a large family of at least 24 endopeptidases whose activities depend on metal ions, such as Zn^{+2} and Ca^{+2}. They play a key role in normal physiology including in connective tissue formation during development, morphogenesis and healing (Klein & Bischoff, 2011). Based on domain organisation and substrates the MMPs are classified into the following groups: collagenases (MMP-1, -8, -13, -18), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), and a heterogeneous group containing matrilysins (MMP-7), metallo elastase (MMP-26) and epilysin (MMP-28). Another class of MMPs are the membrane-type MMPs or MT-MMPs which consist of MMPs-14, -15, -16, -17, -24, -25) (Marco et al., 2013; Nagase et al., 2006). As Figure 1-8 shows MMPs share specific functional and structural components. Most contain a pro-peptide domain of approximately 80 amino acids, a metalloproteinase domain of about 170 amino acids, a linker peptide of variable lengths, and a hemopexin domain of about 200 amino acids (Nagase et al., 2006). The pro-peptide domain contains a conserved cysteine residue that links to the catalytic zinc site and sustains the latency of the MMP. The hemopexin like C-terminal domain is linked to the catalytic domain by a short hinge region and domain binds endogenous TIMPs (Bauvois, 2012). The hemopexin domain is also essential for the collagenolytic activity of collagenases. The catalytic domains of MMPs can hydrolyse non-collagenous proteins but they cannot cleave triple helical collagens without the hemopexin domain (Clark & Cawston, 1989). The gelatinases including MMP-2 and MMP-9 are different from the other MMPs. They contain three repeats of the fibronectin type II motif in the

metalloproteinase domain which facilitates their binding to gelatin and collagen (Klein & Bischoff, 2011).

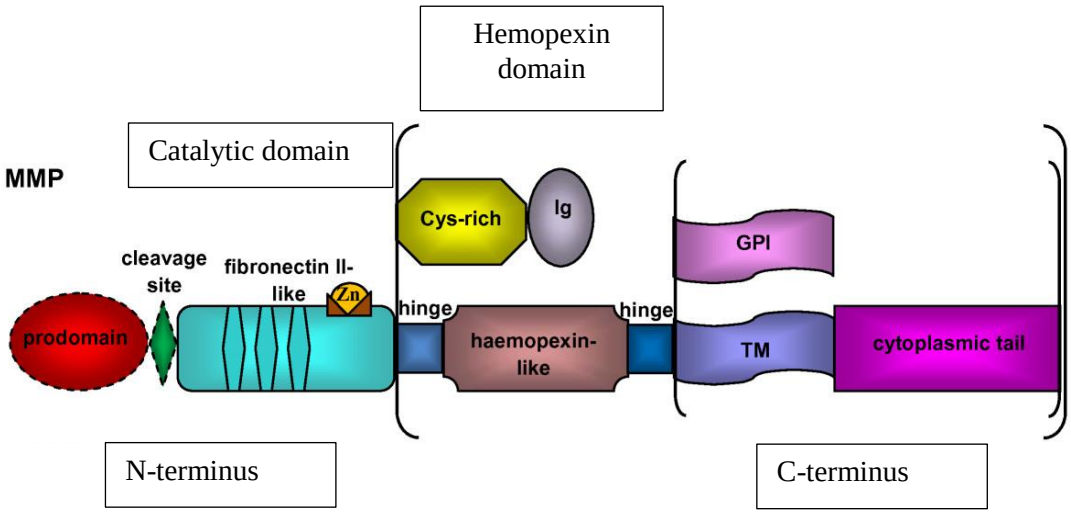

Figure 1-8: Basic structure of MMPs.
The typical structure of MMP is made of a prodomain, a furin cleavage site (all MT-MMPs, MMP-21, -23, and -28), a catalytic metalloproteinase domain with fibronectin type II repeats (MMP-2, MMP-9), a linker peptide and a haemopexin domain (except for MMP-7, -26, and -23), a linker peptide, a transmembrane domain and cytoplasmic tail (MMP-14, -15, -16, -24) or glycosylphosphatidylinositol (GPI) anchor (MMP-17, -25). MMP-23 bears C-terminal cysteine-rich (Cys-rich) and Ig-like (Ig) domains and its propeptide lacks a cystein switch motif. Adapted from (Paulissen et al., 2009)

Most MMPs are secreted as inactive zymogens; they need to be activated usually by proteolytic cleavage of their NH2-terminal pro-domains (Woessner et al., 1991). They are processed to their active forms via several mechanisms all of which lead to disruption of the cysteine switch and cleavage of the pro-peptide. They can be activated by tissue or plasma proteinases by proteolytic cleavage of their NH_2-terminal pro-domains. This proteolytic removal of the prodomain by other serine proteases such as plasmin and furin is one of the most common mechanisms of

activation. Other mechanisms of activation include activation by other MMPs (Klein & Bischoff, 2011). One example is the role played by MT1-MMP in activation of MMP-2 (Brew et al., 2000; Rundhaug, 2003). For activation of MMP-2 by this mechanism pro-MMP-2 forms a complex with MT1-MMP and paradoxically TIMP-2.

As mentioned the activities of MMPs are inhibited by TIMPs, which are secreted by most cell types except neutrophils. There are four family members: TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Brew et al., 2000). All TIMPs have two distinct domains, an N-terminal domain of about 125 amino acid residues and a C-terminal domain with about 65 residues; the conformation of each domain is stabilised by disulphide bonds. The structure of the N-terminal region is highly conserved, it binds to the active site of the MMPs in such a way that the conserved N-terminal Cysteine 1 of the TIMP lies above the catalytic $Zn^{2}+$ of the MMPs (Murphy, 2011). The C-terminal also make some connections with the protease, but these are not important contributors to complex formation (Brew & Nagase, 2010).

All four TIMPs inhibit MMPs but structural studies of TIMP–MMP complexes have explained some differences in inhibitory properties between the different TIMPs. For example, TIMP-1 has inhibitory actions against most MMPs and it has a relatively low affinity for the MT-MMPs, MMP-14, MMP-16, MMP- 24, and MMP-19 (Baker et al., 2002; Brew & Nagase, 2010). Additionally, TIMP-2 and TIMP-3 are weaker inhibitors than TIMP-1 for MMP-3 and MMP-7. The interaction between TIMPs and the various pro-MMPs is relatively specific: TIMP-2, TIMP-3

and TIMP-4 can interact with pro-MMP-2 and TIMP-1 and TIMP-3 can interact with pro-MMP-9 (Brew et al., 2000; Brew & Nagase, 2010; Hamze et al., 2007).

Apart from activation, MMP activities are regulated at the level of transcription. A number of different signalling pathways have been implicated in this process including activation of MAPK, NF-κB or Smad-dependent pathways by growth factors/cytokines, activation of Focal Adhesion Kinase (FAK) by integrin activation, or activation of β-catenin signalling by cadherins (type-1 transmembrane proteins) (Martins et al., 2013).

The main physiological function of the MMPs is the modulation and regulation of ECM quantity (Galis & Khatri, 2002). They selectively degrade most ECM components including collagen, fibronectin, laminins and proteoglycans. The MMPs are also increasingly recognised as having roles in the release and activation of biologically active proteins. Some of these are growth factors such as Insulin-Like Growth (IGF) (Nakamura et al., 2005), VEGF (Bergers et al., 2000) and TGF-β (Yu & Stamenkovic, 2000). Cytokines such as IL-1β are also activated by MMPs (Schonbeck et al., 1998). They can also cleave cell surface proteins such as cytokine receptors, and cell adhesion molecules (Bauvois, 2012; Klein & Bischoff, 2011).

1.1.7.1 **Matrix metalloproteinases in wound healing**

The MMPs have many roles in wound repair being involved in each step of the process, that is inflammation, granulation tissue formation, angiogenesis, wound remodelling and maturation (Muller-Quernheim, 2011). Most cell types involved in wound repair including fibroblasts, keratinocytes, and endothelial cells express the

MMPs. They are also expressed by inflammatory cells such as neutrophils, monocytes/macrophage and lymphocytes (Martins et al., 2013). In general MMPs are constitutively expressed and their synthesis is up regulated by growth factors such as Epidermal Growth Factors (EGF), Keratinocyte Growth Factor (KGF), Fibroblast Growth Factor (FGF), VEGF, PDGF, Hepatocyte Growth Factor (HGF), and TGF-β. These growth factors have all been reported to transcriptionally activate MMPs (Martins et al., 2013). The pro-inflammatory cytokines TNF-α and IL-1α are also known to be potent inducers of both MMPs and TIMPs (McCarty & Percival, 2013) although this effect appears to be concentration dependant. In the low concentration, TNF-α can increase TIMP production and regulate MMP activities whereas higher concentrations have been reported to stimulate the production of MMPs and suppress the production of TIMPs -1, -2, and -3 (Medina et al., 2005).

As discussed above MMPs are activated by the removal of the pro-peptide fragment and this form of MMP can facilitate activation of pathways leading to the regeneration of injured tissues. This activation includes pathways which can increase migration of neutrophils to the wound area and activation of TGF-β to induce granulation tissue formation (McCarty & Percival, 2013). These activation pathways also contribute to the regulation of barrier function, inflammatory cytokines, as well as chemokine activity and gradients, resolution of infection, and tissue repair (Lagente & Boichot, 2010). Controlled expression of MMPs is important to the progression of the normal wound healing process (Martins et al., 2013) but elevated and prolonged expression of MMPs disrupts this balance. The increased MMPs can lead to degradation of ECM components, persistence activation of cytokines and degradation of growth factors. Together these factors have all been associated with

impaired healing (Schultz & Wysocki, 2009). Whether persistence of MMPs leads directly to poor wound healing or is a consequence of persistently high levels of wound pro-inflammatory cytokines is not clear. The high cytokines may induce increased expression of MMPs (Mott & Werb, 2004) and down regulate TIMP expression to collectively cause an environment consistent with an excess of MMPs activity (McCarty & Percival, 2013). This excess MMP activity can subsequently destroy growth factors, receptors, and matrix proteins essential for initiation of the wound healing process (Lobmann et al., 2006). In the diabetic environment gene expression and protein levels of MMP-1, MMP-2, MMP-8, and MMP-9 have all been shown to be increased (Lobmann et al., 2002). On the other hand the level of TIMP-2 is decreased (Rysz et al., 2007). Together these changes support the contention that impairment of wound healing in diabetic ulcers is due to prolonged inflammation and an increase in the proteolytic wound environment (Stechmiller et al., 2006; Weckroth et al., 1996). The biological activities mediated by MMPs during wound healing are shown in Table 1-2.

Table 1-2: The roles of MMPs in wound healing

Biological effect	**Responsible MMPs**
Increase cell migration, proliferation, Keratinocyte migration and reepithelialisation	MMP-1
Mesenchymal cell differentiation with inflammatory phenotype, epithelial cell migration, enhanced collagen affinity, increased bioavailability of TGF-β	MMP-2
Generation of angiostatin-like fragment, enhanced collagen affinity, increased bioavailability of TGF-β	MMP-3
Require for wound closure and generation of antimicrobial activity, Promotes neutrophil activation	MMP-7
Mainly expressed by neutrophils promotes cutaneous wound healing	MMP-8
Generation of angiostatin-like fragment, enhanced collagen affinity, pro-inflammatory, promotes cell migration and re-epithelialisation	MMP-9
Expressed by the keratinocyte at the edge of the wound	MMP-10
Macrophage specific, potential regulator of angiogenesis	MMP-12
Promotes keratinocyte migration and invasion, regulate epithelial cell proliferation	MMP-14
Widely expressed in many organs including proliferating keratinocytes in healing wounds	MMP-19
Expressed by migrating keratinocytes during cutaneous wound healing	MMP-26
Expression is associated with cell proliferation during epithelial repair	MMP-28

Adapted from (Martins et al., 2013; Nagase et al., 2006).

The work described in this thesis focuses on one of the MMPs, the gelatinase called MMP-9. This MMP is of interest as our group has previously shown that its activity is increased in wound fluids in diabetic wounds and that its level can provide information about future wound healing rate in diabetic wounds (Liu et al., 2009). MMP-9 has been shown to directly delay wound healing by interference with re-epithelialisation (Reiss et al., 2010). Unlike most of the other MMPs, which are constitutively expressed, MMP-9 expression is induced in a variety of cell types in response to inflammatory stimuli (Martins et al., 2013).

As previously described (Section 1.1.4) and shown in Figure 1-9 acute wound healing proceeds through several overlapping phases that involve an inflammatory response and associated cellular migration, proliferation, matrix deposition, and tissue remodelling (Nicks et al., 2010). Interruption or disruption of one or more of these phases leads to failure of the wound to heal i.e. a chronic wound. In people with diabetes, some wounds heal albeit at a slower rate than in those without diabetes. In others despite best care the wounds are trapped in a destructive and ongoing cycle in the inflammatory stage. This persistence of inflammation is a common feature of diabetic wounds and can lead to an increase in the number of inflammatory cells in particular neutrophils and macrophages. These inflammatory cells can be induced to increase MMP expression and their presence is likely responsible for the persistence of MMPs (Martins et al., 2013) .

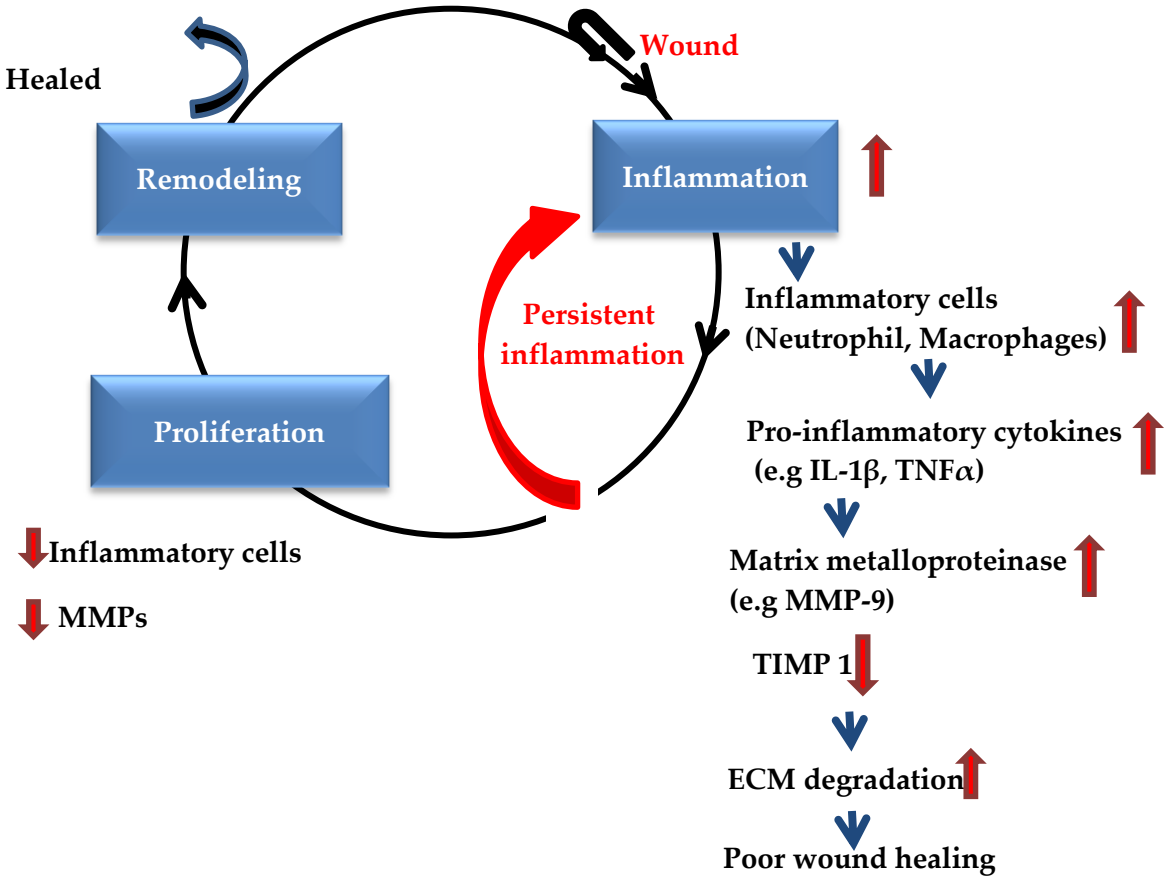

Figure 1-9: Increased level of MMPs in chronic wounds.
The inflammatory phase of healing is exaggerated in chronic wounds leading to an excess of proteases and inflammatory cytokines released by neutrophils and macrophages. Excess of MMPs degrade ECM components and causes tissue damage and poor wound healing.

1.1.7.2 **MMP-9 structure and activity**

Shown in Figure 1-10 at the transcriptional level, MMP-9 is positively regulated by multiple factors, including E-26 (Ets) transcription factors, NF-κB, Polyomavirus Enhancer A-binding protein-3 (PEA3), Activator Protein-1 (AP-1), specificity protein 1 (Sp-1), and Serum Amyloid A-activating Factor (SAF)-1 (Crawford & Matrisian, 1996). MMP-9 is secreted as inactive zymogen (proMMP-9) with a molecular weight of 92 kDa. Cleavage of the prodomain yields the biologically active form of MMP-9 with a molecular weight of 82 kDa (Bauvois, 2012). The pro form of MMP-9 can be activated by a number of proteases including plasmin, trypsin-2, MMP-2, MMP-13, MMP-3, serine elastase and kallikrein (Klein & Bischoff, 2011). Inhibition of MMP-9 is performed by TIMPs binding to the pro forms of the enzyme. TIMP-1 binds to pro-MMP-9, in addition to inhibiting its active form (Brew & H. Nagase, 2010).

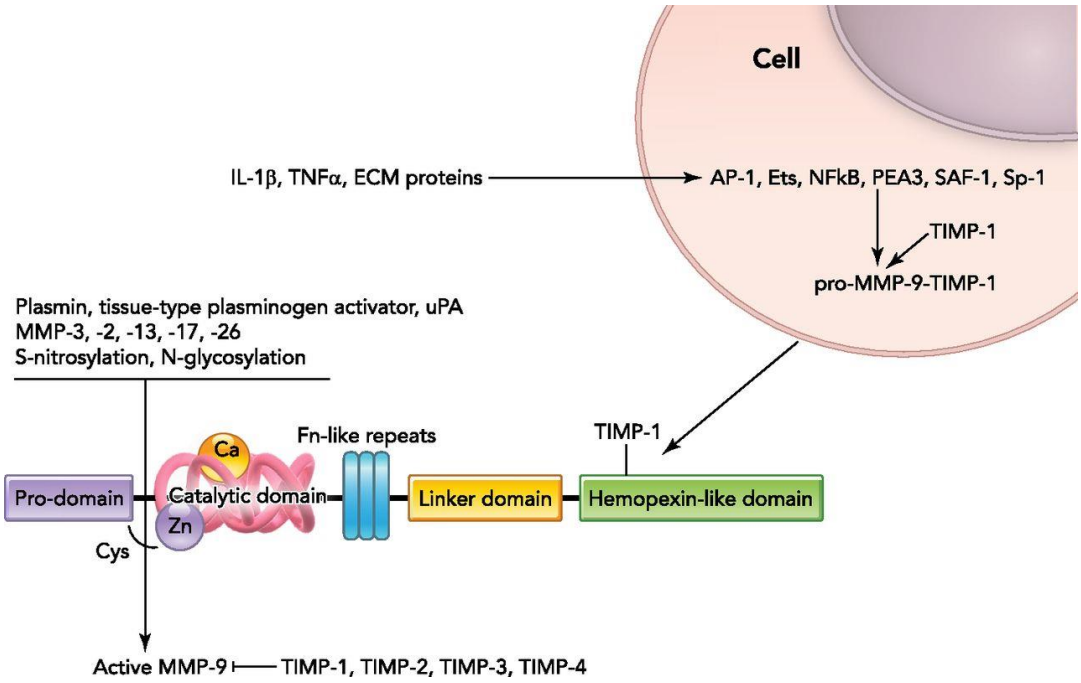

Figure 1-10: MMP-9 structure and factors regulating MMP-9 transcription and translation.
Ets, E-26 transcription factor; NF-κB, nuclear factor κB; PEA-3, polyomavirus enhancer A-binding protein-3; AP-1, activator protein 1; SAF-1, serum amyloid A-activating factor 1; HSP60, heat shock protein 60; TIMP, tissue inhibitor of metalloproteinase; uPA, urokinase plasminogen activator. Adapted from (Yabluchanskiy et al., 2013)

MMP-9 is produced and secreted by a number of different cell types including neutrophils, macrophages, monocytes, fibroblasts, and keratinocytes (Cullen et al., 2002). In neutrophils, MMP-9 is synthesised during the late stage maturation process in the bone marrow and thereafter is stored in the specific and gelatinase granules. MMP-9 is constitutively expressed by mononuclear cells and keratinocytes (Fang et al., 2010) and is an important factor in the remodelling and re-epithelialisation of wounds (Rowe & Weiss, 2008). Increased levels of MMP-9 have been shown to inhibit keratinocyte migration, attachment, and re-establishment of the epidermis (Reiss et al., 2010). In wound fluids high levels of MMP-9 correlate

with the severity of the ulcer (Rayment et al., 2008) and as mentioned our group has shown that wound fluid MMP-9 can predict future poor wound healing of diabetic foot ulcers (Liu et al., 2009). The wound fluid MMP-9 is thought to come mainly from neutrophils or macrophages as these are the main cells present in the fluids (Cullen et al., 2002).

One of the important heteromeric complexs of MMP-9 isolated from neutrophils is a complex between proMMP-9 and neutrophil gelatinase associated lipocalin-2 or NGAL. This composite is a 125 KDa disulphide-bonded complex between cysteine residue 87 (C87) of NGAL and an unidentified cysteine in residue in the hemopexin-like C-terminal domain of MMP-9 (Bouchet & Bauvois, 2014). This formation of the NGAL/proMMP-9 complex precedes the formation of a ternary complex with TIMP-1 which is mediated by the C-terminal of proMMP-9 and the non-inhibitory C-terminal of TIMP-1. When the free N-terminal of TIMP-1 in the NGAL/MMP-9/TIMP-1 complex is activated, a quaternary complex is generated which upon activation of proMMP-9 exhibits a sixfold higher proteolytic activity than the active ternary complex (Kolkenbrock et al., 1996). Interestingly, MMP-9 secreted by neutrophils is different from that secreted by other cells. It is TIMP free and is often covalently associated with NGAL. This special feature of MMP-9 secreted by neutrophils and the ability of the NGAL/MMP9 complex to form extracellulary a complex with TIMP-1 explains its high specific activity when compared to MMP-9 from other cell types (Hibbs et al., 1985; Kjeldsen et al., 1993). In addition to delivering a more highly active MMP-9 the degradation of MMP-9 is significantly inhibited in the presence of NGAL. Together these changes result in the preservation of MMP-9 enzymatic activity and suggest a potential regulatory

role for NGAL in the modulation of MMP-9 activity (Yan et al., 2001). The presence of NGAL-MMP-9 complex has been observed in a number of different diseases in particular cancer (Bouchet & Bauvois, 2014) and diabetic nephropathy (Thrailkill et al., 2010) and its detection in tissues such as plasma, urine, saliva, and tissue lysates has been suggested as a marker of disease (Thrailkill et al., 2010). Whilst these functions of NGAL in regulation of MMP-9 activity are known, little is known regarding its level in wound fluids and its association with poor wound healing.

1.1.8 **Neutrophil Gelatinase a Member of the Lipocalin Family**

The Lipocalin protein family is a large group of small extracellular proteins which fulfil a variety of different functions including roles in the regulation of the immune reaction and the mediation of cell homoeostasis (Flower et al., 2000). They are characterised by their ability to bind small mainly hydrophobic molecules such as retinol, their binding to specific cell-surface receptors and their formation of macro-molecular complexes (Flower et al., 2000). One of these complexes includes the complex formed between NGAL and MMP-9. NGAL is encoded by the LCN2 gene (Chakraborty et al., 2011) and was originally isolated from the specific granules of human neutrophils (Kjeldsen et al., 1993).

The calculated structure of Lipocalins shows NGAL to be a 178-residue protein with a lipocalin fold as depicted in Figure 1-11. The protein sequence has been shown to share similarity with the amino acid sequences of the rat α_2-microglobulin-related protein and the mouse 24p3 protein (Coles et al., 1999; Kjeldsen et al., 1994) with highest degree of similarity 63.5 and 62% identity, respectively. Additionally it is

highly similar to the homologue present in chimpanzees with about a 98% identity (Chakraborty et al., 2011).

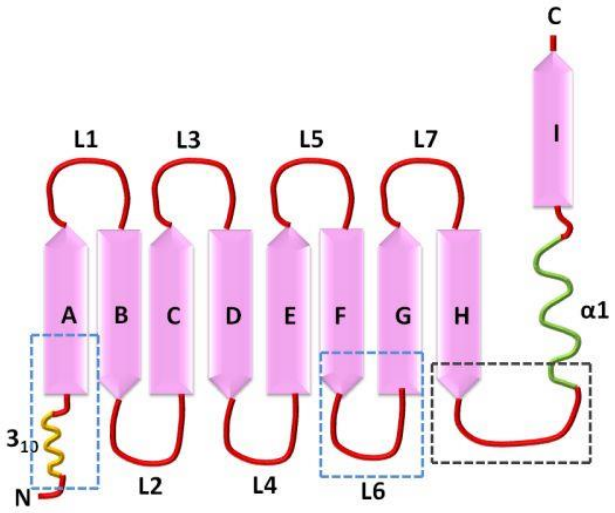

Figure 1-11: Schematic representation of the lipocalin fold.
The characteristic feature of lipocalins is the "lipocalin fold" which comprises of an N-terminal 3-10 helix. This domain which is responsible for binding of lipocalins to their ligands is structurally comprised of an eight stranded β barrel with its loops running in an antiparallel direction. The eighth beta sheet is connected to an alpha helix (α1), which is in turn connected to a C-terminal beta sheet. The beta sheets are connected by loops (L1-L7). Loops L1, L3, L5 and L7 form the open end of the molecule (i.e. the opening to the ligand binding site of NGAL). Adapted from (Chakraborty et al., 2011)

NGAL is resistant to degradation and under non-reducing conditions exist as a 25 kDa monomer, a 45 kDa disulphide-linked homodimer, a 75kDa trimeric NGAL complex, and as mentioned also covalently bound to MMP-9 as a 125 kDa heterodimeric form shown schemmatically in Figure 1-12. This covalent complex can form *in vitro* and studies mixing MMP-9 with NGAL have generated MMP activities with two different molecular sizes of 125 kDa and 115 kDa. These represent complexes of MMP-9 with the monomeric and the dimeric forms of NGAL, respectively (Yan et al., 2001).

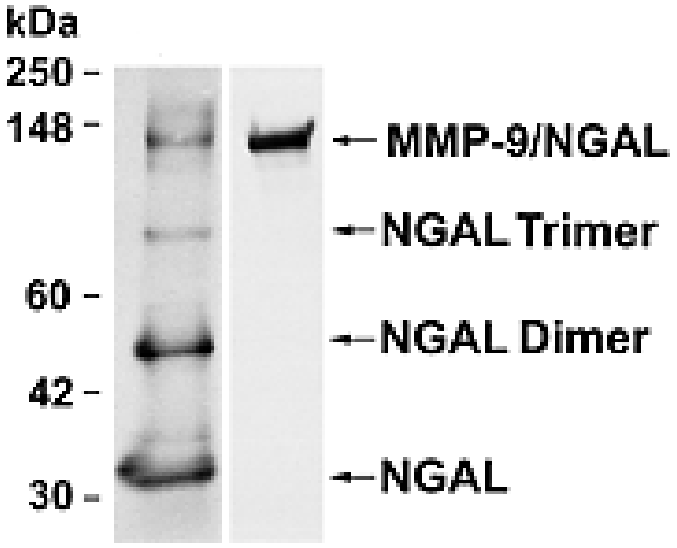

Figure 1-12: A representative sample containing different forms of NGAL
~125-kDa purified human neutrophil NGAL/MMP-9 complex as well as NGAL in monomeric, dimeric, and trimeric forms. Adapted from (Yan et al., 2001)

NGAL is secreted by activated neutrophils and it is also expressed albeit at very low levels by macrophages. Its expression can be induced in epithelial cells, and keratinocytes during inflammation (Borregaard et al., 2005). NGAL has been reported to be expressed in several tissues including the kidney, liver, lungs, trachea, small intestine, bone marrow, thymus, prostate, adipose tissue, hepatocytes, renal tubular cells (Aigner et al., 2007; Chakraborty et al., 2011), and the epidermis of the fetal skin (Mallbris et al., 2002). Measurement of circulating NGAL levels have also been discussed as possible diagnostic markers of neutrophil activity in disease such as chronic obstructive pulmonary disease and asthma (Keatings & Barnes, 1997) and in arteriosclerosis (Elneihoum et al., 1997).

The expression level of NGAL in the circulation may be influenced by the presence of renal diseases, hypertension, inflammatory conditions, hypoxia and malignant diseases (Chakraborty et al., 2011). NGAL is an inflammatory marker which is associated with obesity, insulin resistance, and hyperglycemia (Wang et al., 2007). NGAL is also a marker of renal structural damage and is mainly produced in renal tubules in response to structural kidney injury during diabetes (Bolignano et al., 2008). Elevated NGAL expression in the kidney, as well as elevated urinary NGAL levels have been observed in diabetic kidney injury (Demir et al., 2012; Nielsen et al., 2010). Similarly, elevated NGAL levels have been observed in chronic kidney injuries and correlate with severity of renal impairment (Bolignano et al., 2008).

An important function of NGAL is to act as a bacteriostatic agent with actions against gram-negative bacteria and mycobacteria (Flo et al., 2004). In this role NGAL binds to bacterial proteins and captures iron-laden bacterial siderophores, such as enterochelin and carboxymycobactins (Holmes et al., 2005). This interaction of an iron-siderophore complex with NGAL results in the import of iron into host cells and limits bacterial growth by depriving bacteria of essential nutrients. NGAL deficiency in genetically modified mice leads to an increased bacteremia and bacterial burden in the liver and spleen (Flo et al., 2004). This function is likely important in neutrophils, where iron is required not only during their development in the bone marrow but also for their bacterial activity (Walter et al., 1986).

In skin keratinocytes NGAL levels are increased on wounding and its expression is upregulated in response to microbial stimulation and pro-inflammatory cytokines such as IL-1a (Bando et al., 2007). NGAL expression is also regulated by cytokines

including GM-CSF, IL-α, IL-β, IL-6 IL-17, IL-22 and TNFα. Its expression is also stimulated by growth factors including IGF-1, and EGF (Barresi et al., 2011). NGAL expression can be also regulated by NF-κB, a regulator of several key pathways (Florin et al., 2004) including by TLR ligands and TNFα (Kewcharoenwong et al., 2013).

NGAL is rarely detectable in normal skin cells and only is restricted to the hair follicle compartments in normal tissue (Seo et al., 2006). There are only a few studies which have examined NGAL levels in chronic wounds. Wound fluid NGAL levels in healing wounds which correlate with wound area. In contrast, there was no significant change in the NGAL level in non-healing wounds (Pukstad et al., 2010). Additionally, increased wound and plasma levels of both NGAL and MMP-9 have been reported in persons with chronic ulcers (Serra et al., 2013). High levels of circulating soluble NGAL and MMP-9 have also been shown to correlate with the non-healing venous ulcers (Serra et al., 2013; Serra et al., 2013). However, the effect of diabetes on wound fluid NGAL/MMP-9 complex concentration and its association with overall wound NGAL and MMP-9 levels, has received little attention.

Therefore, in this study the effect of diabetes on the relationship between NGAL and MMP-9 and NGAL/MMP-9 complex in inflammatory cells in wounds and peripheral blood neutrophils in two different wound-healing models was examined in detail. In addition the mechanism of the effect was studied in isolated neutrophils and pilot studies in patients were performed.

1.2 **Hypothesis and Aims**

In this study we investigated the effect of diabetes on the relationship between NGAL and MMP-9 and NGAL/MMP-9 complex in inflammatory cells in wounds and peripheral blood neutrophils in two different wound healing models. Our aims of this study were:

- To examine expression of NGAL in diabetic wounds and its association with MMP-9

- To examine the effect of interventions such as insulin treatment (to improve diabetic control) or Doxycycline (a regulator of MMPs) on NGAL and MMP-9 levels in diabetic wounds

- To examine *in vitro* the mechanism of the effect of glucose on neutrophil NGAL expression

- To examine the expression of NGAL and MMP-9 in circulating white blood cells in people with diabetes

CHAPTER 2: MATERIALS

AND METHODS

2.1 **Animal Studies**

The methods in this chapter describe techniques used in the animal studies and *in vitro* studies. The methodologies specific for the human study have been included in Chapter 5 where the human studies are presented.

2.2 **Experimental Design and Induction of Diabetes**

A summary of the experimental design is depicted in Figure 2-1. Male Sprague-Dawley rats (200-250g) obtained from Australian Laboratory Supply (Perth, Australia) and aged between 5 and 6 weeks were used to investigate the effect of diabetes on wound healing in both an excisional wound model and a sponge implant model. The excisional model enabled investigation of wound closure and tissue morphology, whilst the sponge implant model was used for examination of inflammatory cells.

In both studies diabetes mellitus was induced by intraperitoneal injection of streptozotocin (STZ; 65mg/kg in 0.1M citrate buffer, pH4.5, Sigma, S0130-USA) and confirmed by a tail vein blood glucose level > 11mmol/L from three days after injection. Diabetic animals were treated with a low dose insulin regimen (2-4IU insulin, twice weekly) to maintain their body weight and prevent ketoacidosis. Six weeks later, diabetic and control animals were anesthetised by using a combination of Ketamine (85mg/kg; Provet, Sydney, Australia) and Xylazine-20 (5mg/kg) prior to wounding or implantation of sponges as described in more detail below.

After induction of diabetes, in independent parallel studies, the diabetic animals were divided into different subgroups as shown in Table 2-1.

All animal experiments in this study were approved by the Animal Ethics Committee of this institution (Protocol No: 2010-024).

Table 2-1: Description of animal groups and treatment durations for the various studies

Groups	**Abbreviation**	**Duration of treatment**
• Control animals with no treatment	CON	------
• Control animals treated with oral doxycycline (100mg/kg) by oral gavage	CON+HDOX	6 days from time of wounding
• Control animals treated with doxycycline (20mg/kg) by oral gavage	CON+LDOX	6 days from time of wounding
• Diabetic animals with no treatment	DM	------
• Diabetic animals treated with insulin (10 IU daily)	DM+INS	6 weeks from diabetes induction
• Diabetic animals treated with doxycycline (100mg/kg) by oral gavage	DM+HDOX	6 days from time of wounding
• Diabetic animals treated with doxycycline (20mg/kg) by oral gavage	DM+LDOX	6 days from time of wounding

Figure 2-1: Schematic representation of the experimental design

2.2.1 **Measurements of body weight and blood glucose concentration**

All animals were weighed at the beginning of the study and then once per week throughout the study. Non-fasting blood glucose concentration was measured once per week for the duration of the study using a Freestyle Lite glucometer (Abbott). To obtain the blood the tip of tail was nicked with a scalpel blade and blood glucose was monitored by blood glucose test strip using the blood glucose monitor.

2.2.2 **Wound models**

In the excisional wound healing model four full-thickness circular wounds were created on the dorsum of the rats using an 8mm skin biopsy punch as previously described (McLennan et al., 2008). The wounds were then covered with transparent dressing (Coloplast, Comfeel® Plus Transparent 3533) and redressed as needed. For the wound sponge model PVA sponges ($1cm^2$; KCL) were sterilized by gamma irradiation (5Gy) and were inserted surgically, 4 sponges per animal via 2cm skin incisions (one incision per sponge) on the dorsum of the animal and the incisions were closed with suture. At the time of surgery each animal received a single dose of ampicillin (50 mg/kg) and Buprenorphine (Temgesic 0.03 mg/kg) for prevention of infection and pain relief.

2.2.3 **Excisional wound model**

Macroscopic wound closure was determined at the time points mentioned by placing a transparency film on the wound surface and tracing the wound area. Measurements were made at day 0 (time of incision), day 3 and at termination day 6. The wound tracings were scanned and wound area was measured using Image J software (NIH Image) and then the change in wound area was calculated and the

results were expressed as a change in area from day 0. Wound closure rate was evaluated based on the following formula:

% wound closure = 100 × (wound area at day 0 – wound area at day x) /wound area at day 0.

At termination (day 6), the skin containing the wound tissue was excised and treated as follows. Two wounds were snap frozen in liquid N_2 for later measurement of gene expression and protein levels. The other two wounds were divided in half, one half was fixed in formalin (10%) for histological and immunohistological studies and the other half was placed in OCT and frozen for immunofluorescence studies.

2.2.4 **Sponge implant wound model**

Animals were terminated at days 3, 6 and 12 after the sponge implantation. At termination blood was obtained by cardiac puncture and collected into heparin blood collection tubes for quantification of neutrophil and monocyte number, isolation of neutrophils and measurement of plasma MMP activity as described in following sections. The implanted sponges were removed and the fluids and cells removed by gentle squeezing. This process removed the majority of the cells. An aliquot (100 µL) of the extract was used for determination of neutrophil and monocyte number by flow cytometry. The remaining fluids and cells were placed in a tube and the cellular component was pelleted by centrifugation at 800 ×g for 10 min using a swing out rotor (Eppendorf 5810R). The supernatant was collected and stored at -80^0C for later analysis of protein content by BioRad DC Protein assay as well as the measurement of NGAL protein by ELISA and MMP activity by zymography.

The red blood cells were removed from the pelleted cells by incubation in red blood cell (RBC) lysis buffer (Appendix-1) prior to centrifugation at 800 ×g for 10 min. The supernatant was discarded and the pellet containing the cells was divided as follows: re-suspended in Tri-reagent for later RNA extraction and measurement of gene expression or spun onto slide for histological examination.

2.3 **Isolation of Rat Neutrophils**

Neutrophils were isolated from rat blood using a modification of the histopaque and dextran segmentation methods as described by (Kumar et al., 2010). Briefly, blood samples were centrifuged at 800 ×g for 15 minutes and the plasma was removed. The pelleted cells were then re-suspended in sterile Hanks' balanced salt solution (HBSS; 1:1 vol/vol, Gibco), and applied to an equal volume of Histo-Pacque (Sigma, Histopaque-1083) prior to centrifugation at 400 ×g for 45 min. At the end of centrifugation four distinct phases were observed Figure 2-2. The mononuclear cells were removed using sterile pipette from the upper layer.

The lower layer from the Histo-Pacque gradient, containing the neutrophils was then pipetted onto 2 ml of Dextran (6%, Sigma) and incubated at 37°C. After 20 min the supernatant containing the neutrophils was removed and centrifuged at 270×g for 10 min. The neutrophil pellet was then washed using RBC lysis buffer, 5 min later an equal volume of HBSS was added and the solution was centrifuged at 480×g for 10 min. The neutrophil pellet was washed with HBSS (10mL) and the neutrophils were again pelleted by centrifugation (480g for 10 min). The pellet was then resuspended in HBSS (1mL) and the isolated neutrophils were examined for purity (>90%) using May-Grunwald-Gimsa or DAPI stain (Figure 2-2). The neutrophils were then stored

as follows; i) Tri-reagent for later RNA extraction ii) RIPA buffer (Appendix-2) for protein analysis or ii) spun onto slides for histological examination of purity or protein expression by immunofluorescence.

Purification of Neutrophils from Rat Blood

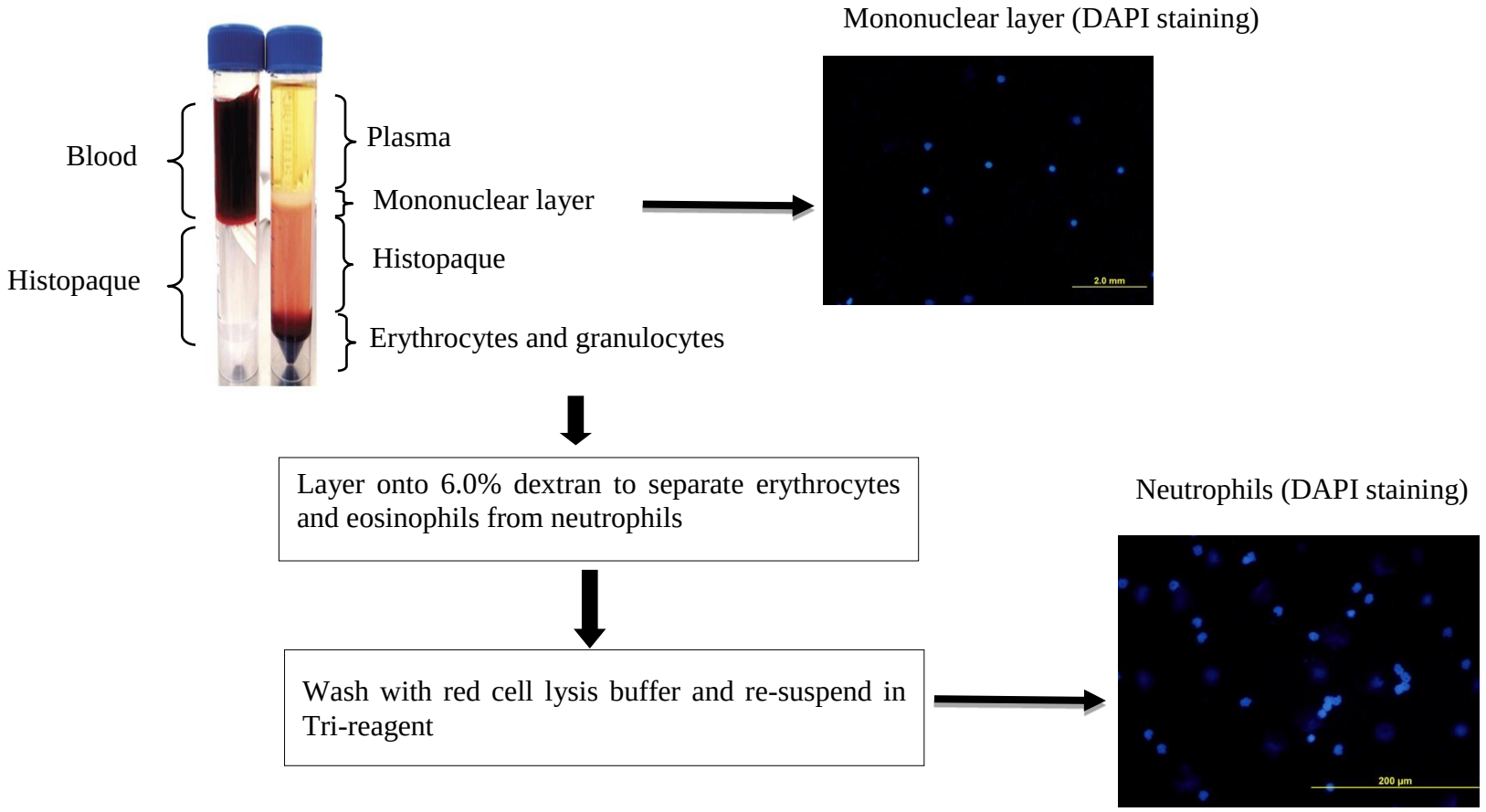

Figure 2-2 : Purification of neutrophils from rat blood

For the primary cell culture studies the isolated neutrophils were washed with RPMI and then plated in 6-well-plates at a density of 1×10^6. The neutrophils were then cultured in RPMI medium containing either normal glucose (5mM) glucose, a high glucose environment (25mM), or high glucose and 10 IU/ml insulin. The plated neutrophils were then incubated at $37^{\circ}C$ in 95% O_2 and 5% CO^2 for 1, 2, and 6 hours. At each time point the cells were harvested and centrifuged at 1000 rpm for 5 min. The cell pellet was then resuspended in TRI-reagent for later RNA extraction. In addition the media was stored at -80°C for later analysis.

2.4 **Preparation and Analysis of Protein**

2.4.1 **Measurement of protein concentration**

The protein concentration of all samples was measured using the BioRad DC protein assay with BSA as a standard (0-5mg/ml). For this assay, 5µl of the diluted samples in PBS (1:20) were incubated in 20µl of reagent A and 200 µl of reagent B and then were shaken on for 15 sec with 5 sec break for a total 15 min. The resulting colour developed was then read using a Multiskan plate reader at 690nm filter. The protein concentration was calculated from the BSA standard curve.

2.4.2 **Western blot analysis**

Western blot analysis was used to examine the specific proteins semi quantitatively in plasma and wound fluid. For these measurements the total protein concentration was determined as described. Equal amount of proteins (50µg) from each sample were loaded onto 10% non-reducing SDS-PAGE gels and the gels were run at 135V until the dye reached the bottom of the gel. The proteins on gel were then transferred to a Nitrocellulose membrane (Hybond-C Extra) for 7 min using the Trans-Blot

Turbo Transfer System (Bio Rad). After transfer the Nitrocellulose membrane was incubated for 1 hour with 5% non-fat milk in Tris Buffered Saline containing Tween 20 (TBS-T) (Appendix) at room temperature to block non-specific protein binding. Excess milk was removed by washing three times (5 min each) in TBS-T. Specific proteins were then detected by incubation in the following antibodies: anti-NGAL (1:500, ab63929, Abcam) or anti-MMP-9 (1:1000, ab38898, Abcam). The membranes were then washed with TBS-T buffer 3 times for 5 min at room temperature followed by incubation with horse radish peroxidase (HRP) conjugated anti-rabbit secondary antibody (1:10,000, SIGMA A-0545) in TBS-T buffer for 1 hour at room temperature. Proteins were visualised using the Enhanced Chemiluminescence (ECL) detection kit according to the manufacturer's instructions. Images were captured using the Syngene GBox-HR Gel Doc System.

2.4.3 **ELISA to measure soluble NGAL concentration**

The soluble form of NGAL was measured in plasma and wound fluids of experimental animals using an ELISA (Enzyme-linked immune-Sorbent Assay) kit (R&D Systems). Briefly, the primary monoclonal antibody (goat anti-rat Lipocalin-2, 0.8µg/mL in PBS pH 7.0, 100µL) was coated onto 96 well microplates by overnight incubation. The excess antibody was removed by washing three times in 400µL of washing buffer. Non-specific binding sites were blocked by incubation in 300µL of blocking buffer (1% BSA in PBS, pH 7.2) for 1 hour. The wells were then washed (3 times) and 100 µl of sample (1:20) or standards diluted in reagent diluent was added per well in duplicate and incubated for two hours at room temperature. The wells were again washed and then the detection antibody (150 ng/ml) (biotinylated goat anti-rat Lipocalin-2) was added to each well (2 hours, room

temperature). Colour development was performed after addition of 100µl of HRP-conjugated streptavidin for 20 min. Followed by removal of excess HRP-conjugated streptavidin and 100µL of 1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) were added. The optical density of each well was measured using a microplate reader set to 450 nm and also 570nm for correction for variation in plate optical density. As shown in Figure 2-3 results were calculated by comparison with absorbance for known standards.

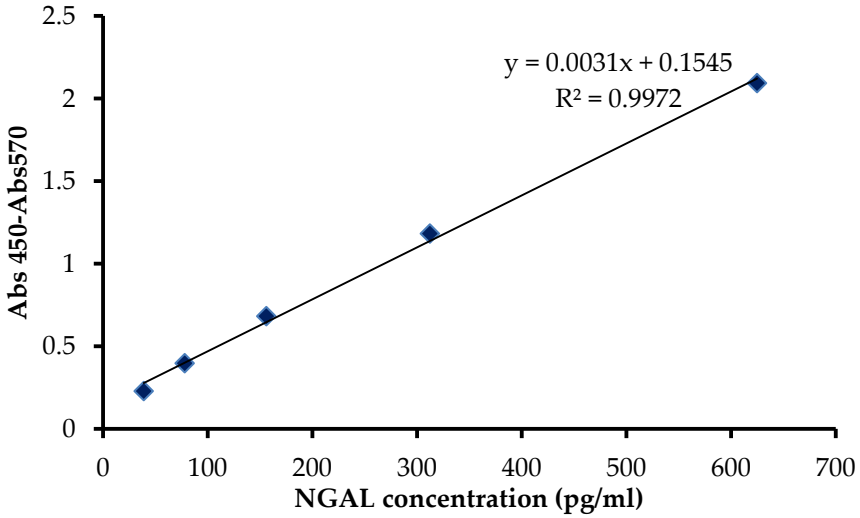

Figure 2-3: A representative standard curve for NGAL ELISA

2.4.4 Gelatin zymography for the measurement of MMP-9 and NGAL/MMP-9 complex

The pro and active forms of MMP-9 as well as the NGAL/MMP-9 complex were quantified in wound fluid and plasma using MDPF-labelled gelatin zymography as previously described (Min et al., 2006). By this technique, proteins are separated by size and MMP-9 activity is identified by their ability to degrade the gelatin substrate

contained within SDS-PAGE (Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis) gel. For this study, the diluted samples (wound fluid: 1:10, plasma; 1:20) were mixed with SDS loading buffer (Appendix) and then loaded into an 8% polyacrylamide gel (Appendix). The gel was then run at a voltage of 135V for 1.5 hours. Gels were then incubated in a series of 3 wash buffers all at pH 7.5 with constant shaking at room temperature for 30 min each wash (see Appendix for Buffers recipes). The gels were then incubated in buffer III overnight at 37°C. A sample containing a known concentration of MMP-9 was included on each gel as control and a pre-stained molecular weight marker (BenchMark Pre-Stained Protein Ladder, Invitrogen) was used to confirm protein size.

For quantitation, the gels were scanned using the Bio-Rad ChemiDoc MP Imaging system and band intensities were determined using Phoretix 1D Advanced version 3.01 densitometric software. Bands corresponding with NGAL-MMP-9 complex (~125 kDa), pro MMP-9 (~92kDa) and active MMP-9 (~82 kDa) were observed and their identity was confirmed by western blot analysis using anti NGAL (ab63929) and anti MMP-9 (ab38898) antibodies respectively.

2.5 Extraction of RNA and Quantification of Gene of Interest by RT-PCR

2.5.1 Primer design

All primers which are listed in Table 2-2 were designed using primer-blast at Blast (Basic Local Alignment Search Tool).NIH http://www.ncbi.nih.gov

Table 2-2: Primer sequences of the genes of interest

Target gene (rat)	Forward primer (5-3)	Reverse primer (5-3)
NGAL(Lipocalin-2)	TCCATCCTCGTCAGGGGCCA	AGTGTCGGCCACTTGCACATCG
36B4	GTACCATTGAAATCCTGAGCGA	GCCATTGTCAAACACCTGCT
MMP-9	GCTTAGATCATTCTTCAGTGCC	GTTTAGAGCCACGACCATACAG
MMP-8	CAAGACTCCAAGAATTACAACCTG	TCTCTGTAACCATAGTTTGGGT
TLR4	ATTGTTCCTTTCCTGCCTGAG	CTAGGTTCTTGGTTGAATAAGG
TLR2	AGGTCTCCAGGTCAAATCTC	CTTTGTCTTTGCTGTGAGTCC
TNFα	GCCTCTTCTCATTCCTGCTC	AAGATGATCTGAGTGTGAGGGT
MCL-1	TTGTAAGGACGAAGCGGGAC	GCCACTTTCTTTCTGCCGTG

2.5.2 RNA extraction from cells

RNA was isolated from infiltrated cells obtained from the sponge implant or wounded skin after homogenisation using TriReagent (Sigma). Tissue samples were homogenized in 1ml Tri-reagent using a tissue homogeniser. The homogenised suspension was centrifuged for 10 min at 14000 rpm to remove cellular debris and large tissue fragments. A 200µl 1-Bromo-3 chloropropane was then added to the supernatant for each 1ml Tri-reagent and mixed well for 15 seconds. The samples were left at room temperature for 5 min until the two phases were separated. The samples were then centrifuged (14000 rpm) at 4°C for 15 min. After centrifugation three clear layers were observed. The top and clear layer containing RNA was removed and transferred into a new microcentrifuge tube containing 500µl isopropanol and incubated at -20°C overnight.

After incubation, the samples were centrifuged at 14000 rpm for 15 min to pellet the RNA from the isopropanol. The obtained pellet was washed twice in 70% cold ethanol (14000rpm, 15min, and 4°C). The pellet was then left at room temperature to dry and then dissolved in 20µl RNase- free water. RNA concentration and purity was determined using the Nanodrop (BioRad) and only those samples with 260/280 ratio greater than 1.80 were used. Total RNA was stored at -80°C for later analysis.

2.5.3 **Reverse transcription of RNA**

RNA was reverse transcribed into cDNA using oligo-dT 18 and Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturers' instructions. The following RNA/Primer mixture was prepared: 10 µL RNA, 0.5µl Random Hexamer (RH) (20 nM), 0.5µl Oligo (dT)13-18 with the remaining volume of up to 12.5µl provided by addition of RNase- free water. The mixture was then incubated at 70°C for 10 min using a PCR machine (Bio Rad).

The following reaction mixture was then prepared: 4µl 5x RT-Buffer (PCR buffer), 2µl 0.1M DTT and 1µl dNTPs, and 0.5µl of Superscript III RT (except for the negative controls). For the reverse transcription reaction 7.5µl of reaction mixture was added to each tube, mixed gently and collected by spinning down. The mixture was then incubated at 25°C for 10 min. The reaction was completed by increasing the temperature to 50°C for 60 min, and then 70°C for 10 min followed by cooling to 4°C. cDNA was stored at –20°C for later quantitative gene expression.

2.5.4 **Polymerase chain reaction (PCR)**

To confirm the PCR products of gene of interest as a single band with the correct size, PCR reaction was set using the following mixture: 0.5µl 100mM dNTP mix, 0.8µl Red Taq DNA Polymerase PCR enzyme, 19.2µl RNAse free water, 1µl cDNA template (see2.5.3), 0.5µl NGAL or 36B4 Forward and 0.5µl NGAL or 36B4 Reverse primer (20µM). The samples were run using the following PCR cycle: denaturing at 95°C for 3min, and amplified through 40 cycles with denaturing at 95°C for 15sec, annealing at 58°C for 30sec, followed by extension at 72°C for 30 sec and final elongation at 72°C for 10 min.

The size of the amplicons products were examined on agarose gels as follows: the PCR products and 1 kb DNA ladder were run at 110V on 1.8% agarose gels using 1x Tris-Acetate-EDTA Buffer (TAE) buffer. Gels were viewed using the Syngene GBox-HR Gel Doc System. As shown in the representative gel for NGAL amplicons (Figure 2-4) a single band at 100bp was observed for NGAL obtained from neutrophils and wound fluid cells, the kidney tissue was used as a positive control. Amplicons for other genes studied were also examined in a similar manner and were also shown to produce amplicons of the expected size (not shown).

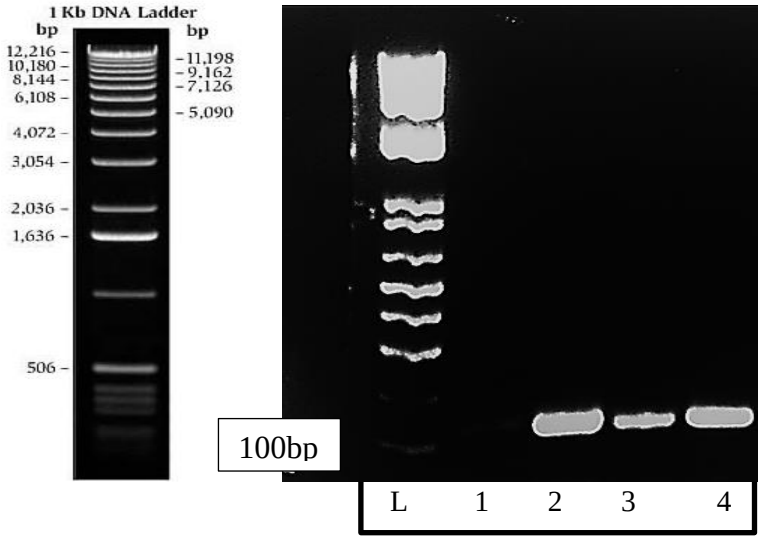

Figure 2-4: The amplified NGAL qPCR product.
NGAL qPCR product was confirmed as a single band (135 bp for NGAL in agarose gel (1.8%). The loading order is: (L) DNA ladder (1) no template control, and NGAL in (2) neutrophil (3) kidney (4) wound fluid cells.

2.5.5 **Quantitative real time polymerase chain reaction**

Quantitative RT-PCR was used to detect relative or absolute gene expression level. The cDNA was amplified in duplicate using a Rotor-Gene 6000 (Corbett) and SensiMix SYBR Hi-ROX kit (Bioline) and primers specific for MMP-8, MMP-9, NGAL the inflammatory markers TNFα, TLR4 and TLR2, and the anti-apoptosis marker MCL-1, 36B4 was used as a housekeeper (Table 2-2). All PCR products were shown to amplify in parallel with 36B4, the no template control (NTC) failed to amplify in the range of Ct studied. The melt curve analysis showed a unique peak for all primer pairs. For each gene the linear range of amplification was determined. Representative example for NGAL is shown in Figure 2-5.

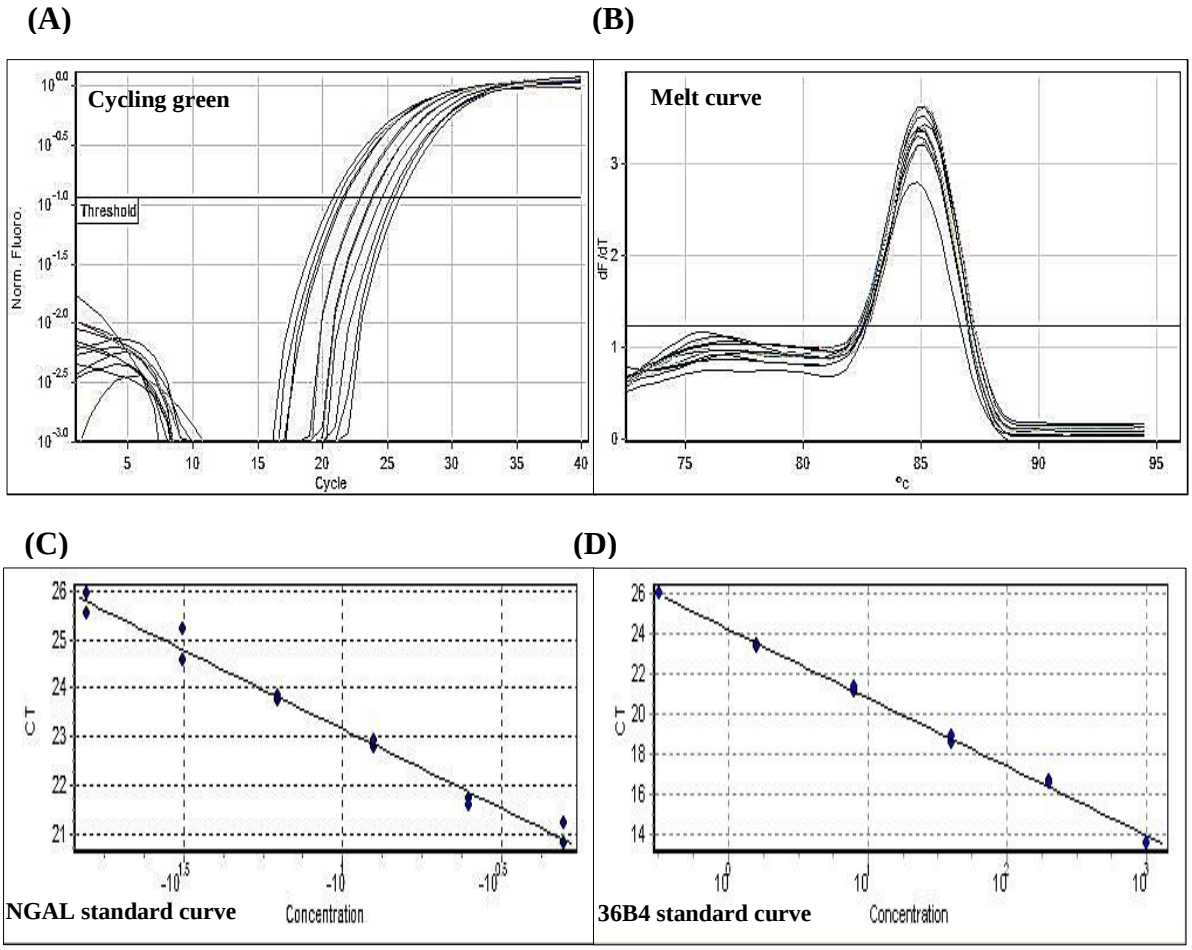

Figure 2-5: A representative images for establishment of linear range of amplification of NGAL.
Shown in (A) amplification plots (B) melt curve (C) and standard curve showing linear amplifications over ½ dilution range for NGAL and (C)1/5 dilution for 36B4.

2.6 **Tissue Preparation**

As mentioned skin samples containing wounds were obtained at termination and wounds were divided in half and snap frozen in OCT and liquid nitrogen. Other wounds were fixed in 10% Formalin for 24 hours at room temperature. The fixed tissues were then incubated in 70% ethanol at 4°C overnight and then dehydrated using a series of alcohols (70%, 80%, 90%, and 100%, respectively). The tissues were then cleared in

xylene and embedded in paraffin. Each sample was embedded perpendicular to the surface and serial sections of 5μm thickness were cut using a microtome, placed on a slide, dried and stored at 4°C for latter histological and immunohistological studies.

2.6.1 **Histological analysis**

For morphological analysis of wound tissue, samples were stained with Hematoxylin (H), and counterstained with Eosin (E). To stain for collagen I and III, sections were stained with hematoxylin for nuclei and then in PSR (Picro-Sirius Red Stain) for one hour. For both types of stain the slides were washed and dehydrated and coverslipped.

Analysis of histological staining was achieved by 2 independent observers who were blinded to the source of tissue. The scoring system used was based on a score of 0-3 where 0 represented light colour intensity and 3 were intense staining. Additionally, as there are three distinct zones in the granulation tissue each zone (top, middle, and bottom) was independently scored (Figure 2-6). Lesions relating to histopathological changes were scored in the granulation tissue of stained slide shown in Table 2-3. For analysis the score for each zone was added and the data was analysed with a staining intensity cut off at > 6, by Chi squared analysis.

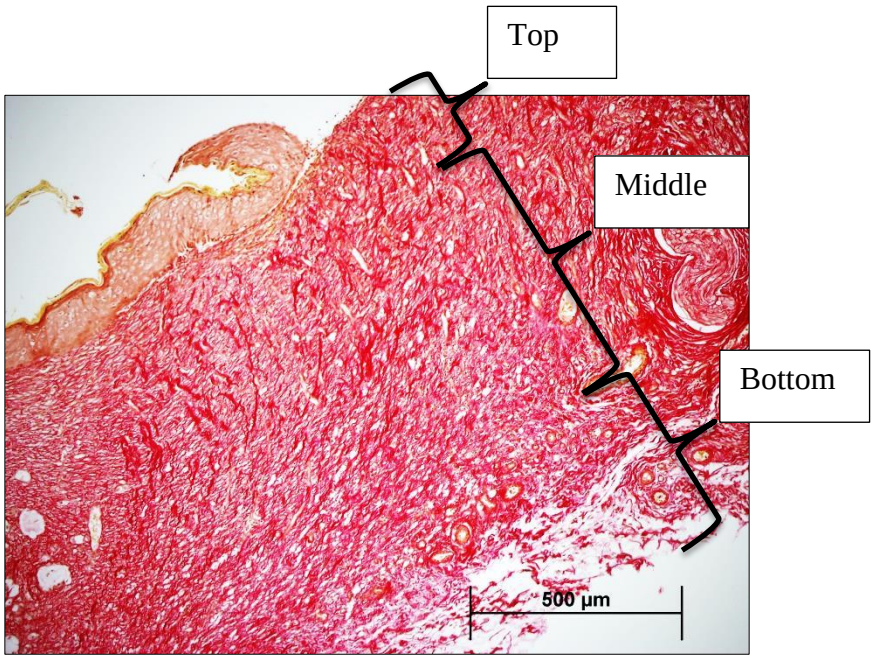

Figure 2-6: Three distinct zones in the granulation tissue for PSR staining

Lesions relating to histopathological changes were scored in the granulation tissue of stained slide shown in Table 2-3.

Table 2-3: Scoring of PSR results for collagen deposition in skin wounds.

	Granulation tissue
PSR Scores	**Zone (Top Middle Bottom)**
0	No staining
1	Low staining
2	Moderate staining
3	Intense staining

2.6.2 **Immunohistochemical staining**

All antibodies used for immunohistochemical staining were first titrated and then used at an appropriate concentration. Isotype controls, a control to measure the level of non-specific background staining were used at the same concentrations as the respective primary antibodies listed in the Table 2-4.

To determine the NGAL, and MMP-9 positive cells, immunohistochemical staining was performed on the skin sections of the experimental animals. The wounded skin tissue was prepared as previously described in section 2.6. Antigenicity was retrieved by boiling (20 min) in target retrieval solution (Tris EDTA, PH 9.0) in a microwave; the slides were cooled for 20 min and placed in TBST. The slides were incubated with a peroxidase block solution (3% H_2O_2) in TBS-T for 10 min and then immersed 3 times with TBST for five min each. Non-specific binding was then prevented by incubation of the slides in a blocking reagent (10% Normal Goat Serum NGS) for 10 min. The sections were then incubated with the appropriate primary antibody overnight at 4°C. The rest of the staining was performed at room temperature. The slides were washed 3 times in TBST and incubated with the appropriate secondary antibody (Table 2-4). After 40 min the slides were washed in TBST (3×5) and incubated with Vectastin ABC kit (Vector, PK-4000) for 60 min. To develop the color, the slides were incubated with 3, 3-diaminobenzidine (DAP) substrate for 1-2 min. The slides were then counterstained with hematoxylin to stain the nuclei and mounted with coverslips. All slides were examined by light microscopy (× 200) (Olympus AX-70 - Fluorescence).

Table 2-4: Antibodies used in the immunohistochemical study.

Primary Ab	Application	Description	Secondary Ab
NGAL	1:800	ab41105, Abcam	1:200 goat anti rabbit, Vector
MMP-9	1: 4000	ab76003, Abcam	1:200 goat anti rabbit, Vector

All samples stained for NGAL expression were examined in the epithelial (top), granulation tissue (middle) and inflammatory (basal) zones. Scoring was based on a scale of 0-3 where 0 represented no staining above isotype control and 3 were intense staining (Table 2-5). For MMP-9 scoring the percentage of positive stained cells was calculated using ImageJ software.

Table 2-5: scoring method for NGAL expression in skin wounds.

	Epidermis			Dermis			Hypodermis
NGAL scores	**Tip**	**Top**	**Bottom**	**Inflammatory cells**	**Endothelial cells**	**Fibroblasts**	**inflammatory cells**
0	No staining						
1	Less staining						
2	Moderate staining						
3	Intense staining						

2.6.3 **Immunofluorescence staining**

NGAL localisation and expression was studied in the wound tissue, sponge inflammatory cells and isolated neutrophils. The cells expressing NGAL (M145, Santa Cruz) were identified by co-localisation with MPO (ab45977, Abcam) for neutrophils and CD68 (ab31630, Abcam) for macrophages. The co-localisation of

NGAL and MMP-9 in isolated circulating neutrophils was also investigated as a marker of neutrophil activation.

For these studies, cryo sections of wound tissue and cytospun cells were fixed with cold methanol for 15 min and permeabilised with 0.2% TritonX-100. After washing in PBST, the slides were incubated overnight in NGAL antibody (1:500) at 4°C. The slides were then washed (PBST) and incubated in secondary antibody (1:200, Alexa Fluor 488, goat anti rabbit, Invitrogen). After removal of excess antibody by washing in PBST, the slides were incubated with antibodies to MPO, CD68, or MMP-9 (at 1:1600, 1:400, 1:4000 respectively) for 1 hour at RT. After 3 washes (5 min) the MPO, CD68 or MMP-9 were visualised by incubation with secondary antibody (1:200, Alexafluor 594, goat anti rabbit/or goat anti mouse Invitrogen). Stained cells were then mounted and the nuclei were visualised by staining with DAPI. Images were captured at magnification 200× using a fluorescence microscope and the number of positive cells per 100 cells was determined.

2.7 **Flow Cytometric Studies**

Flow cytometry was used for determination of the number of neutrophils and monocytes as well as neutrophil apoptosis in whole blood and also in the samples collected from the implanted sponges. For flow cytometric analysis, blood or sponge infiltrate (100 µL) was added to an equal volume of RBC lysis buffer. After lysis, the reaction was stopped by addition of PBS (900 µL) and the pellet was collected by centrifugation at 300g for 5 min. The pellets were washed by resuspension in FACS buffer (1% BSA, PBS, and 0.1% sodium azide) and re-pelleted by centrifugation (5min at 300g). The supernatant was then decanted and the cell pellet

was re-suspended in 100-µL FACS buffer for flow cytometric analysis (FACS Canto flow cytometer).

Neutrophil and monocyte numbers were calculated based on their morphology by forward scatter (FSC) and side scatter (SSC). Neutrophil viability and apoptosis was determined after staining with PE-conjugated mouse anti-rabbit RP-1 (BD Biosciences) as a rat neutrophil marker and annexin V (Biolegend) and Near-IR Dead Cell Stain (LIVE/DEAD® Fixable Near-IR Dead Cell Stain Kit: Life Tech.) as live and dead cell markers respectively. For this analysis, cells were incubated with Annexin V and Near-IR prior to fixation with 1% paraformaldehyde for 10 min at 4^{o} C. The cells were then permeabilised by incubation with saponin buffer (0.5% saponin, 0.5% BSA, 0.05% NaN_3 in PBS) for 15 min at room temperature. After permeabilisation cells were spun for 5 min at 300g and the pellets were washed with FACS buffer and spun (300g, 5min). The cells were then stained with RP-1 (3µL). Cells stained with PE-conjugated mouse anti-rabbit isotype (PE Mouse IgG2a, BD Biosciences) were used as negative controls. Analysis of data was performed using Flowjo software (Treestar Flowjo V.X10).

2.8 **Statistical Analysis**

Datasets were tested for their conformance to normality and subjected to the analysis of variance. Analyses were carried out with the Statistical Package for the Social Science (SPSS for Windows, version 20). The effects of treatments on diabetic animals were then compared across normal, diabetic, and treatment groups using one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons. Values are expressed as mean ± standard error (SEM) and all statistical tests are

conducted at 95% confidence level ($P<0.05$). Independent sample T-Test was used for statistical comparison of two groups. Chi square test was used for comparison of the histopathological scores.

CHAPTER 3: Effect of Diabetes on Wound Healing and Neutrophil Activity

3.1 **INTRODUCTION**

There are several factors that influence wound healing in a diabetic patient. Some of these factors include hyperglycemia, macrovascular and microvascular disease, immune system deficiency and infection may contribute to wound infection and delayed wound healing in diabetic patient. In addition, leukocytes in particular neutrophils are not only involved in protection against invading environmental microorganisms but they also participate in the inflammatory stage of wound healing (Wilgus et al., 2013). Matrix metalloproteinase that are proteases, are stored in neutrophil granules in their latent form and are activated on release. Of the MMPs present in neutrophil granules, the functions of MMP-8 (Pirila et al., 2007), and MMP-9 (Liu et al., 2009) are associated with chronic wounds. NGAL which is secreted from activated neutrophils can bind to MMP-9 to prevent its degradation (Kjeldsen et al., 2000) but whether it plays a role in increased MMP activities in poorly healing wounds in diabetes is not known. Our previous studies have shown that increased level of MMP-9 can predict poor wound healing in diabetic foot ulcers (McLennan et al., 2008) and diabetes can affect circulating neutrophil function. Whether neutrophil activities increase MMP-9 activity in diabetic wounds is not known.

Insulin plays a critical role in regulation of wound healing and impairment of insulin signalling has been shown to contribute to impaired wound healing. It is well documented that insulin signalling pathways such as Insulin receptor substrate 1 (IRS-1), SHC, Protein kinase B (AKT), and Extracellular Signal-Regulated Kinases (ERK1/2) are significantly decreased (Lima et al., 2012). Insulin treatment can accelerate wound healing rate and is accompanied by decreased inflammation,

increased collagen deposition (Madibally et al., 2003) and increased proliferation and migration of keratinocytes (Brem & Tomic-Canic, 2007). In terms of the effect of insulin on inflammatory cells, insulin treatment decreased the number of infiltrating neutrophils by inhibiting expression of macrophage inflammatory protein-2 and stimulating neutrophil function (Sunahara et al., 2012). For instance, insulin improves neutrophil migration, phagocytosis and ROS production in the diabetic environment (Alba-Loureiro et al., 2007) and prevents the increased neutrophil numbers in the peripheral blood (Walrand et al., 2004). Insulin has a potent anti-inflammatory effect by reduction of pro-inflammatory transcription factors, suppression of MMP concentration (P. Dandona et al., 2002) and inhibition of the pro-inflammatory transcription factor early growth response gene-1 expression in mononuclear cells (Aljada et al., 2002).

In this chapter to investigate the effect of diabetes on the relationship between MMP-9 and neutrophil activities in wound healing, two wound healing models were used. In addition, a small pilot study at 3 week diabetes duration time point served to establish the methodology for circulating neutrophil isolation. The data from this pilot study are included in this chapter.

The studies described in this chapter were designed to investigate:

- The effect of diabetes on wound NGAL and MMP-9 levels
- The effect of diabetes on gene expression of NGAL and MMP-9 in inflammatory cells in the wound and circulation
- The association between NGAL and MMP-9 activity in wound fluid and plasma
- The effect of insulin on NGAL and MMP-9 levels in inflammatory cells and fluids

3.2 MATERIALS AND METHODS

As previously described in Chapter 2, male Sprague-Dawley rats were used to investigate the effect of diabetes on wound healing in both an excisional wound and a wound sponge implant model. Diabetes mellitus was induced using STZ (65 mg/kg) as described in Chapter 2.2. To investigate the effect of diabetes and the combination of diabetes and wounding on circulating inflammatory cells, a sub study was performed in animals with 3 weeks duration of diabetes and the results were compared with results obtained from a larger study in animals with 6 weeks duration of diabetes.

For the study at 3 weeks duration of diabetes, the study animals were divided into control and diabetic groups as shown in Figure 3-1.

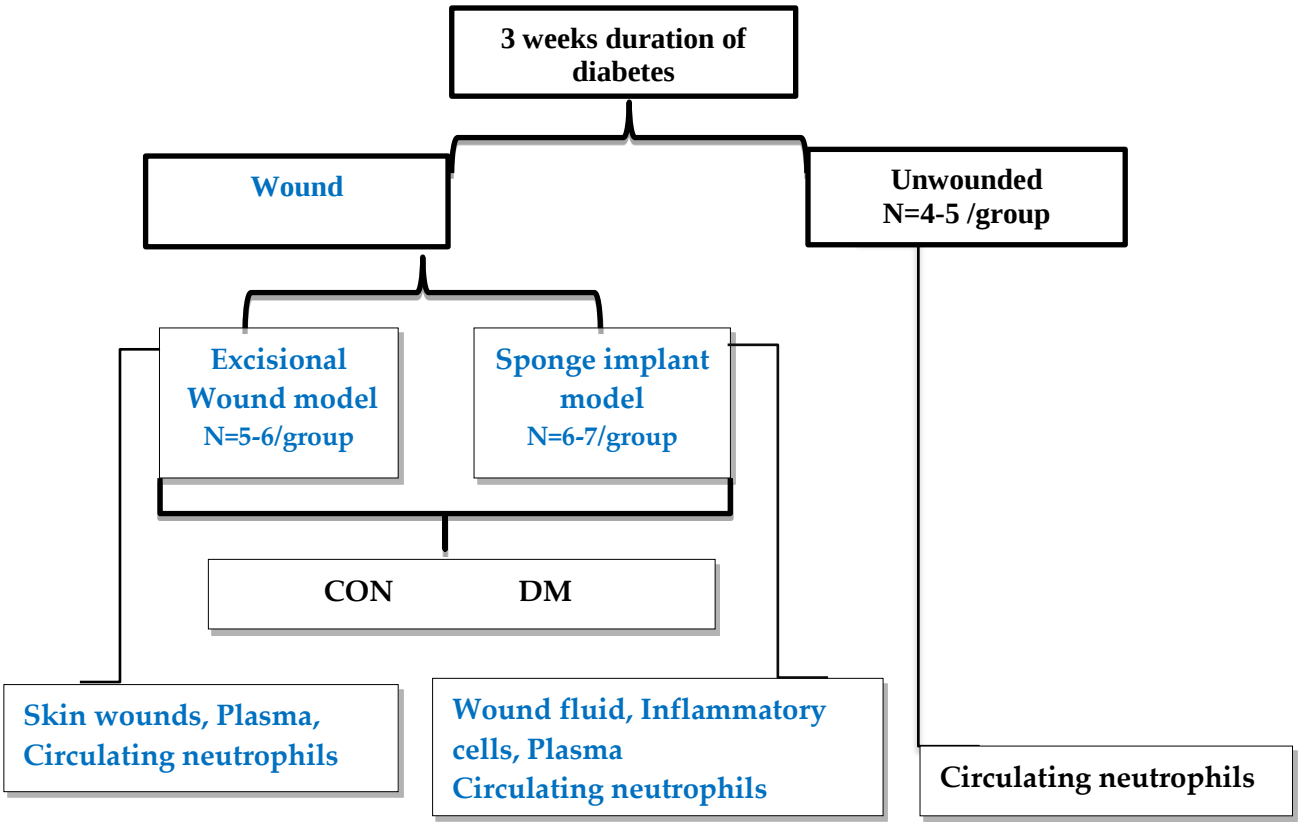

Figure3-1: Experimental design for 3 weeks duration of diabetes studies. All animals were terminated at Day 6 and the wound tissue and sponge implants were removed for analysis.

Shown Figure 3-2 is the study design for animals with 6 weeks duration of diabetes. All animals were divided into following groups.

1) Control animals that did not receive any treatment (CON)
2) Diabetic animals were treated with a low dose insulin regimen (2-4IU insulin, twice weekly) to maintain their body weight and prevent ketoacidosis (DM)
3) Diabetic animals that were intensively treated with a dose of insulin (10IU/day) (DM+INS), which aimed to maintain near normal glycemia.

Samples studied included i) wound tissue from the excisional model, ii) sponge inflammatory cells and wound fluid from the sponge implant model, and iii) circulating neutrophils which were isolated from whole blood.

For these studies the wounds in the excisional wound healing model four full-thickness circular wounds were created on the dorsal of the rats and at termination (day 6) the skin containing the wound tissue was excised for further experiments.

For the wound sponge model sterilised PVC sponges were inserted surgically on the dorsum of the animals and the incisions were closed. Animals in this model were terminated at day 3, 6, and 12 days after sponge implantation. The implanted sponges were removed and inflammatory cells were removed by centrifugation from wound fluid. In both animal models blood was obtained by cardiac puncture and collected into the heparinised blood tubes for isolation of neutrophils, quantitation of the number of neutrophil and monocyte and detection of the plasma MMP activity as described in Chapter 2.

Neutrophil specific genes MMP-8, MMP-9, NGAL and other pro-inflammatory markers TNFα and TLR4 and TLR2 were measured from the sponge implant inflammatory cells as well as in circulating neutrophils. The NGAL/MMP-9 complex and pro and active forms of MMP-9 in the wound fluid, and plasma were also quantified. Flow cytometry was used to investigate the effect of diabetes on inflammatory cell numbers in blood and wound fluid.

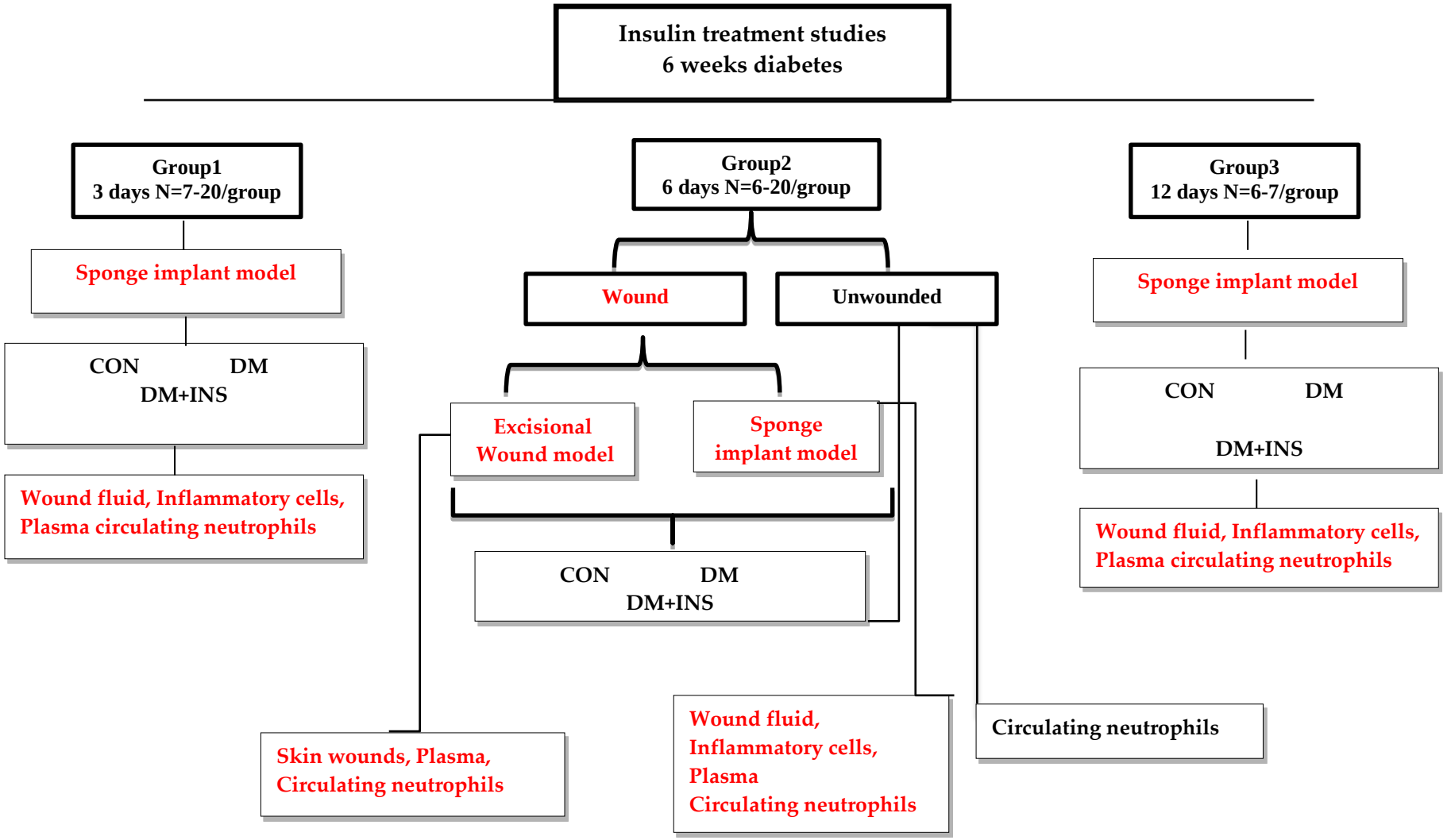

Figure 3-2: Experimental design for insulin treatment studies

3.3 RESULTS

3.3.1 Effect of 3 Weeks Duration of Diabetes on Wound Healing

3.3.1.1 Blood glucose concentration in experimental animals

Body weight and blood glucose level was measured after 3 weeks diabetes. The body weight in diabetic animals (396.1± 8.7 g) was significantly lower than control (471.5 ± 7.1 g). Diabetic animals had also a significantly higher non-fasting blood glucose level than control non-diabetic animals (22.7 mmol/L in diabetic vs. 5.1mmol/L in control animals; $P<0.05$).

3.3.1.2 Effect of diabetes on excisional wounds

In this series of studies wounds were placed dorsally after 3 weeks duration of diabetes. Wound healing was monitored as described (Chapter 2.2.3). Six days later the animals were terminated and the skin tissue containing the wound was excised and treated for MMP and NGAL measurement by RT-qPCR, zymography, IHC and IF as described (Chapter 2.6.1).

3.3.1.2.1 Effect of diabetes on wound closure

Wound area was measured at day 3 and 6 and the percentage wound closure was determined. Shown in Figure 3-3 the wounds in diabetic animals displayed delayed healing in particular at day 3 when it was significantly slower than controls (11% vs 26%, diabetic and control respectively). They also tended to be delayed at day 6 but the difference was not significant (49% vs 42% respectively).

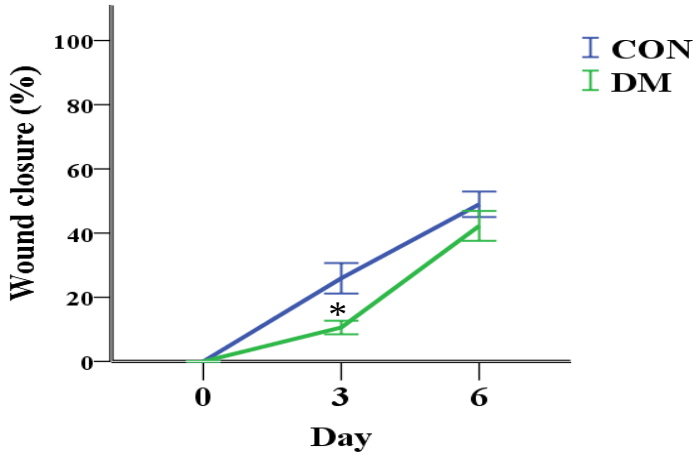

Figure 3-3: Percentage wound closure in animals with 3 weeks duration of diabetes compared with age matched controls.
Control (CON: n=5) and diabetic (DM: n=5) animals at 3 and 6 days after wounding.
*Results are expressed as Mean ± SEM.*P<0.05 compared to control.*

3.3.1.2.2 **Effect of diabetes on wound NGAL expression by IHC**

The expression and cellular localisation of NGAL was examined by IHC staining. As shown in Figure 3-4 even at this relatively early time point of 3 weeks, the expression of NGAL was increased in the diabetic animals compared with the controls (Figure 3-4-A). NGAL expression appeared to be localised to the inflammatory cells, and to lesser extent fibroblasts, endothelial cells and keratinocytes (Figure 3-4 B).

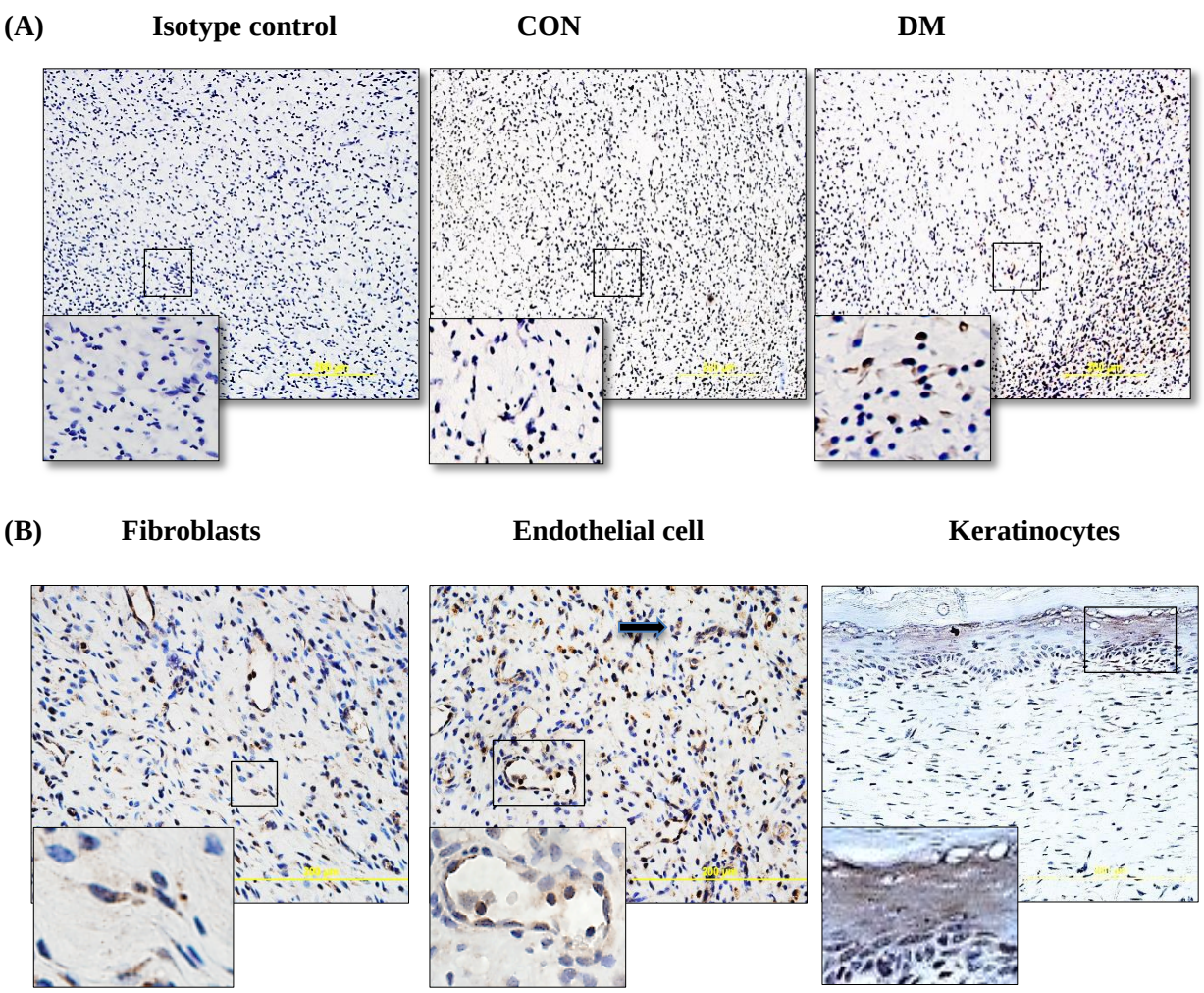

Figure 3-4: Representative images of skin wounds showing NGAL expression in skin wounds of control and diabetic rats with 3 weeks duration of diabetes.
(A) NGAL expression in skin wounds control (CON) and diabetic (DM) animals at 3 weeks study. Expression of NGAL in the fibroblasts, endothelial, and keratinocyte cells in the diabetic skin wounds shown in (B). Black arrow shows some inflammatory cells. Image magnification (A) ×200, (B) ×400.

3.3.1.2.3 **Effect of diabetes on wound MMP-9, NGAL and TLRs gene expression**

The gene expression of markers of neutrophil activity NGAL and MMP-9 as well as pro-inflammatory markers TLR4 and TLR2 were measured in the RNA extracted from skin wounds. As shown diabetes increased NGAL mRNA level (by 1.8 fold) and but had no effect on MMP-9 mRNA. The effect of diabetes on TLRs was also measured as a marker of differences in bacterial load and /or altered inflammatory pathways. At the mRNA level, no differences in the expression of these markers were observed (Figure 3-5).

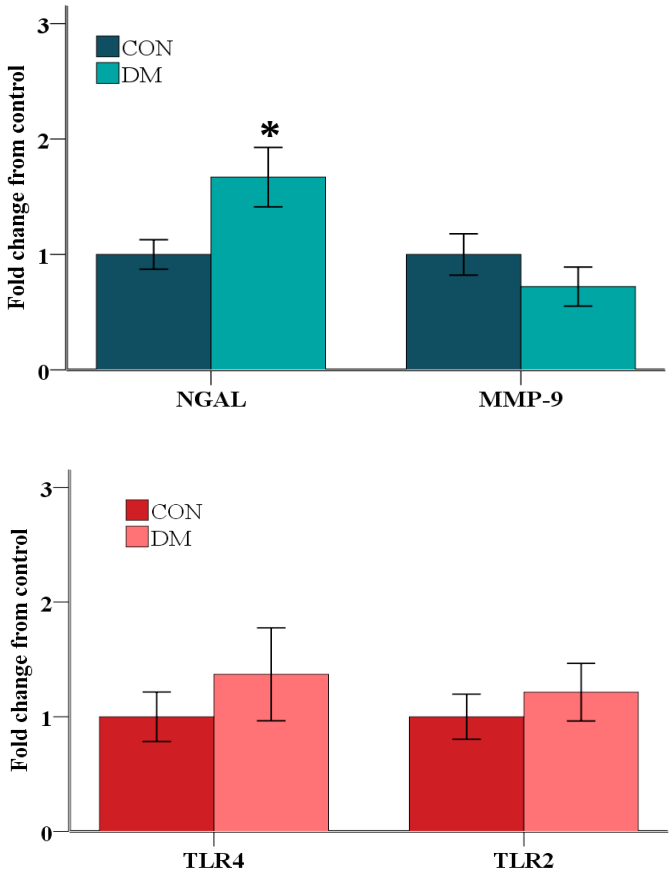

Figure 3-5: Gene expression of NGAL, MMP-9, TLR4 and TLR2 in the skin wounds at 3 weeks duration of diabetes.
*Control (CON: n=5), and diabetic (DM: n=5) animals. Values are corrected for the expression of 36B4 as a housekeeper gene and expressed as fold change from control. Data are Mean ± SEM.*P <0.05 vs. control.*

3.3.1.3 **Effect of diabetes on the sponge implant inflammatory cells**

The effect of diabetes on the sponge implant inflammatory cells was studied at two different durations of diabetes, i.e. 3 and 6 weeks diabetes duration. In this series of studies sponges were implanted sub-dermally in the flanks of animals, at defined time points (3, 6, and 12 days after sponge implant) the sponges containing the cells and fluids were removed and the cells and fluids isolated as described in Chapter 2.2.4.

3.3.1.3.1 **Effect of diabetes on the sponge implant inflammatory cell NGAL gene expression**

In the animals with 3 weeks duration of diabetes, animals were terminated at day 6 after implantation of the sponge. The cells and fluids were removed and the cellular NGAL mRNA level was determined by qRT-PCR. Results in Figure 3-6 showed that the NGAL mRNA was significantly increased (4 fold) in the diabetic animals compared to controls.

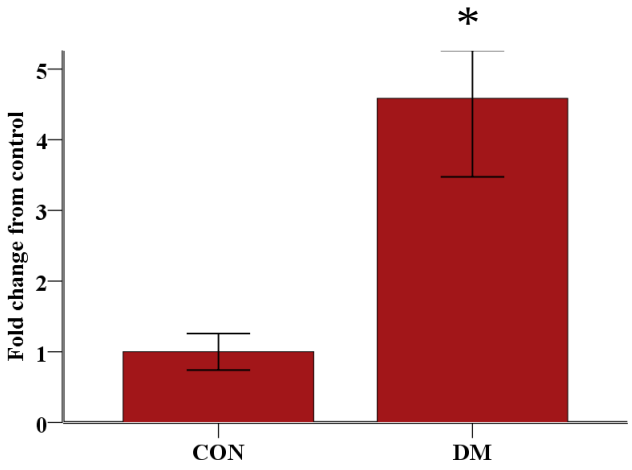

Figure 3-6: NGAL gene expression in the sponge implant inflammatory cells at day 6 in animals which had 3 weeks duration of diabetes.
*Control (CON, n=5) and DM (diabetic, n=5) animals. Values are corrected for the expression of 36B4 as a housekeeper gene and expressed as fold change from control. Data are Mean ± SEM.*P <0.05 vs. control.*

3.3.1.4 **Effect of diabetes on the circulating neutrophils**

To investigate the effect of diabetes and/or wounding on circulating neutrophils, the neutrophils were isolated from whole blood obtained at termination from the control and diabetic animals in both wounding models. In a parallel experiment neutrophils from unwounded animals with the same duration of diabetes were also studied. The cellular protein concentrations of NGAL were examined by immunofluorescence and their gene expression of markers of activated neutrophils and inflammation by qRT-PCR. Plasma MMP-9 activity and NGAL/MMP-9 was also measured by zymography.

3.3.1.4.1.1 **Effect of diabetes on the circulating inflammatory cells**

The profile of expression of NGAL was examined in the circulating neutrophils and monocytes by IF staining. As shown in the representative photos in the Figure 3-7 neutrophils were the main source of NGAL.

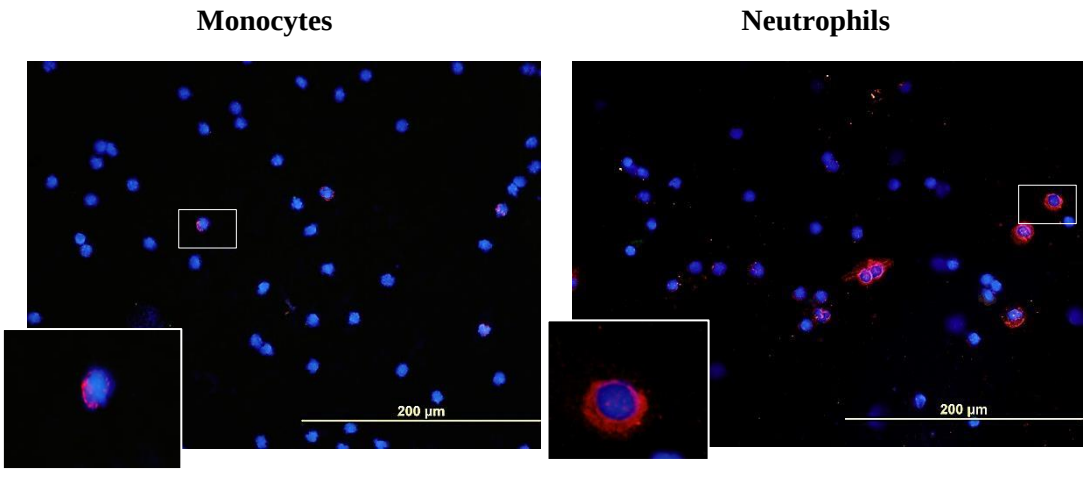

Figure 3-7: Representative images showing NGAL expression in monocytes and neutrophils isolated from blood in animals which had 3 weeks duration of diabetes. Red colour shows the expression of NGAL in the circulating monocytes and neutrophils.
Image magnification ×400.

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3.3.1.4.2 **Effect of diabetes on the circulating neutrophils by qRT-PCR**

In the short duration of diabetes, gene expression of markers of activated neutrophils NGAL, MMP-8 and MMP-9 and inflammation markers TLR2 and TLR4 and TNFα was measured in the circulating neutrophils by qRT-PCR. As shown in Figure 3-8 in unwounded animals diabetes tended to increase the gene expression of neutrophil NGAL and MMP-8, but neutrophil MMP-9 was not altered. On the other hand analysis of circulating neutrophils obtained from animals six days after wounding showed increased expression of NGAL, MMP-9, and MMP-8 mRNA in the diabetic animals. However no change in expression of these genes was observed in circulating neutrophils isolated from animals in which the sponges had been implanted.

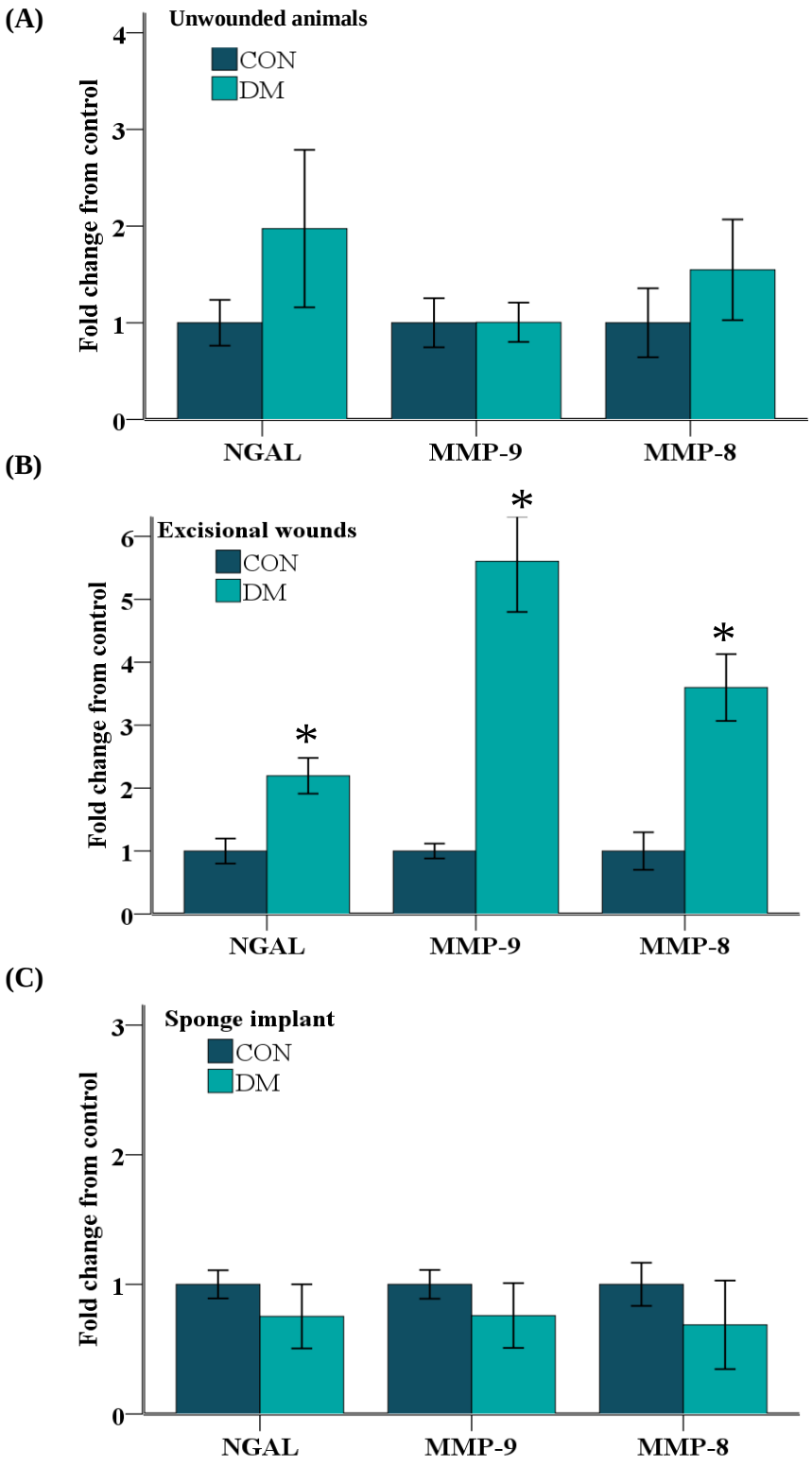

Figure 3-8: NGAL, MMP-9, and MMP-8 gene expression in the circulating neutrophils from (A) unwounded, (B) excisional and (C) sponge implant models in animals which had 3 weeks duration of diabetes.
*Control (CON, n=5) and diabetic (DM, n=5) animals. Values are corrected for expression of 36B4 as a housekeeper gene and expressed as fold change from control. Data are Mean ± SEM.*P <0.05 vs. control.*

3.3.1.4.3 **Effect of diabetes on neutrophil apoptosis by gene expression**

Neutrophil clearance is important for the resolution of inflammation and their apoptosis is critical for their uptake by macrophages. Whether diabetes and wounding can alter circulating neutrophil apoptosis is not known. In this study neutrophil apoptosis was examined by measurement of the anti-apoptotic marker MCL-1 in the neutrophils. As shown in Figure 3-9 diabetes alone significantly decreased MCL-1 gene expression by 0.5 fold compare to control. In contrast, diabetic animals wounded with an excisional wound, had significantly increased neutrophil MCL-1 gene expression (by 2.2 fold, $P<0.05$) compared to control. Consistent with a lack of change in expression of neutrophil activation markers neutrophil MCL-1 mRNA was not altered in the circulating neutrophils obtained from the sponge implant model.

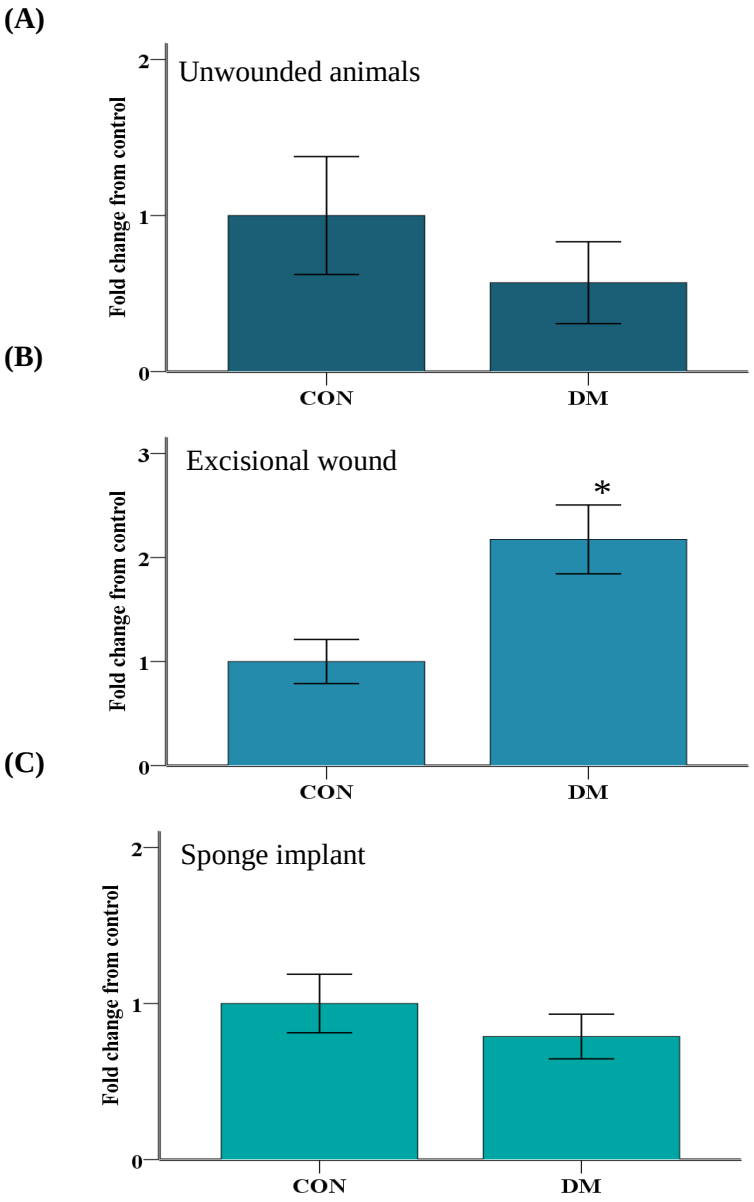

Figure 3-9: MCL-1 gene expression in the circulating neutrophils from (A) Unwounded, (B) excisional wounds and (C) sponge implant models in animals which had 3 weeks duration of diabetes.
*Control (CON, n=5) and diabetic (DM, n=5) animals. Values are corrected for expression of 36B4 as a housekeeper gene and expressed as fold change from control. Data are Mean ± SEM.*P <0.05 vs. control.*

3.3.2 **Effect of Longer Duration of Diabetes (6 Weeks) on Wound Healing**

3.3.2.1 **Blood glucose concentration in experimental animals**

As expected the diabetic animals had a higher non-fasting blood glucose level and lower body weight than control animals. Insulin treatment at a dose of 10IU/ day partially normalised these parameters. To establish that the insulin treatment regimen was effective in normalisation of blood glucose levels we performed serial measurements of blood glucose levels and showed near normal levels 4 hours and 12 hours post injection Table 3-1.

Table 3-1: Blood glucose level and body weight in the experimental animals

		Week 6			
Groups	Start weight (g) Week1	Termination weight (g)	BGL (mmol/L) 4hr after insulin injection	BGL (mmol/L) 12 hr after insulin injection	BGL (mmol/L) 24hr after insulin injection
CON	338.5±7.4	488.3±9.9	5.01±0.1	5±0.1	5.07±0.1
DM	334.8±6.3	373.8±10.2*	19.1±0.8*	21.3±0.8*	21.2±1.4*
DM+INS	329.1±11.2	421.7±9.6*	3.4±0.4#	9.2±1.6*#	17.12±0.6*#

*Results are expressed as Mean ± SEM, *P<0.05 different from Control (CON)*
#P<0.05vs diabetes. n=8/group

3.3.2.2 **Effect of diabetes on skin wounds**

Excisional wounds were created on the dorsal of rats and monitored during wound healing for 6 days. Six days later the animals were terminated and skin wounds were excised for measurement of gene and protein levels as described.

3.3.2.2.1 **Effect of diabetes on wound closure**

At day 6 the wounds were not fully closed in any of the groups. However the wounds in the control animals were significantly smaller than those in the diabetic group with a reduction wound area from day 0 (time of incision) of 65% ± 2% and 50% ± 2% in control and diabetic animals respectively ($P \leq 0.05$). Intense insulin treatment prevented the diabetes associated decrease in wound closure such that the reduction in wound area was not different to control at day 6 (Figure 3-10).

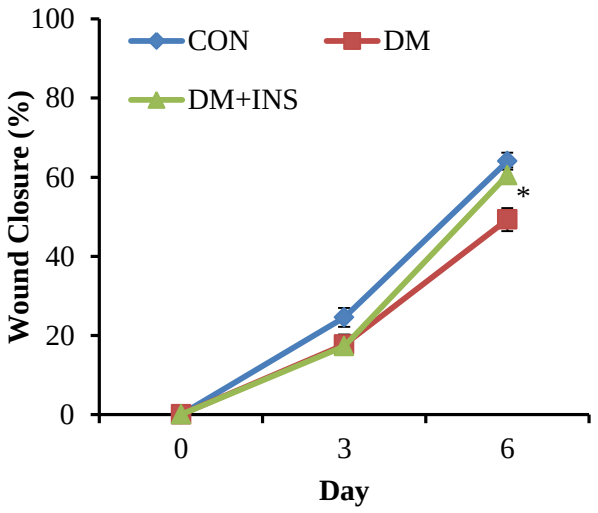

Figure 3-10: Percentage of wound closure in 6 weeks experimental animals after 6 weeks duration of diabetes.
Wound area was measured in control (CON, n=8) and diabetic (DM, n=8) and insulin-treated (DM+INS, n=8) animals at 3 and 6 days post wounding. Results are expressed as percentage of the initial area and expressed as Mean ± SEM. P<0.05 vs CON.*

3.3.2.2.2 **Effect of diabetes on skin wounds (Morphology)**

The histological profile of the wounds was also studied and representative H&E results are shown in Figure 3-11. As shown, in the wound from the diabetic animal the extracellular matrix was less well formed, (i.e. less thickness and less intense staining, suggesting less matrix volume), than that seen in the wound from the control animal. Insulin treatment partially prevented these changes.

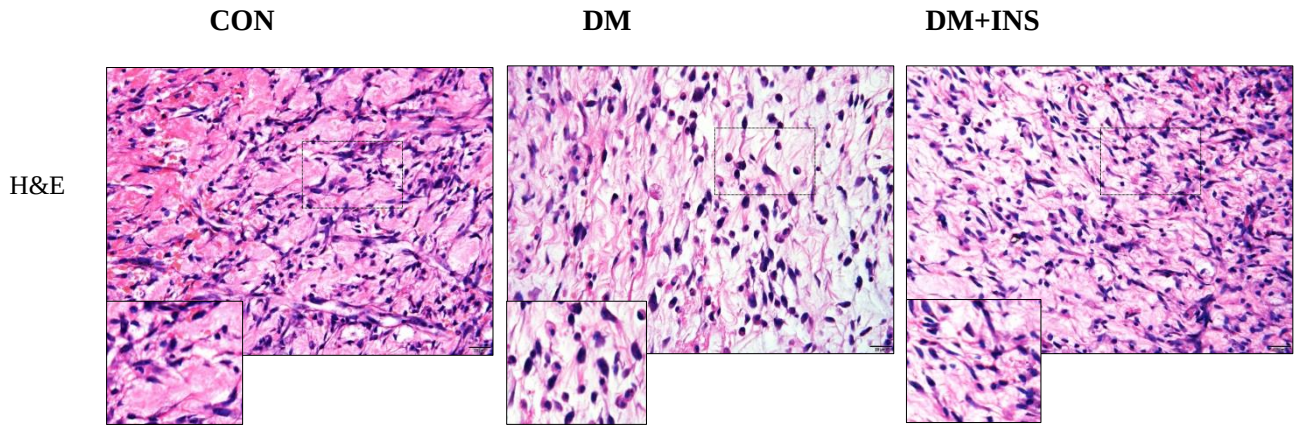

Figure 3-11: Representative images of skin wounds from excisional wounds.
Wound morphology by H&E staining in control (CON, n=4) diabetic (DM, n=3) and insulin-treated (DM+INS, n=4) groups. Image magnification ×400.

The sections were also stained with PSR and the PSR sections were also examined using polarised light microscopy, to detect and analyse collagen I and III fibres. Using the PSR stain the collagen deposition was scored from light (0) to dark (3) in the dermis of skin wounds and analysed using Chi-Square Tests (Table 3-2). Results showed the mature collagen bundles in control animals are long, thick, and wavy. The collagen bundles were appeared thin and less formed in diabetic wounds ($P<0.01$). Insulin treatment partially improved collagen deposition (Figure 3-12-A).

In the present study, picrosirius red stained tissues under polarising microscopy showed different colour and orientation of collagen fibres between different groups. Control groups showed predominantly yellow-orange birefringence combined with some amount of yellowish-green birefringence towards hypodermis. In contrast, in diabetic group the predominant birefringence was red yellowish and yellowish-green was less formed. Yellow-orange birefringence was observed mainly in thick fibres and red-yellowish was localised in thin fibres (Figure 3-12-B).

Table 3-2: Chi-Square tests for analysis of PSR staining in the skin wounds.

	Dermis	
	Scores < 2	**Scores ≥ 2**
CON	**0**	**4**
DM	**3**	**0**
DM+INS	**3**	**1**
Asymp. Sig. (2-sided)	**0.01***	

**P<0.05 different between groups*

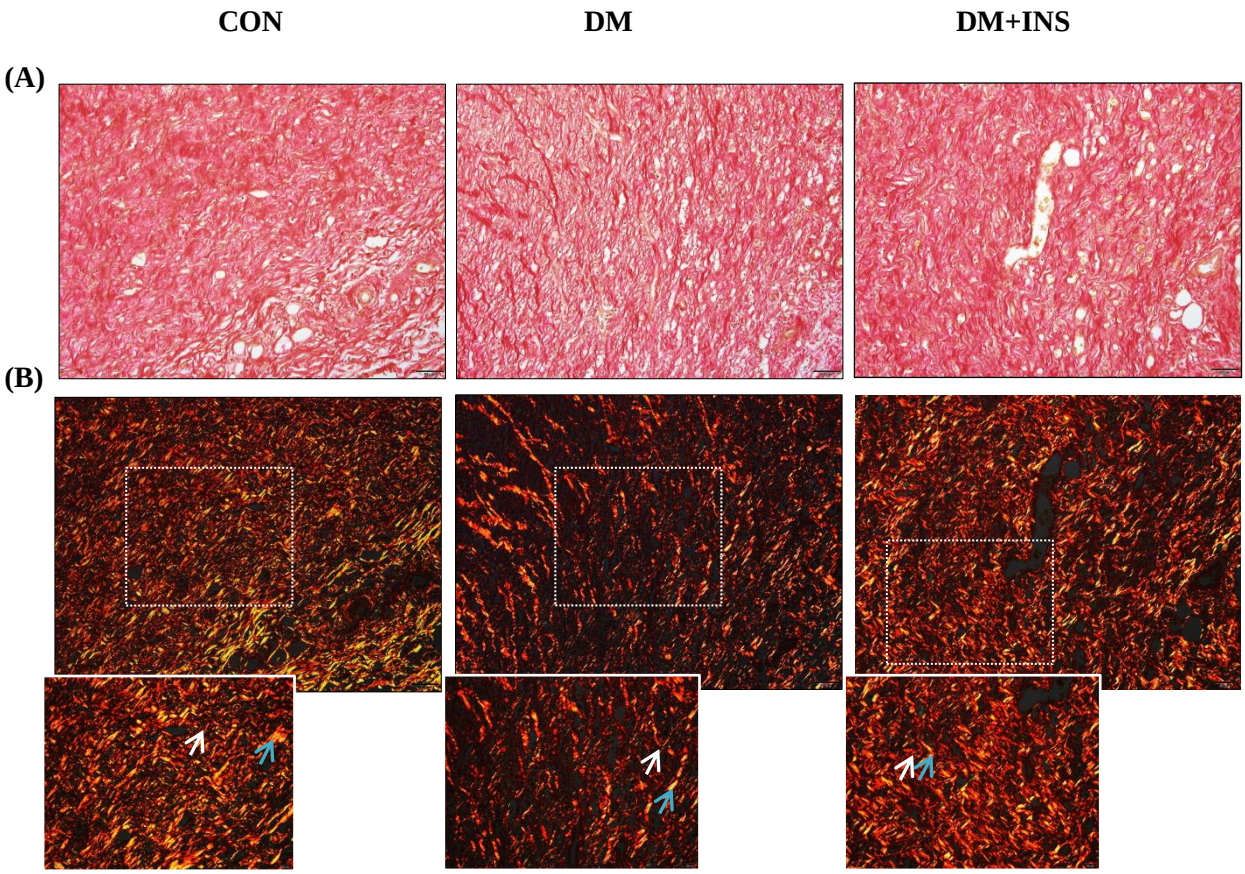

Figure 3-12: Representative images of skin wounds from excisional wounds.
Showing collagen deposition by (A) PSR staining and (B) polarised light microscopy in control (CON, n=4) diabetic (DM, n=3) and insulin-treated (DM+INS, n=4) groups. Image magnification ×400. White arrow thin fibres. Blue arrow thick fibres.

3.3.2.2.3 **Effect of diabetes on wound NGAL and MMP-9 expression**

The expression and localisation of NGAL and MMP-9 in wounded skin was studied by IHC and in a parallel series NGAL co-localisation with MMP-9 was examined by IF staining.

As shown in Figure 3-13 the representative photos NGAL staining was increased in the diabetic wounds tissue (Chi-Square Tests, $P<0.01$). Histopathology changes were scored from none (0) to intense brown (3) based on the isotype control as 0. The average of scores were then grouped into scores less than 2 and scores greater

than 2 for Chi-Square Tests. As shown in Table 3-3 significant changes were only observed in the dermis area, where the count number greater than 2 was higher in the diabetic group than the control group.

Table 3-3: Chi-Square tests for analysis of NGAL staining in the skin wounds.

	Epidermis		**Dermis**		**Hypodermis**	
	Scores < 2	**Scores ≥ 2**	**Scores < 2**	**Scores ≥ 2**	**Scores < 2**	**Scores ≥ 2**
CON	4	1	5	0	1	4
DM	5	0	1	4	0	5
DM+INS	4	0	4	0	1	3

	Asymp. Sig. (2-sided)
Epidermis	.37
Dermis	**.006***
Hypodermis	.5
Overall	**.045***

**P<0.05 different between groups.*

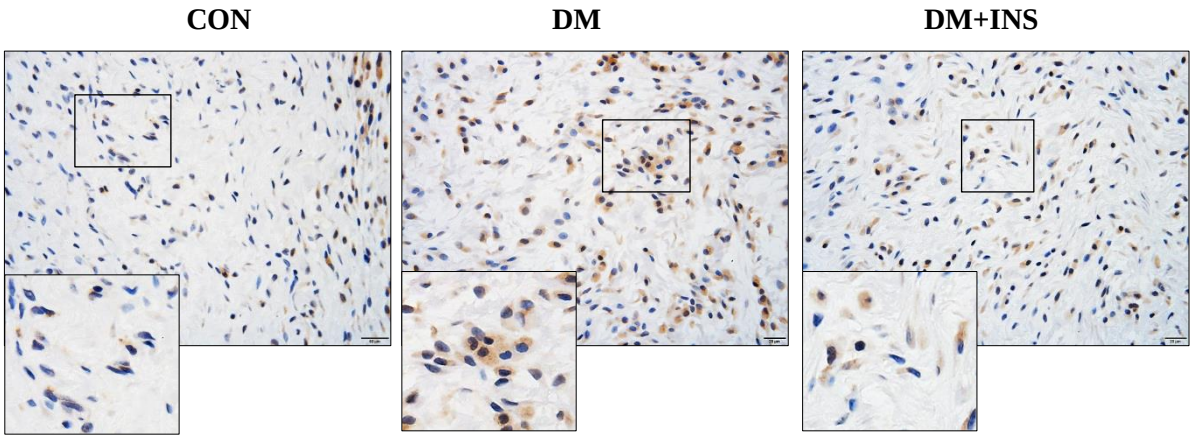

Figure 3-13: IHC representative images of skin wounds for NGAL expression.
Showing NGAL in control (CON, n=5) diabetic (DM, n=5) and insulin-treated (DM+INS, n=4) animals by IHC in the wound granulation tissue. Image magnification×400.

MMP-9 expression was also examined in the wound skins by IHC staining and representative images are shown in (Figure 3-14). The number of MMP-9 positive cells which were calculated using ImageJ was significantly higher in diabetic animals compared to control. These changes were prevented by insulin treatment.

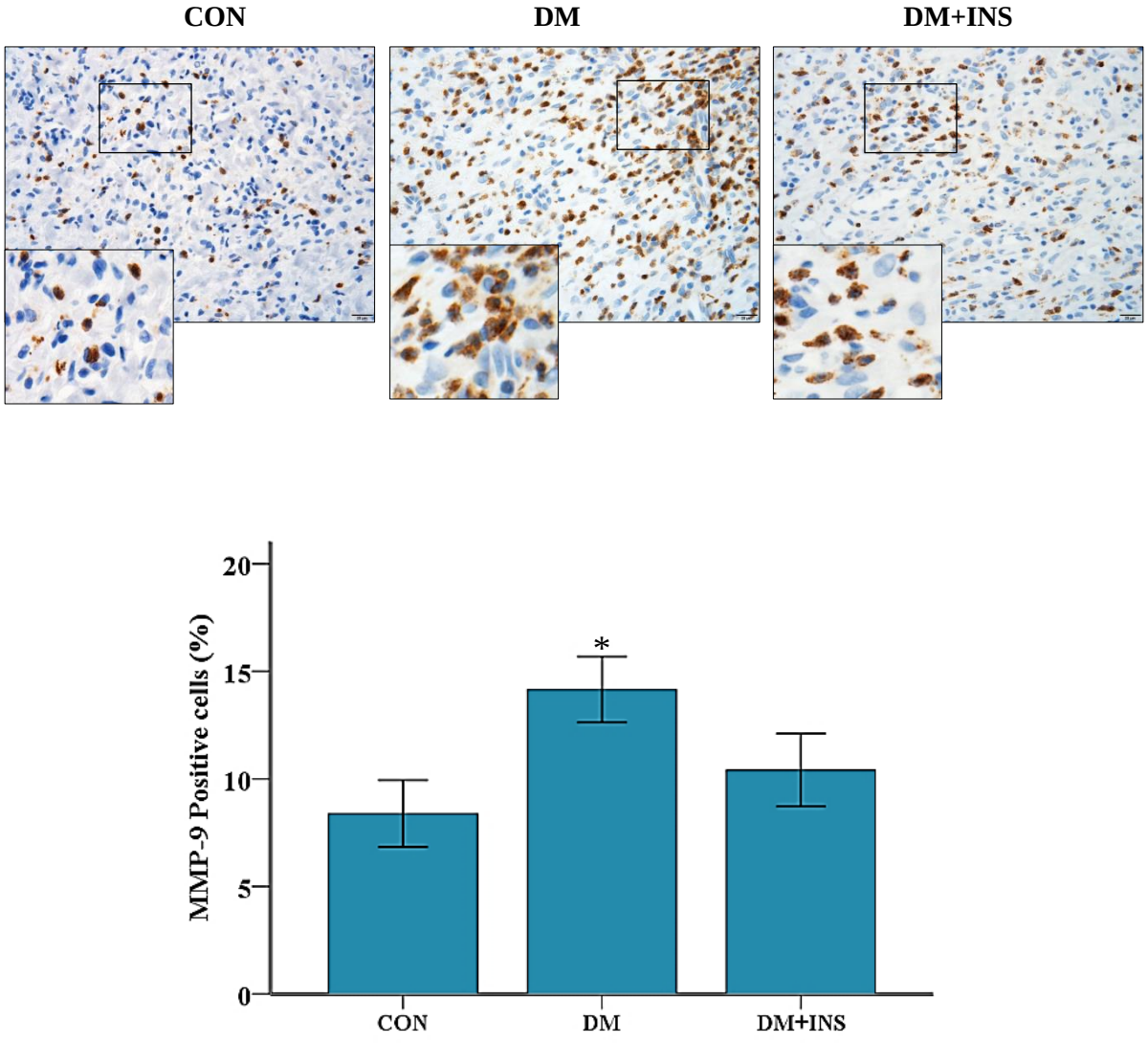

Figure 3-14: IHC representative images and group data for MMP-9 staining in skin wounds.
*Representative images of skin wounds showing MMP-9 staining and the localisation as well as the group data in control (CON, n=5) diabetic (DM, n=5) and insulin-treated (DM+INS, n=4) animals. Data are Mean ± SEM. Image magnification ×400.*P<0.05 vs. control.*

The results from IF staining for NGAL/MMP-9 co-localisation showed that the majority of NGAL positive cells were co-localised with MMP-9 in the skin wounds (Figure 3-15).

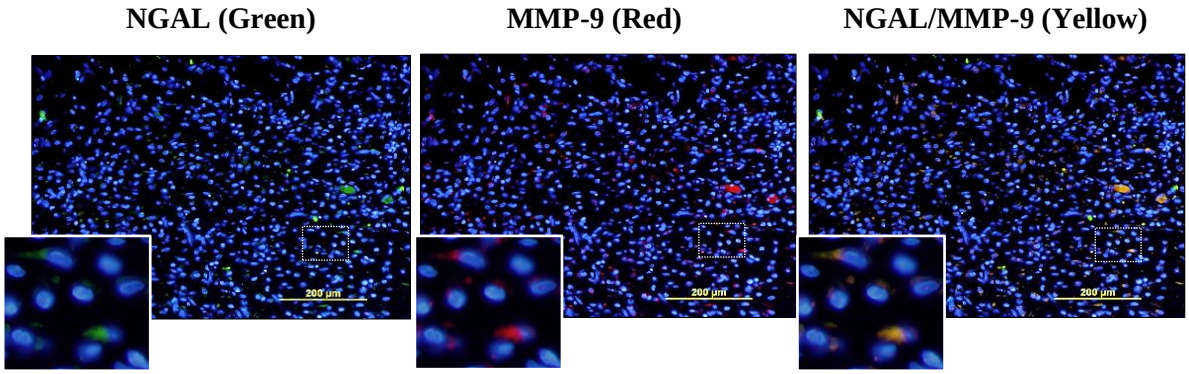

Figure 3-15: IF representative images of skin wounds for NGAL/MMP-9 co-localisation. *Showing IF pattern of expression of NGAL and MMP-9, and NGAL/MMP-9 co-localisation in the wound granulation tissue in diabetic animals. Image magnification ×200.*

3.3.2.2.4 **Effect of diabetes on skin wound gene expression**

The skin wounds were obtained at termination (day 6) and frozen at -80°C until use. The wound tissue was then homogenised in Tri Reagent and RNA was extracted as described in Chapter 2. As shown at the gene level diabetes increased NGAL (by 2 fold compared to control) and this change were prevented by insulin treatment Figure 3-16. The expression of MMP-9 was not different across the groups and MMP-8 tended to be increased in the diabetic and insulin-treated animals but this failed to reach statistical significance. Pro-inflammatory mediators TLR-4 and TLR-2 were increased in diabetic animals. Insulin treatment significantly prevented the increase in expression of TLR-2 in diabetic animals ($P<0.05$, T-test) and partially normalised TLR-4.

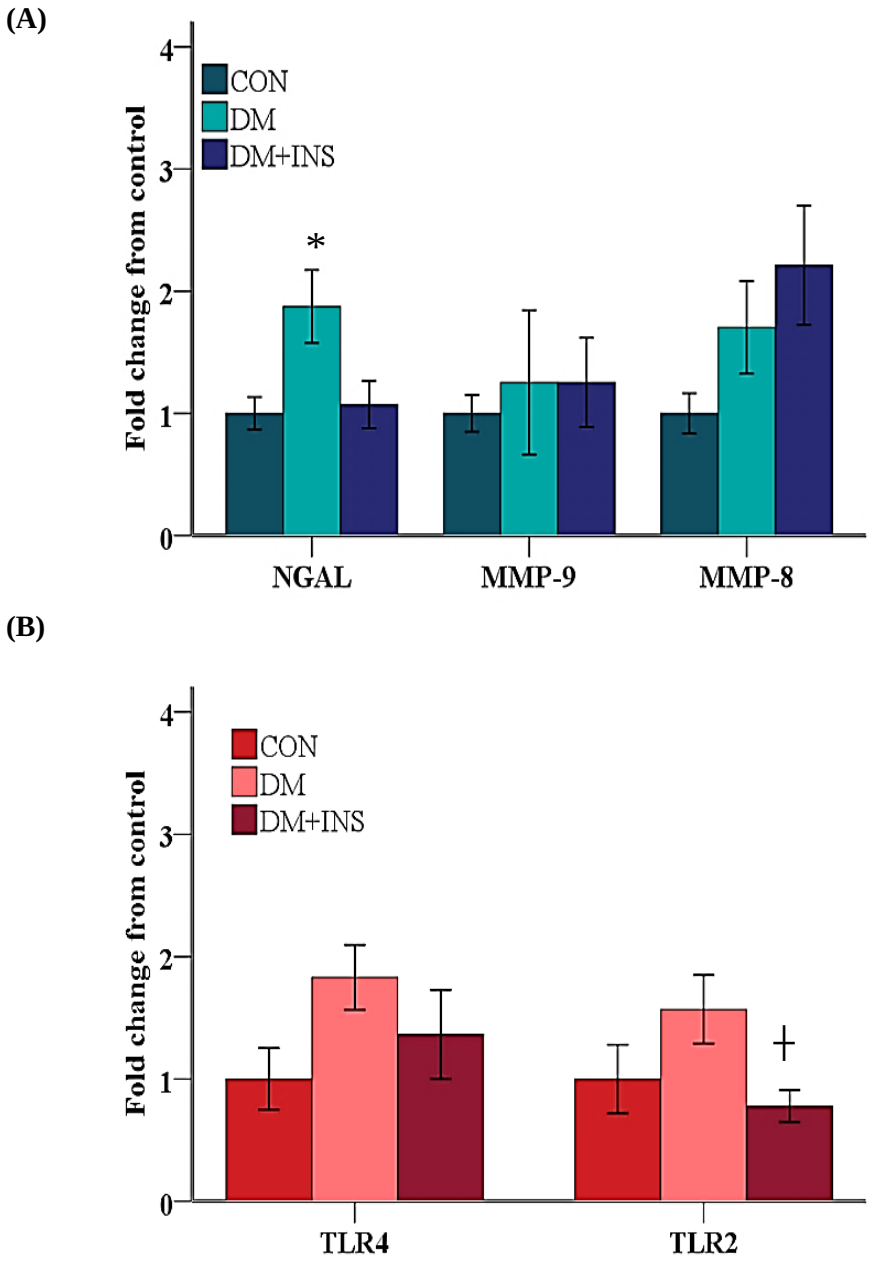

Figure 3-16: Gene expression of (A) NGAL, MMP-9, MMP-8, (B) TLR4 and TLR2 in the skin wounds.
*Control (CON, n=12), diabetic (DM, n=5) and insulin-treated (DM+INS, n=6) animals. Values are corrected by 36B4 as housekeeper gene and expressed as fold change from control. Data are Mean ± SEM.*P<0.05 vs. control. †P<0.05 vs. diabetic by T-test.*

3.3.3 **Effect of diabetes on sponge implant wound cells**

To determine the effect of diabetes on wound inflammatory cells a wound implant model was used. The cells and fluids were extracted from the implant at days 3, 6 and 12 post implant and the expression of NGAL, MMP-9 and MMP-8 as well as some pro-inflammatory mediators was analysed.

3.3.3.1 **Effect of diabetes on wound inflammatory cell gene expression**

Shown in Figure 3-17 the gene expression of NGAL was significantly up regulated in the diabetic animals at day 6 and persisted at day 12 (each approximately 3.5 fold vs. control, $P<0.05$). MMP-8 expression was increased at day 6, but not day 3 and day 12. In contrast the expression of MMP-9 was not altered at any of the time points studied. Additionally, diabetes had no effect on the expression of TLR2, TLR4, and TNFα (Figure 3-18). The diabetes associated increase in NGAL and MMP-8 gene expression was prevented by insulin treatment. Interestingly in the insulin-treated animals the expression of MMP-9 was significantly increased at day 12 ($P<0.05$).

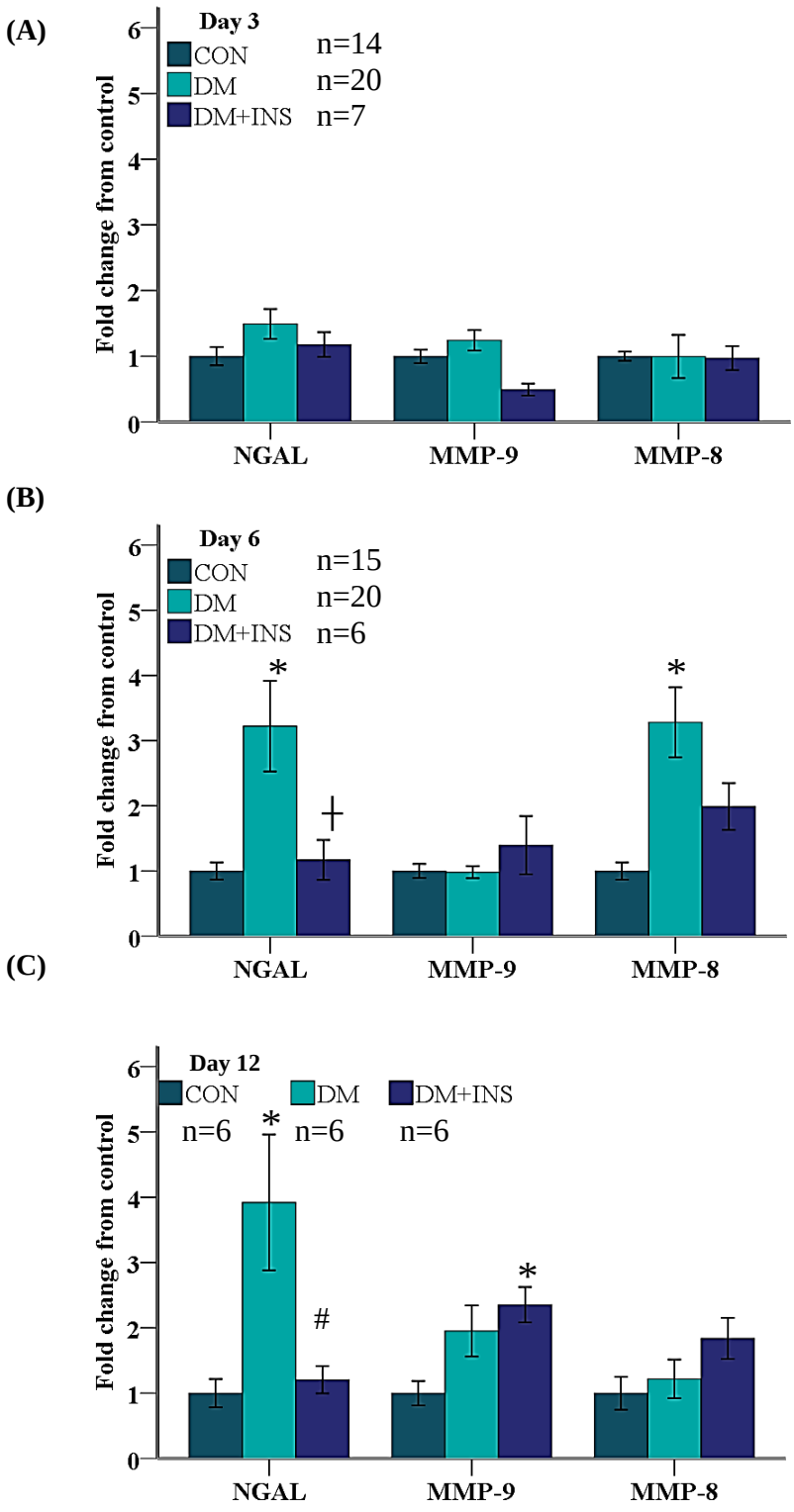

Figure 3-17: Gene expression of NGAL, MMP-9, and MMP-8 of sponge implant inflammatory cells.

*Control (CON), diabetic (DM) and insulin-treated (DM+INS) animals (A) 3days, (B) 6 days, and (C) 12 days after wounding. Values are corrected for the expression of 36B4 as the housekeeper gene and expressed as fold change from control. Data are Mean ± SEM. *P<0.05 vs control. #P<0.05 vs. diabetic. †P<0.05 vs. diabetic by T-test.*

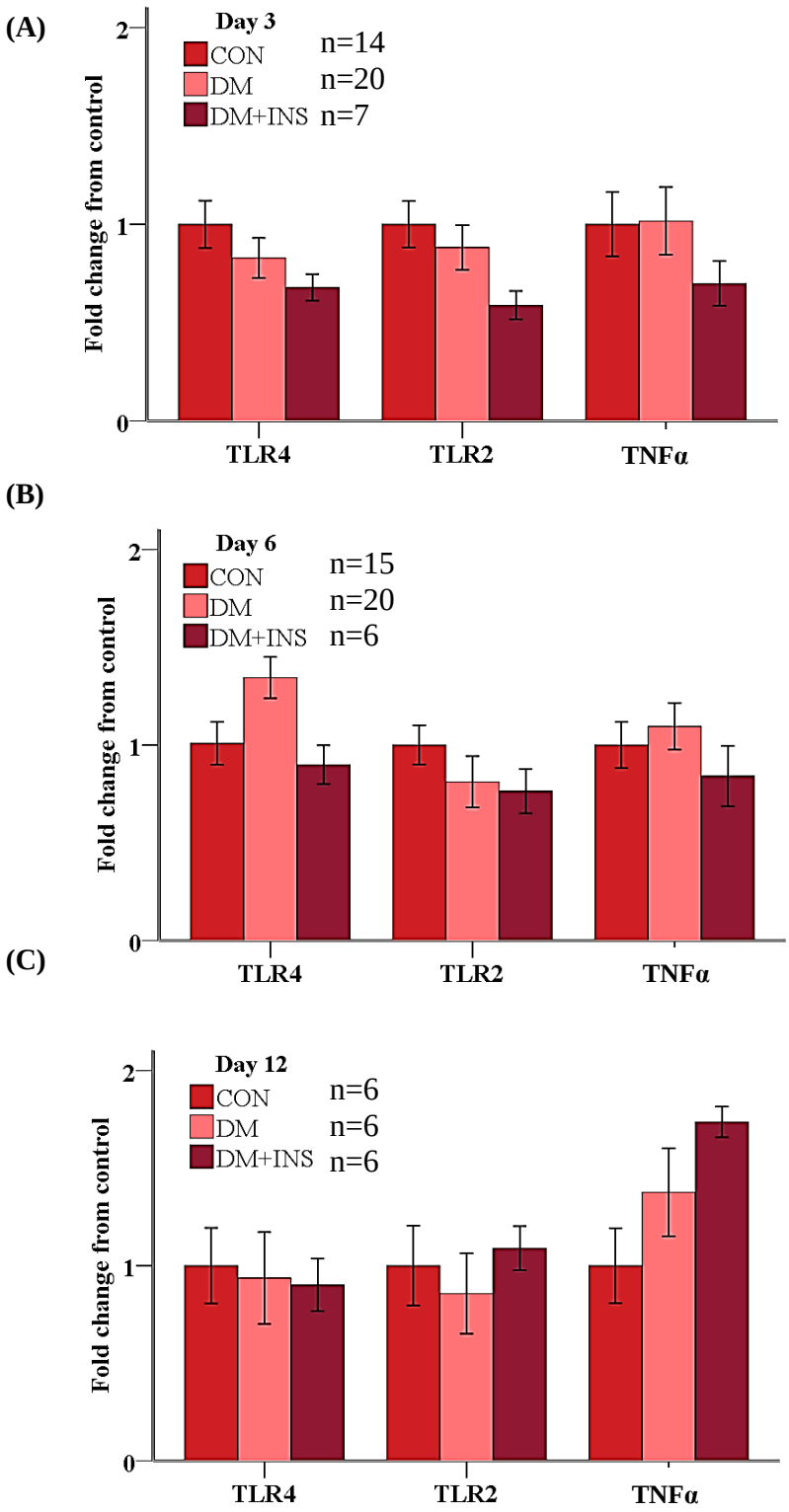

Figure 3-18: Gene expression of TLR4, TLR2, and TNFα in sponge implant inflammatory cells.

*Control (CON), diabetic (DM) and insulin-treated (DM+INS) animals (A) 3days, (B) 6 days, and (C) 12 days after wounding. Values are corrected for expression of 36B4 as housekeeper and expressed as fold change from control. Data are Mean ± SEM.*P<0.05 vs. control. # P<0.05 vs. diabetic.*

3.3.3.2 **Effect of diabetes on wound inflammatory cell number**

The implant inflammatory cell number and in particular the numbers of neutrophils and macrophages was counted at day 6 by immunofluorescence using MPO and CD68 (red) respectively. Shown in Figure 3-19-A compared with controls, in diabetic fluids the percentage of MPO positive cells were significantly increased (by 2 fold). A similar trend was observed for the macrophages but this failed to reach significance. Insulin treatment prevented these changes.

Co-localisation studies showed that whilst some macrophages stained positively for NGAL (yellow) it was predominantly associated with neutrophils. The percentage of NGAL positive cells (green) was significantly increased ($P<0.05$) in the diabetic animals and this increase was seen in both neutrophils and macrophages. The increase in the percentage of NGAL positive cells was not observed in the insulin-treated animals (Figure 3-19).

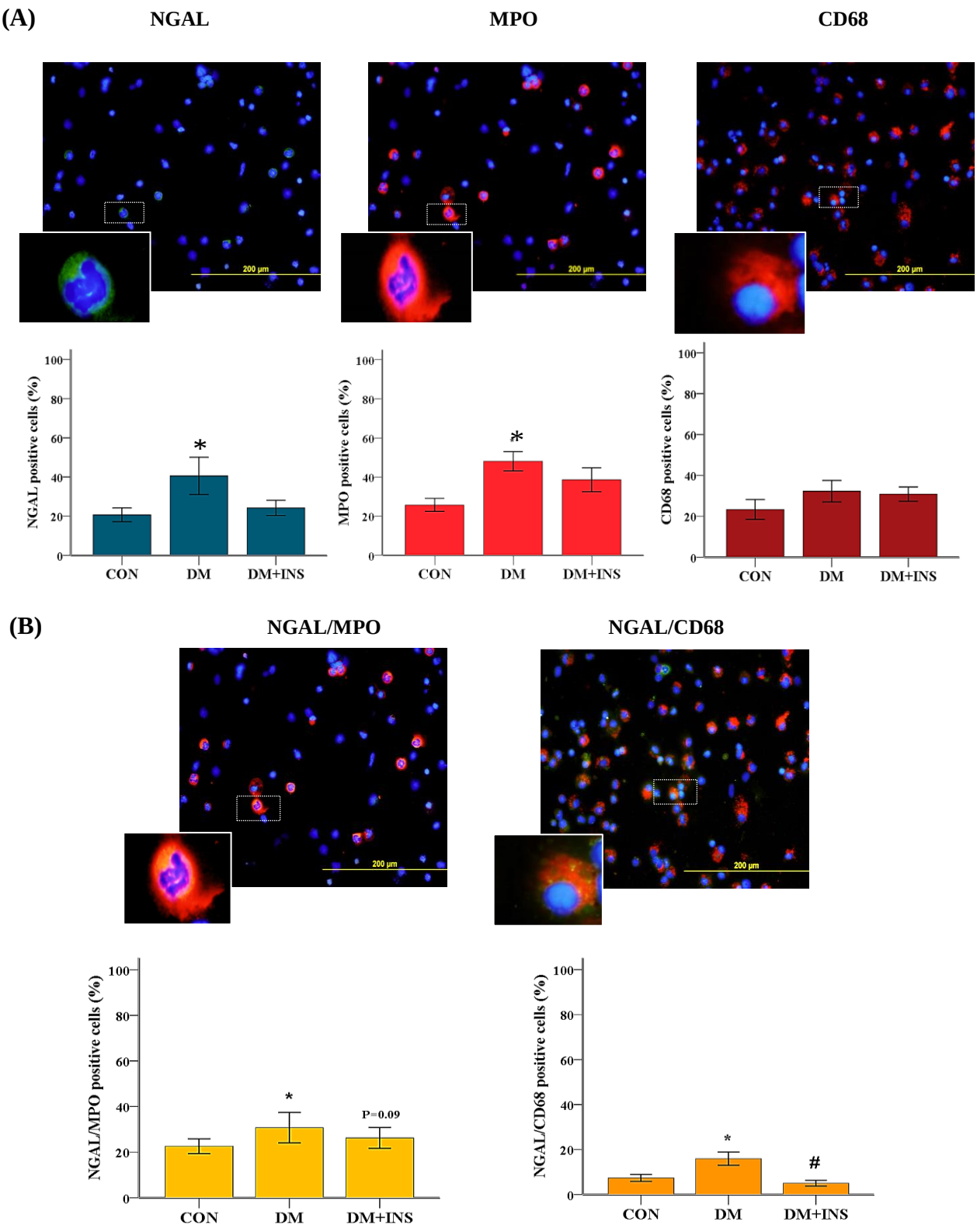

Figure 3-19: Representative images of sponge inflammatory cell infiltrates.
*Showing (A) NGAL (green), MPO (red), and CD68 (red) expression and percentage (B) NGAL/MPO (yellow) and NGAL/CD68 (yellow) co-localisation. Representative images from control (CON) diabetic (DM) and insulin-treated (DM+INS) animals by IF. Image magnification ×400. Group data is percentage positive cells. Data are Mean ± SEM. *P<0.05 vs control. #P<0.05 vs diabetic.*

3.3.3.3 **Flowcytometric identification of wound fluid neutrophils**

Flow cytometry was also used to examine the wound fluid for neutrophil number. In these studies the RP-1 antibody a specific rat neutrophil markers was used.

Figure 3-20-A shows a representative dot plot of inflammatory cells in the rat wound fluid. Neutrophil population was first identified by size and granularity and the percentage of neutrophils calculated. Using this approach and by their staining with RP-1 no difference in the number of neutrophils as a percentage of the total cell population was observed (Figure 3-20-B).

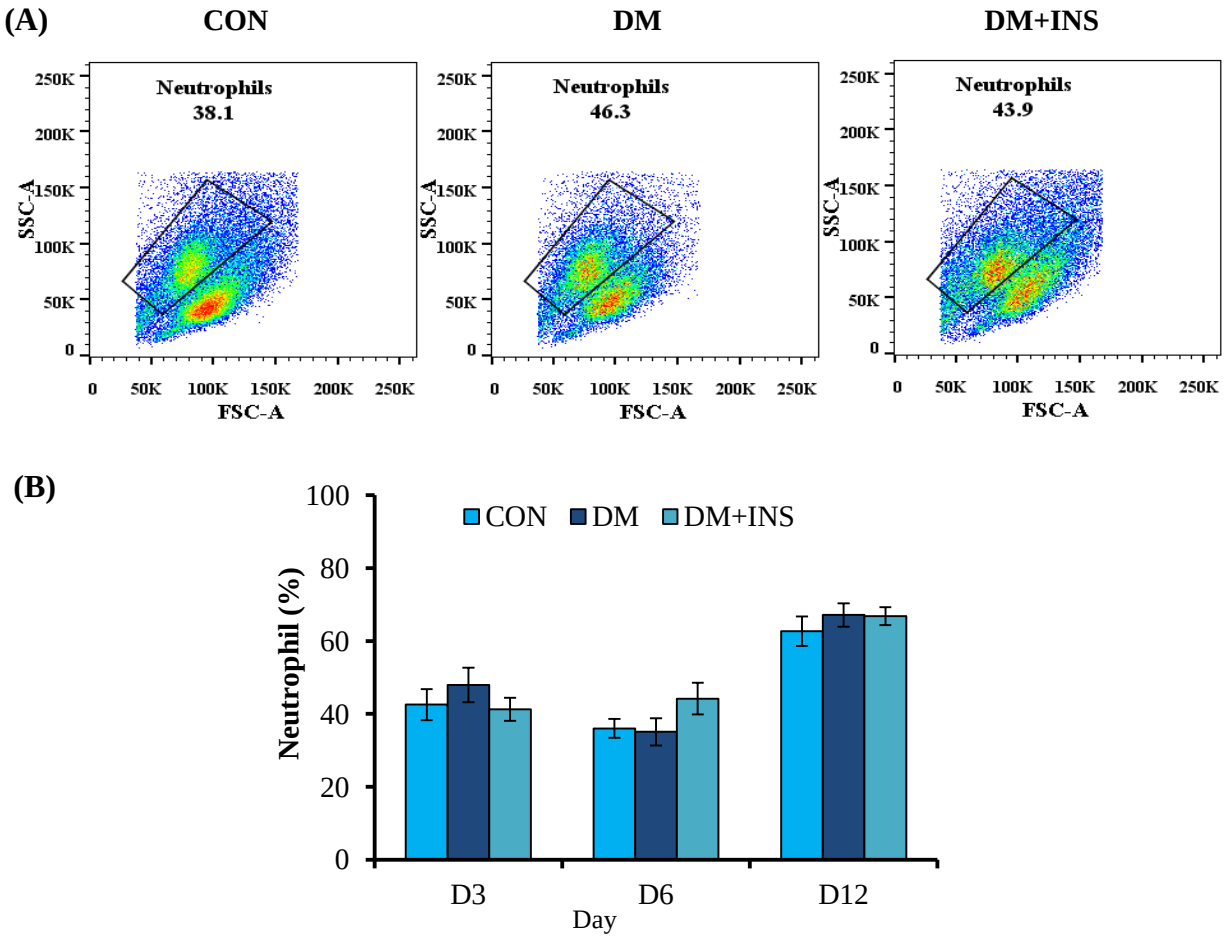

Figure 3-20: Sponge implant inflammatory cells numbers by flow cytometry.
Control (CON, n=6), diabetes (DM, n=6) and insulin-treated (DM+INS, n=6) animals 3, 6, 12 days after wounding. Data are Mean ± SEM.

3.3.4 **Western blot analysis**

To identify the NGAL/MMP-9 complex, plasma samples were first subjected to western blot analysis using an antibody against NGAL, as well as an anti-MMP-9 antibody. Under non-reducing conditions a high-molecular-weight form of NGAL at 115kDa was detected with the anti-NGAL antibody and a band at the same molecular weight was detected with the anti-MMP-9 antibody (Figure 3-21-A). These results suggested that the band at 115kDa is a complex between NGAL/MMP-9. A similar complex was also observed in wound fluid samples (Figure 3-21-B). In wound fluids there was an additional NGAL/MMP-9 complex that was identified by both NGAL and MMP-9 antibodies at approximately 125kDA.

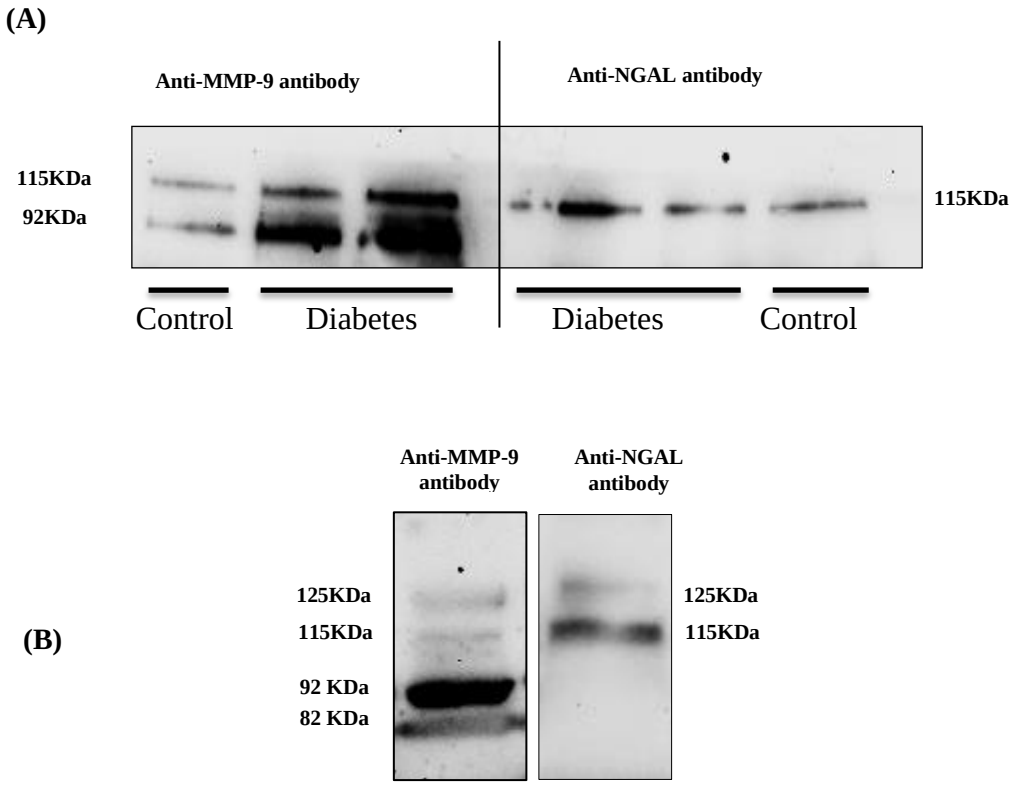

Figure 3-21: Representative images showing western blot analysis of NGAL and MMP-9 in (A) plasma and (B) wound fluid.

3.3.5 **Effect of diabetes on implant fluid MMP-9 activity and NGAL levels**

Gelatin zymography was used as a tool to measure MMP-9 protein in its pro as well as biologically active forms. Bands of gelatinolytic activity at 115 and 125 kDA were also observed and from our western blot analysis this band corresponded with the NGAL/MMP-9 complex, enabling us to also examine NGAL/MMP-9 complex formation in these samples. Results are shown in (Figure 3-22). In diabetic animals there was a transient increase in total MMP-9 (TMMP-9) at day 6 ($P<0.001$) but this was not apparent at day 3 and day 12. This increase in TMMP-9 was due to an increase in the active form of MMP-9 at day 6 and was prevented by insulin treatment. Dysregulation between NGAL and MMP-9 levels were observed. In diabetic animals increased levels of NGAL/MMP-9 complex were observed at day 6 and unlike the levels of MMP-9, which decreased, the increase in NGAL/MMP-9 complex persisted at day 12. Interestingly compared with both non-treated diabetic and control animals insulin treatment decreased total MMP-9 (both pro and active forms) at day 3 and increased its levels at day 12. Insulin treatment prevented the diabetes associated increase in NGAL/MMP-9 complex becoming significant at day 12.

By ELISA the free NGAL concentration tended to be decreased in the implant fluids from diabetic animals but this failed to reach significance (Figure 3-22-B).

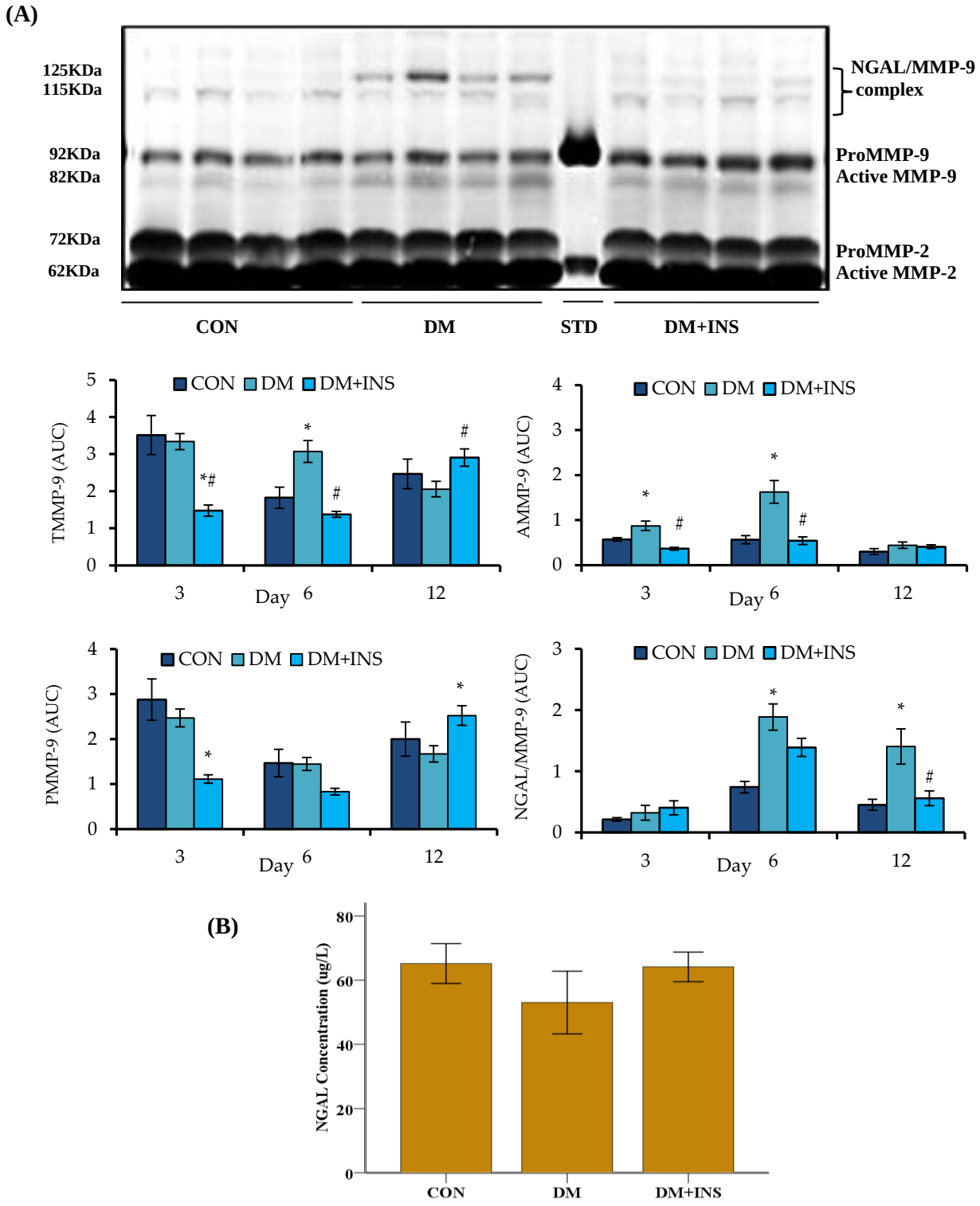

Figure 3-22: The effect of diabetes on MMP-9 activity and NGAL level in wound fluid. *A representative zymograph showing the enzymatic activity of MMP-9, (total: TMMP-9, pro: PMMP-9 and active: AMMP-9) as well as NGAL/MMP-9 complex. Whole group data 3, 6, 12 days after wounding are shown graphically.(B) NGAL in wound fluid, measured by ELISA 6 days after wounding in control (CON), diabetic (DM) and insulin-treated diabetic (DM+INS) animals. AUC (Area Under Curve). Data are Mean ± SEM,*P <0.05 vs control. #P<0.05 vs diabetic.*

3.3.6 **Effect of Diabetes on Circulating Neutrophil Gene Expression**

3.3.6.1 **Effect of diabetes on the circulating neutrophils gene expression in unwounded animals**

To investigate the effect of diabetes on circulating neutrophil activation, the gene expression of NGAL, MMP-9, MMP-8 as well as TLR4, TLR2, and TNFα was measured in the plasma of blood obtained at termination from unwounded normal and diabetic animals with 6 weeks duration of diabetes.

As shown in Figure 3-23, in diabetic animals the gene expression of NGAL, MMP-9, MMP-8 was increased, however this was only statistically significant for MMP-9 ($P<0.05$, T-test). The gene expression of TLR4 tended to increase, and TLR2 mRNA was not changed across the groups. The expression of TNFα gene was significantly increased in the diabetic animals by 4 fold. Insulin treatment did not prevent the diabetes-induced changes but interestingly compared to control significantly increased the circulating neutrophil NGAL and TNFα gene expression by 3 and 25 fold respectively.

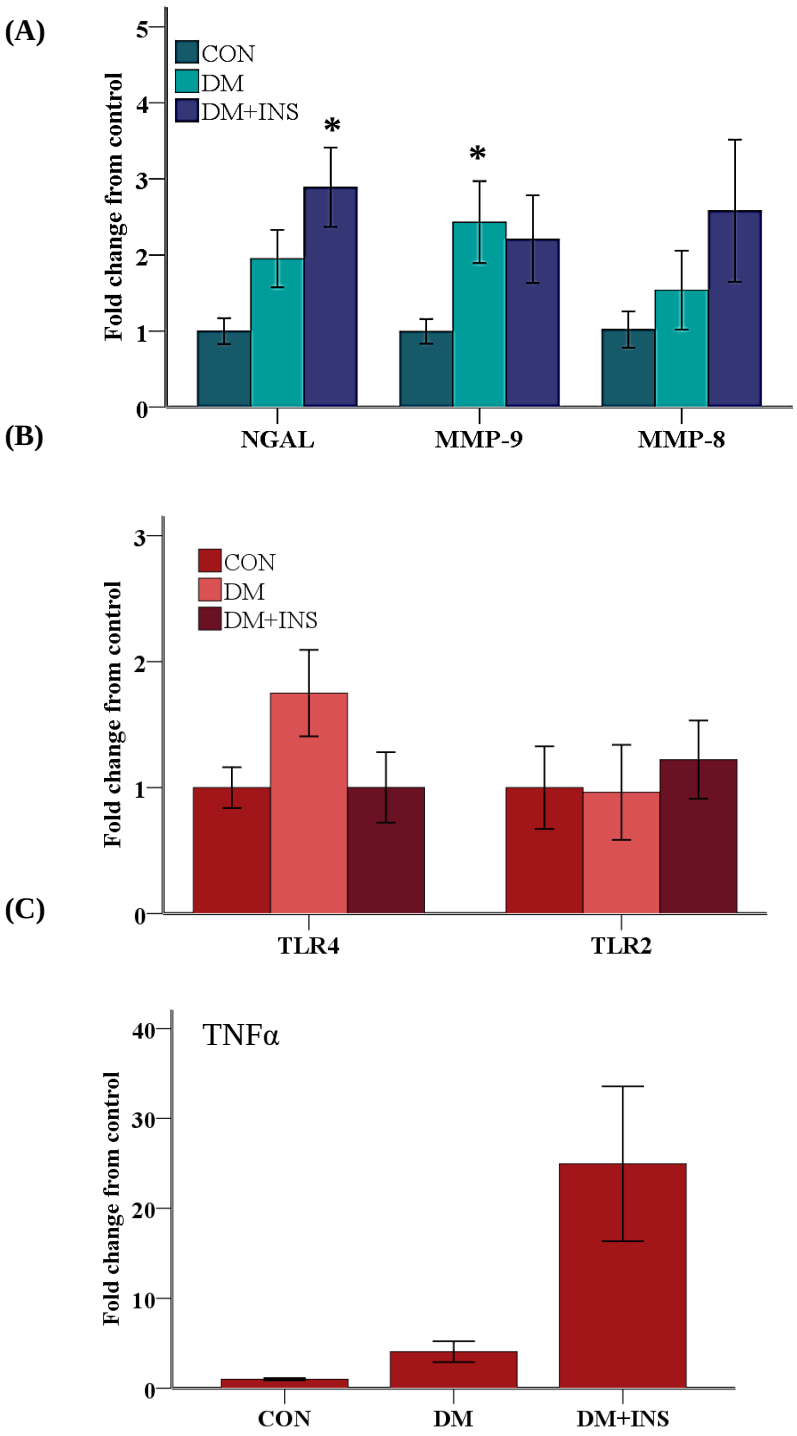

Figure 3-23: Gene expression of (A) NGAL, MMP-9, MMP-8, (B)TLR4, TLR2 and (C) TNFα in circulating neutrophils from unwounded animals.
*Control (CON, n=5), diabetic (DM, n=4) and insulin-treated (DM+INS, n=5) animals. Values are corrected by 36B4 as housekeeper gene and expressed as fold change from control. Data are Mean ± SEM.*P <0.05 vs. control.*

3.3.6.2 **Effect of wounding on the circulating neutrophils gene expression in diabetic animals**

The effect of diabetes on circulating neutrophil mRNA, the gene expression of NGAL, MMP-9, MMP-8 as well as TLR4, TLR2, and TNFα was first measured in the animals in the excisional wound models. As shown in Figure 3-24, compared to similarly treated controls, diabetes and wounding increased the gene expression of the activated neutrophil markers NGAL, MMP-9, and MMP-8, as well as TLR2, and TNFα, but this increase was only statistically significant for TLR2. Insulin treatment prevented the increase in NGAL.

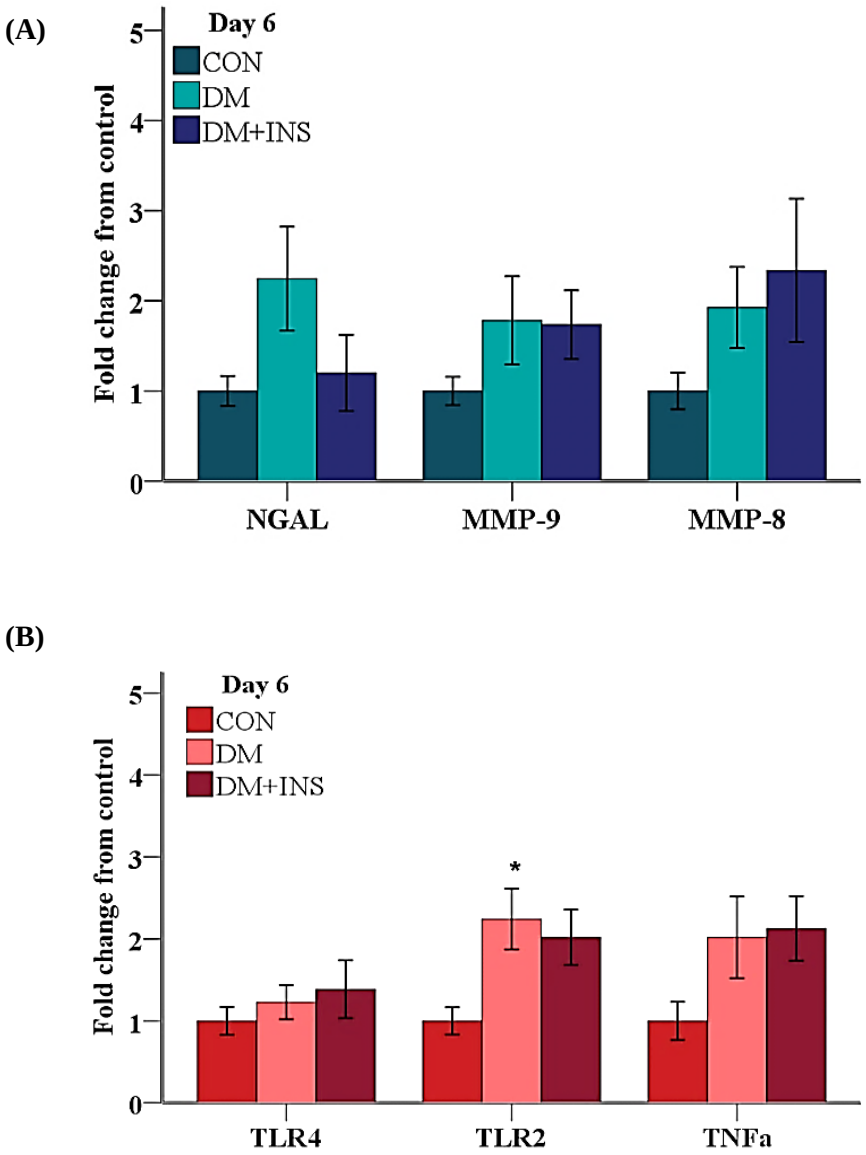

Figure 3-24: Gene expression of (A) NGAL, MMP-9, MMP-8, (B) TLR4, TLR2 and TNFα in circulating neutrophils from the excisional wound models.
*Control (CON, n=7), diabetic (DM, n=9) and insulin-treated (DM+INS, n=6) animals. Values are corrected by 36B4 as housekeeper gene and expressed as fold change from control. Data are Mean ± SEM.*P<0.05 vs. control. n=4-5*

We had previously in the pilot study in rats with 3 weeks duration of diabetes observed a different pattern of expression of neutrophil activation markers in the different wound models. Therefore, we also investigated circulating neutrophil activation markers in the animals that had received the sponge implant. In this group of animals neutrophils were isolated from blood obtained at termination i.e. 3 days, 6 days, and 12 days after wounding.

As Figure 3-25 shows, compared to similarly treated controls the neutrophil expression of MMP-9 and MMP-8 was significantly increased in diabetic animals at day 3 and day 6 after wounding, and similar trend was observed at day 12 but this failed to reach significance. The expression of NGAL, TLR4, TLR2, and TNFα were also increased in diabetic animals at day 6 but returned to control levels at day 12 (Figure 3-26). Insulin treatment prevented these changes.

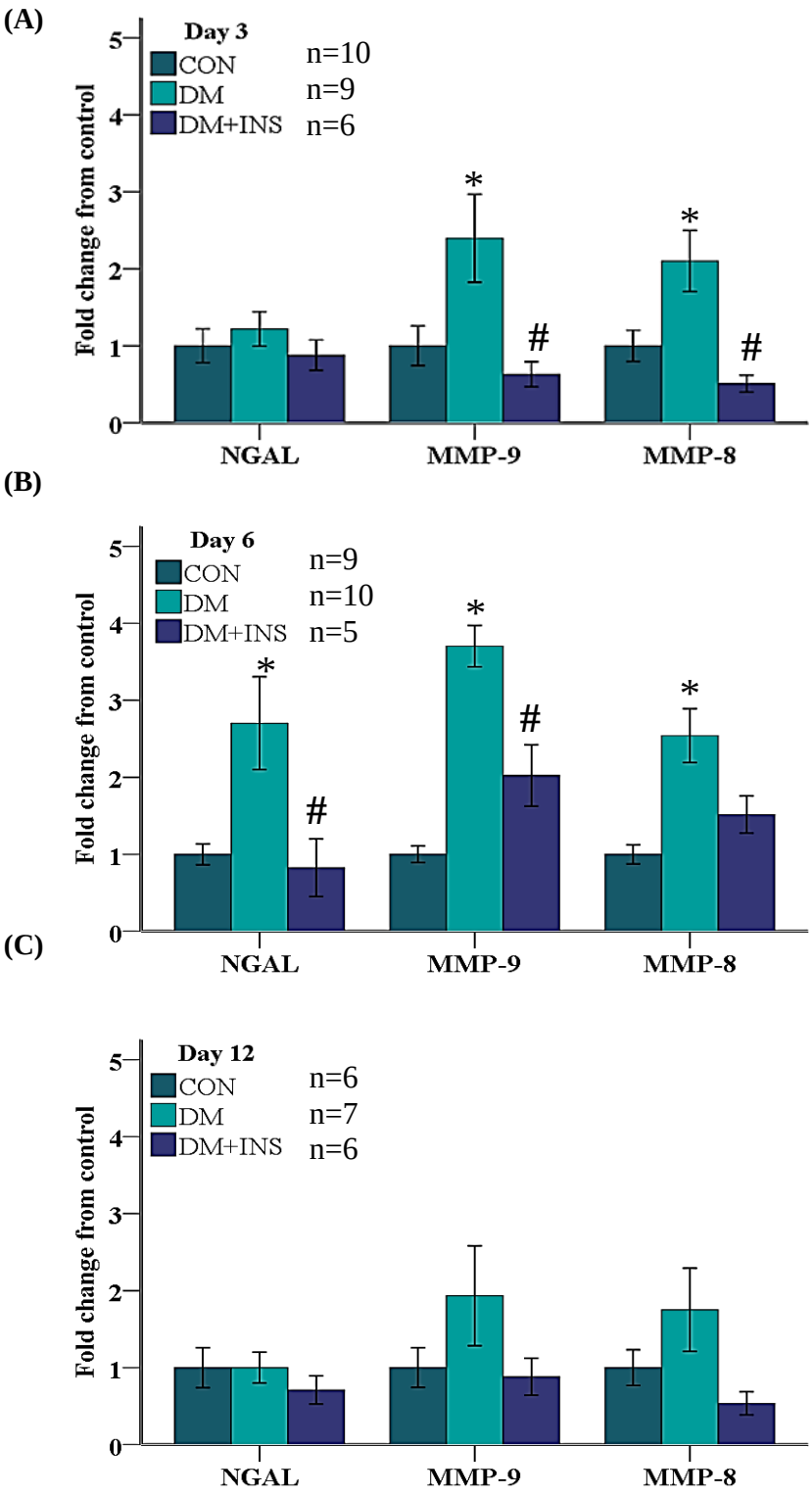

Figure 3-25: Gene expression of NGAL, MMP-9, and MMP-8, in circulating neutrophils from sponge implant model (A) 3, (B) 6 , and (C) 12 days after wounding.
*Control (CON), diabetic (DM) and insulin-treated (DM+INS) animals. Values are corrected by 36B4 as housekeeper gene and expressed as fold change from control. Data are Mean ± SEM.*P<0.05 vs. control. #P<0.05 vs. diabetic.*

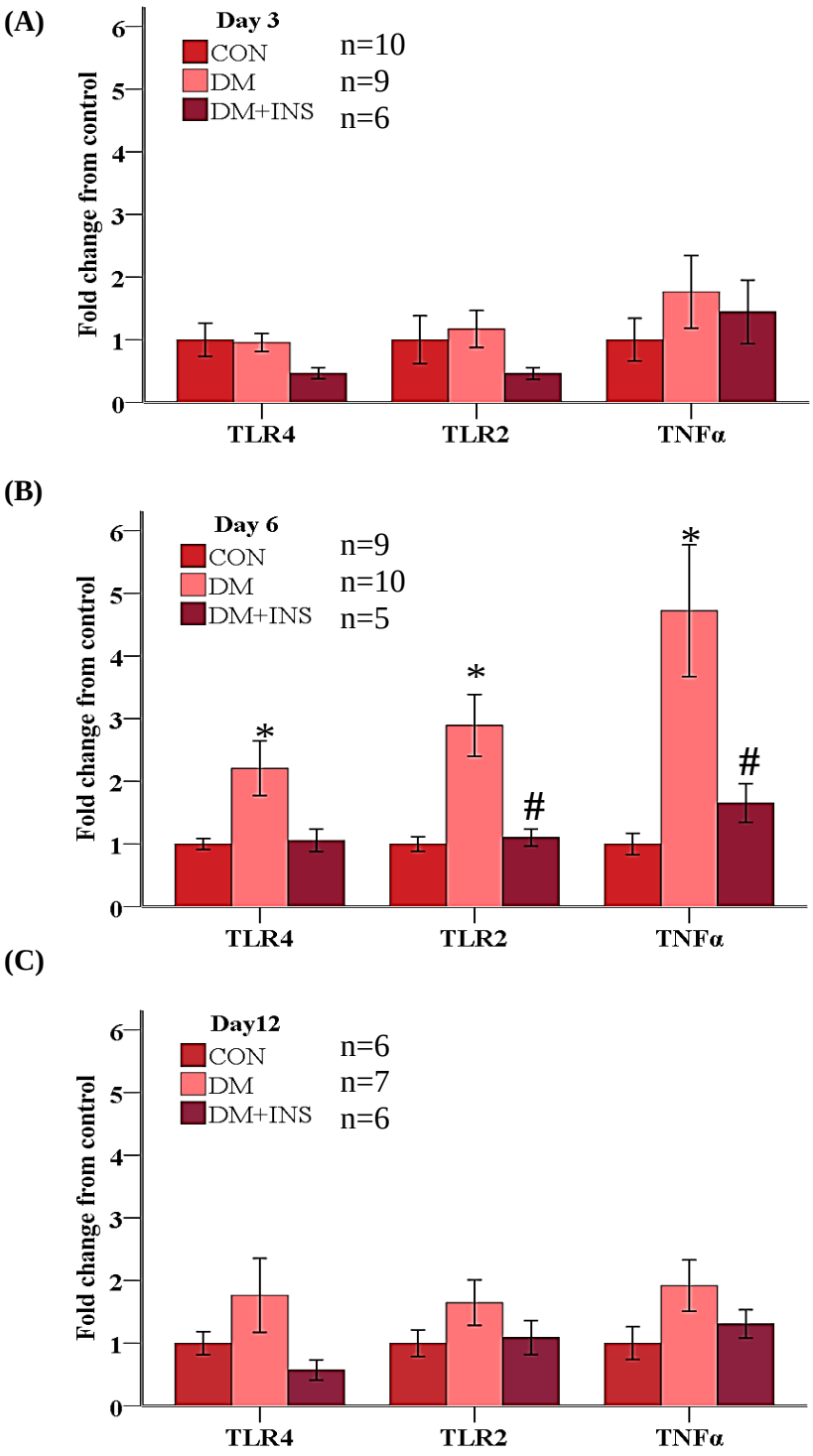

Figure 3-26: Gene expression of TLR4, TLR2, and TNFα, in circulating neutrophils from sponge implant model (A) 3, (B) 6 , and (C) 12 days after wounding.
*Control (CON), diabetic (DM) and insulin-treated (DM+INS) animals. Values are corrected by 36B4 as housekeeper gene and expressed as fold change from control. Data are Mean ± SEM. *P <0.05 vs. control. #P<0.05 vs. diabetic.*

3.3.6.3 **Effect of diabetes on NGAL and MMP-9 protein in circulating neutrophils**

Representative immunofluorescence staining of circulating neutrophils obtained from sponge implant model at day 6 after wounding showed that approximately 20% of all neutrophils were NGAL positive (green) whilst 40% were MMP-9 positive (red). Diabetes significantly increased both the NGAL positive cells and the MMP-9 population. Insulin treatment attenuated the increase in NGAL but had no effect on MMP-9. Double-immunofluorescence staining showed that NGAL was co-localised with MMP-9 predominantly in the peri nuclear regions of the neutrophils irrespective of the presence of diabetes. The diabetes associated increase in NGAL/MMP-9 co-localisation (yellow) was not altered by insulin treatment (Figure 3-27).

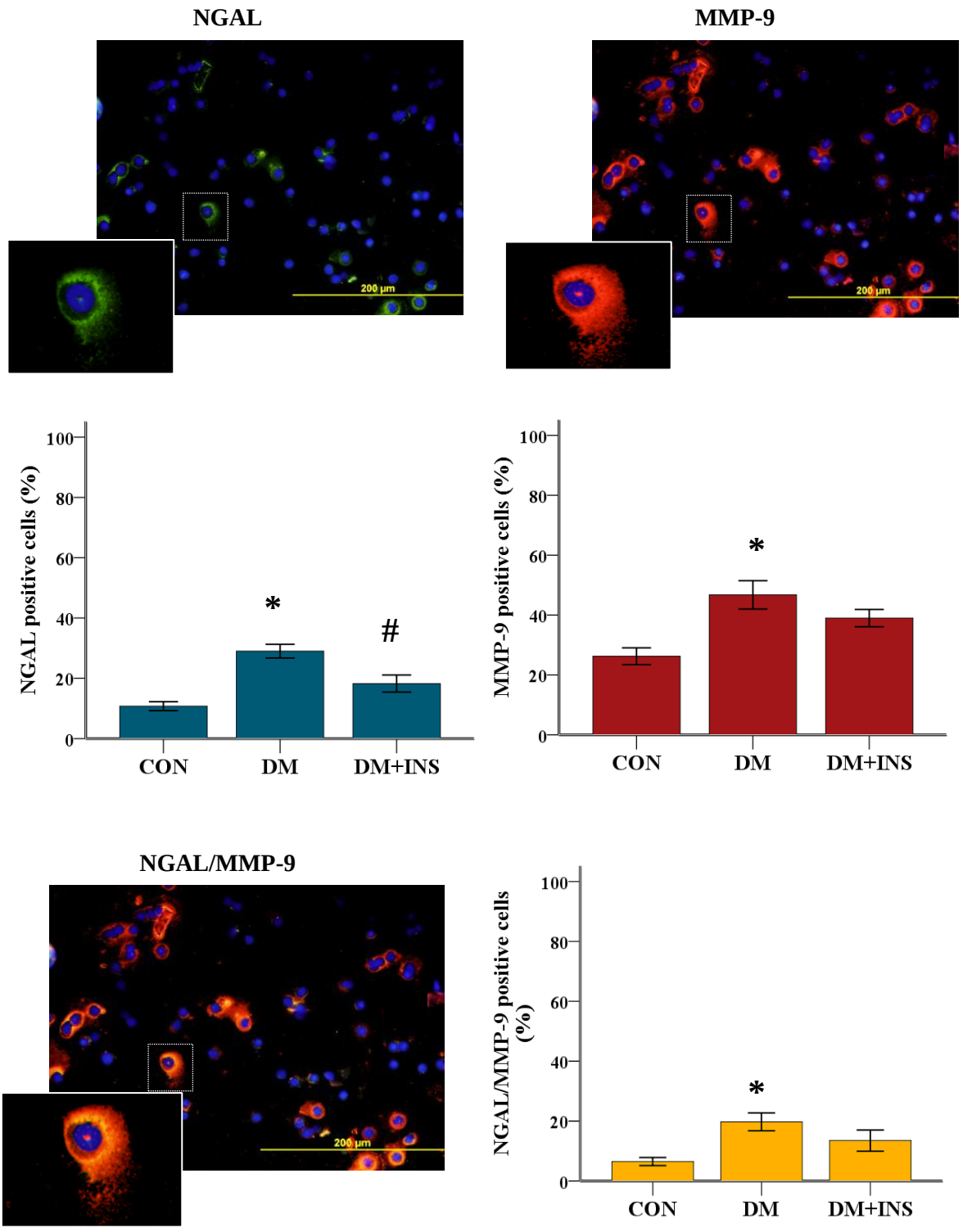

Figure 3-27: Representative images of Neutrophil NGAL (green), MMP-9 (red) expression and percentage and NGAL/MMP-9 (yellow) co-localisation in circulating neutrophils.

*Control (CON), diabetes (DM) and insulin-treated (DM+INS) animals. Data are presented as Mean ± SEM. *P<0.05 vs control. #P<0.05 vs diabetic.*

3.3.7 **Effect of diabetes on MMP-9 activity and NGAL levels in the circulation in the wounded animals**

The effect of diabetes on the circulating levels of MMP-9 showed no change at the plasma levels at day 6. The concentration of NGAL/MMP-9 complex in the circulation was also determined and it was significantly decreased in the circulation of diabetic animals compared to controls (2.26 ± 0.33 vs 3.76 ± 0.03, $P<0.05$ respectively) (Figure 3-28). By ELISA the free NGAL concentration tended to be decreased in the diabetic animals but this failed to reach significance level.

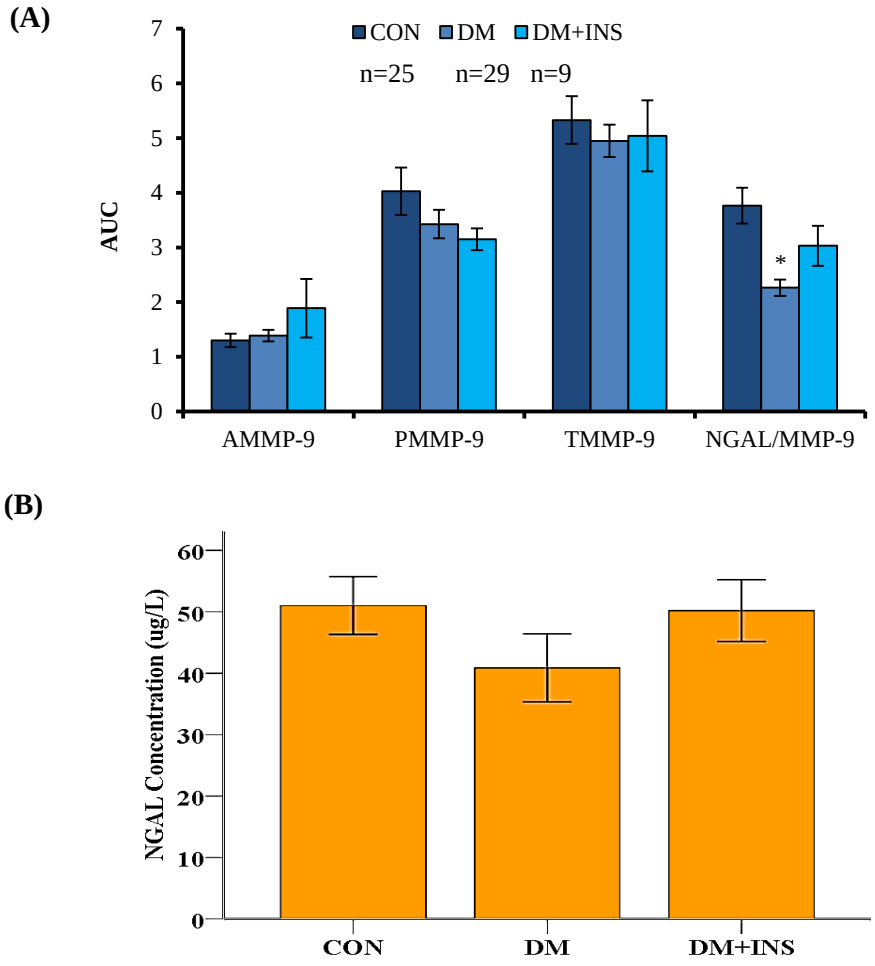

Figure 3-28: The effect of diabetes on MMP-9 activity and NGAL level.
*Plasma level of AMMP-9, PMMP-9, and TMMP-9 and NGAL/MMP-9 complex measured by zymography and (B) soluble form of NGAL 6 days after wounding. Control (CON), diabetic (DM) and insulin-treated (DM+INS) animals. Data are Mean ± SEM. *P <0.05 vs. control.*

3.3.8 **Effect of diabetes on the neutrophil apoptosis by gene expression**

To investigate the effect of diabetes on neutrophil apoptosis MCL-1 gene expression was measured in the circulating neutrophils. As shown in Figure 3-29 in the unwounded group no changes were observed in the diabetic animals in compared to control. Insulin increased MCL-1 gene expression but it failed to reach a significant level. MCL-1 gene expression also did not alter in circulating neutrophils in excisional wound model, whereas, significantly increased levels of MCL-1 were observed in the circulation of diabetic animals in the sponge implant model. This increase was improved by insulin.

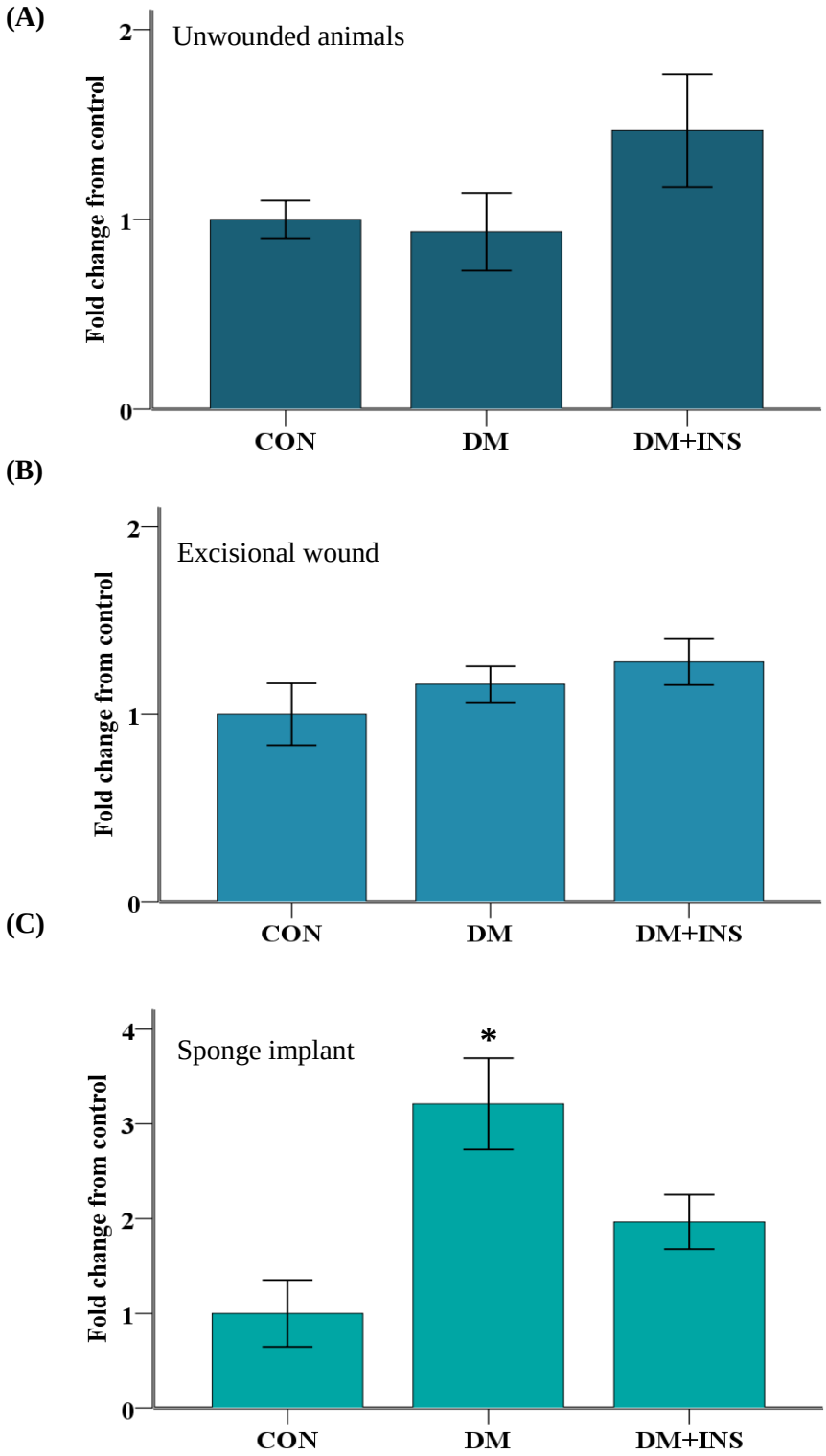

Figure 3-29: Gene Expression of MCL-1 in the circulating neutrophils from (A) unwounded, (B) excisional wounds, and (C) sponge implant model.
*Control (CON), diabetic (DM) and insulin-treated (DM+INS) animals. Values are corrected for the expression of 36B4 as housekeeper and expressed as fold change from control. Data are Mean ± SEM. *P <0.05 vs. control.*

3.3.9 **Effect of diabetes on the inflammatory cell number (Flow Data)**

In order to measure the neutrophil concentration in rat blood; a flow cytometric method was developed and the effect of diabetes and wounding was investigated.

As shown in Figure 3-30 by flow cytometry, circulating neutrophil and monocyte numbers were not altered by diabetes. In contrast, in the sponge implant model, wounding significantly increased circulating neutrophil number at days 3 and 6 and monocyte number at day 3 and day 12. These changes were not seen in the insulin-treated group Figure 3-31.

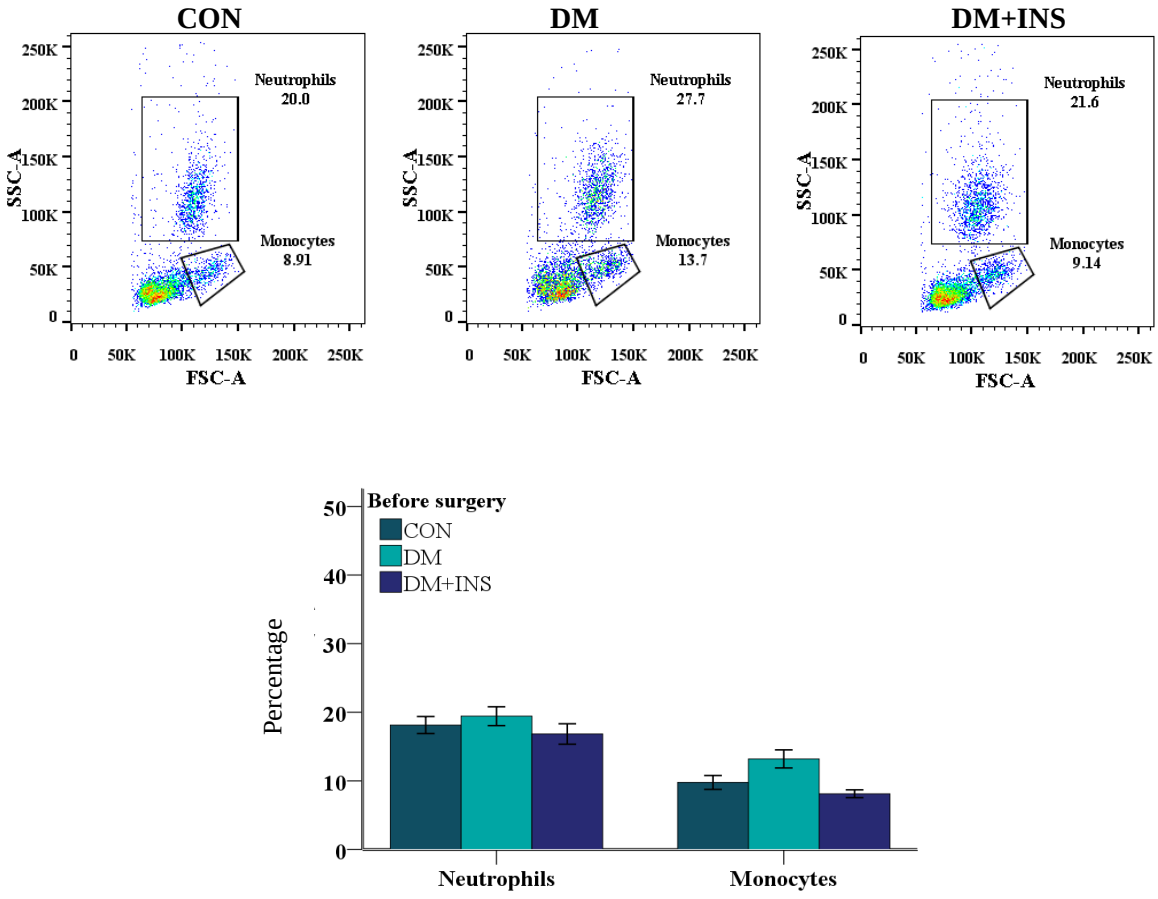

Figure 3-30: Analysis of circulating cell morphology and numbers by flow cytometry. *Control (CON, n=8), diabetes (DM, n=10) and insulin-treated (DM+INS, n=8) animals before wounding. Data are Mean ± SEM. *P<0.05 vs. control. #P<0.05 vs diabetic.*

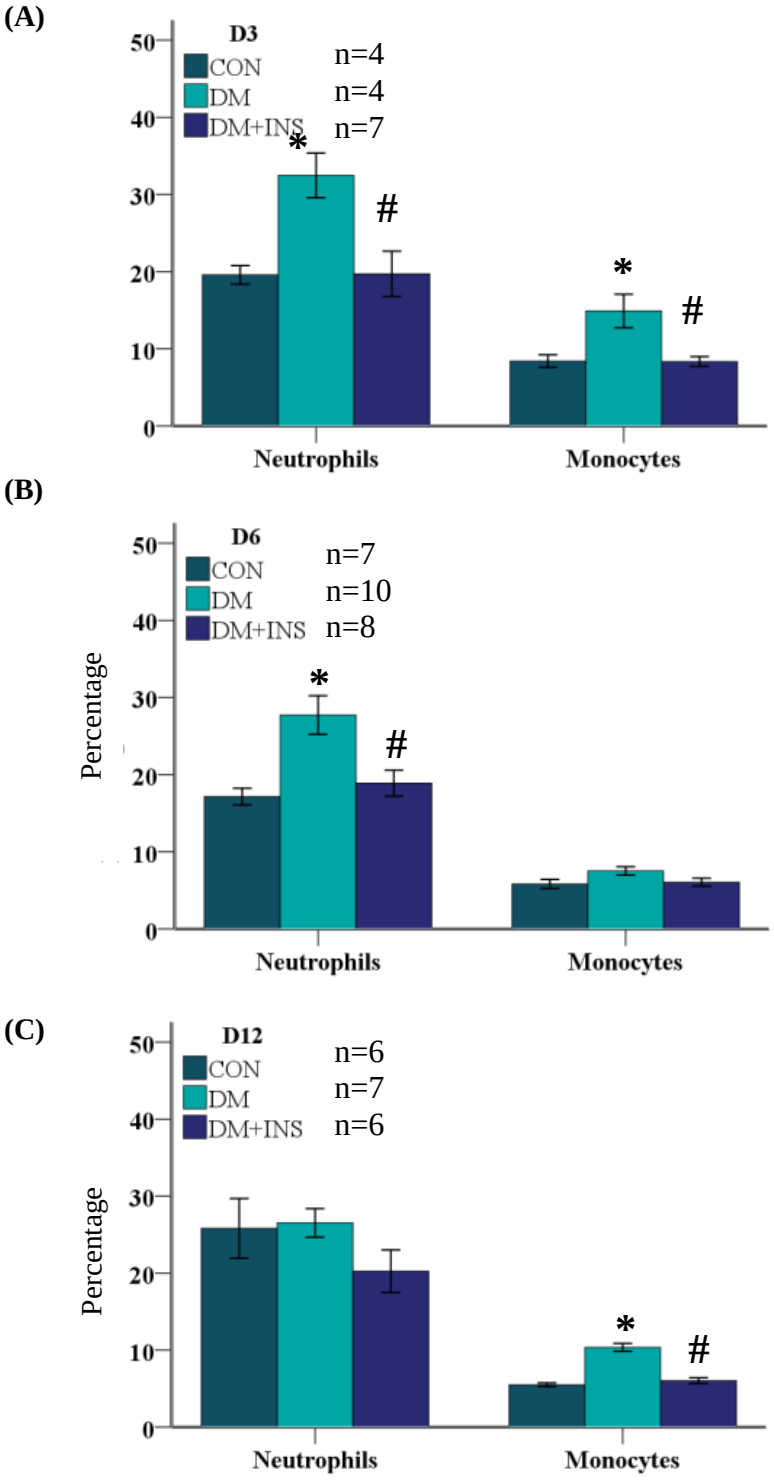

Figure 3-31: Analysis of circulating cell morphology and numbers by flow cytometry from animals after wounding.
*Control (CON), diabetes (DM) and insulin-treated (DM+INS) animals (A) 3, (B) 6, and (C) 12 days after wounding. Data are Mean ± SEM. *P<0.05 vs. control. #P<0.05 vs diabetic.*

In the circulating neutrophils from the sponge implant model we also used flow cytometry with a combination of AnnexinV and near IR to analyse neutrophil apoptosis. Rat neutrophils were identified by RP-1 which was positive in 78%, 82% and 77% of neutrophils from control, diabetic and insulin-treated animals respectively (Figure 3-32). Early apoptosis ($AnnexinV^{+}$, near IR^{-}) was significantly higher (by 9 fold) in the diabetic animals ($P \leq 0.05$) compared with control and was abolished by insulin treatment (Figure 3-33).

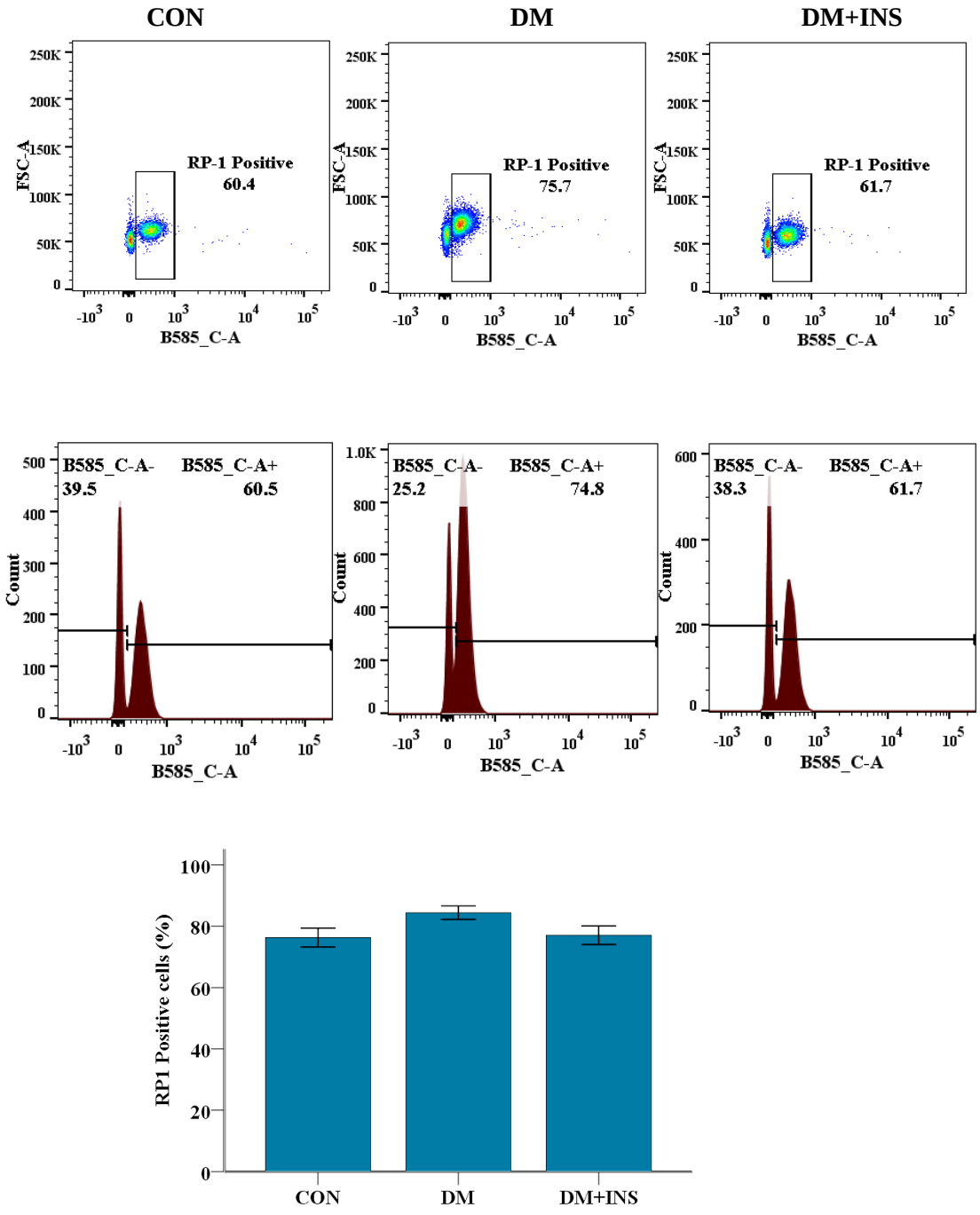

Figure 3-32: Analysis and percentage of circulating neutrophil positive RP-1 by flow cytometry.
In control (CON), diabetes (DM) and insulin-treated (DM+INS) animals before wounding. No significant change across the groups.

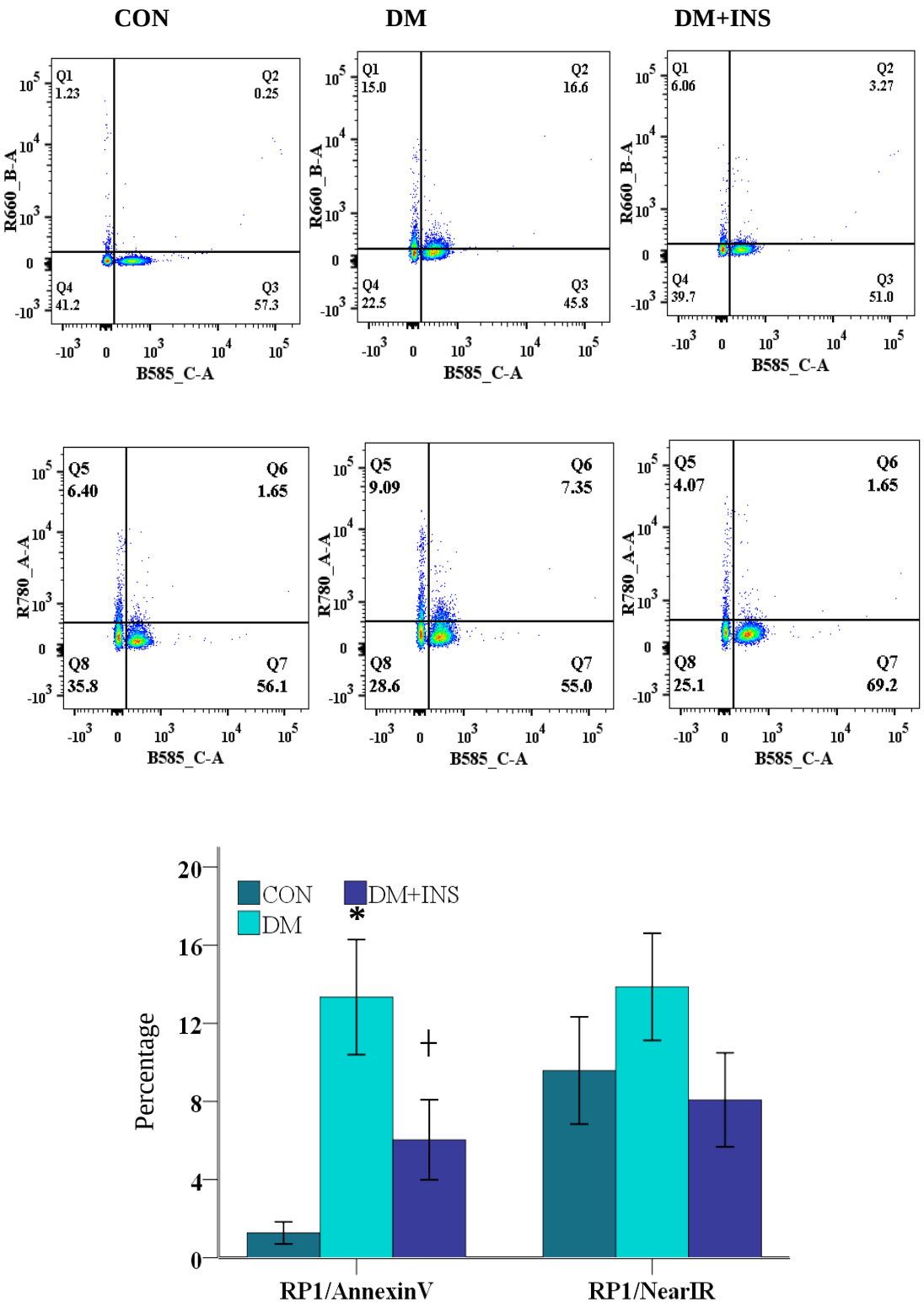

Figure 3-33: Analysis of dead and apoptotic neutrophils before wounding.
*Control (CON, n=6), diabetes (DM, n=9) and insulin-treated (DM+INS, n=9) animals. Data are Mean ± SEM. *P<0.05 vs. control by ANOVA. †P<0.05 vs diabetic by T-test.*

3.3.10 **Primary Cell Culture Studies**

Some in vitro studies were performed to examine the direct effect of high glucose concentration and insulin on neutrophil activation. In this study neutrophils were isolated from whole blood as described in detail in Chapter 2 2.3 and cultured in RPMI media for 1, 2, and 6 hours. The gene expression of activated neutrophil markers NGAL, MMP-9, and MMP-8 as well as TLR4, TLR2, and TNFα as pro-inflammatory mediators was measured by qRT-PCR. Neutrophil apoptotic state was also examined by measurement of the anti-apoptotic marker MCL-1 by qRT-PCR.

As shown in Figure 3-34, high glucose did not change NGAL, MMP-9, and MMP-8 gene expression after 1 hour of incubation. But, these genes were significantly increased after 2 hours. This increase was only observed for NGAL expression after 6 hours. Insulin partially prevented increased NGAL gene expression. High glucose had no effect on the expression of TLR2, TLR4, and TNFα after 1 hour incubation but significantly increased TNFα gene expression after2 hours of incubation. Interestingly, gene expression of TLR2 and TNFα tended to be decreased in high glucose environment (Figure 3-35). Neutrophil apoptosis, which was measured by MCL-1 gene expression, was not altered by high glucose 1 and 2 hour after incubation, but was significantly increased after 6 hours (0.73 fold decrease in MCL-1 gene expression) (Figure 3-36).

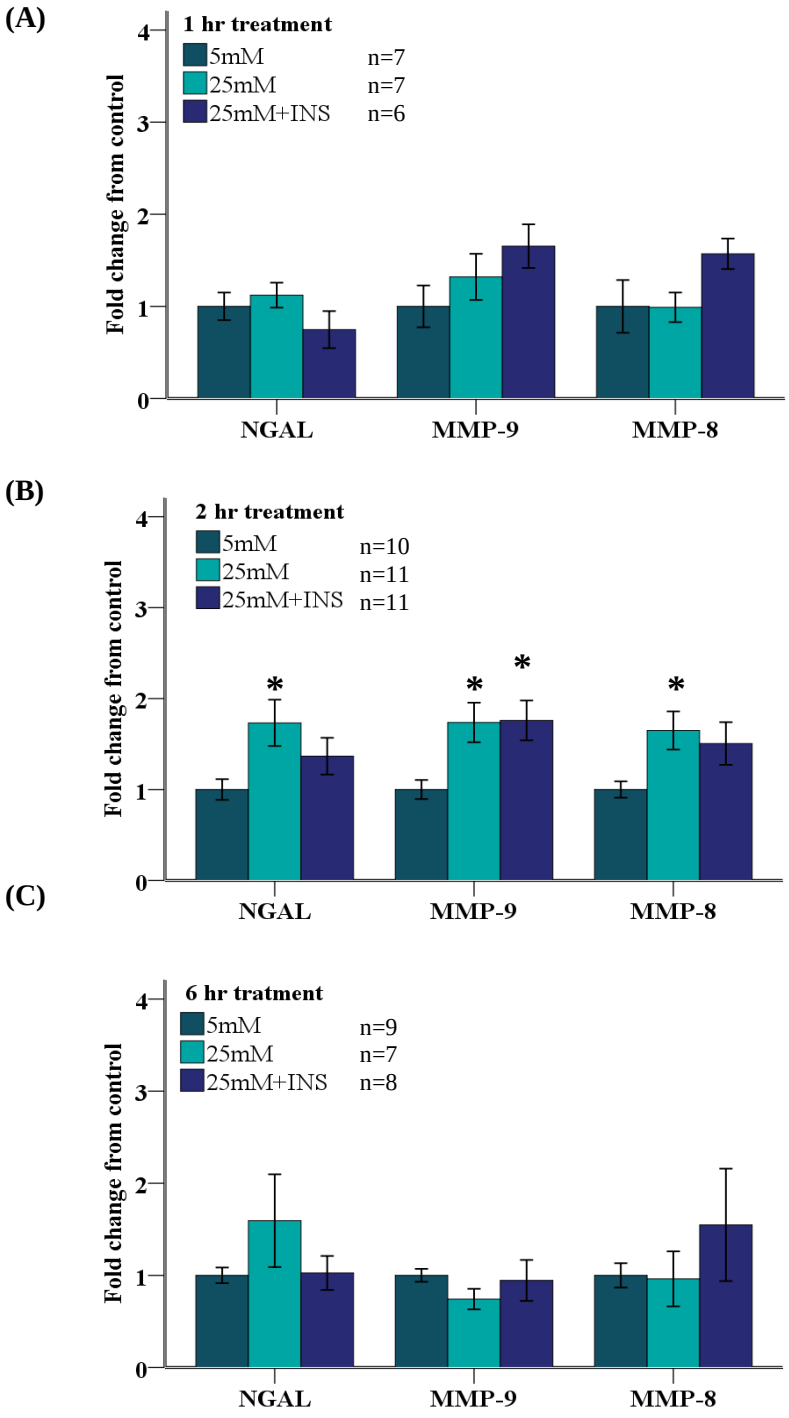

Figure 3-34: The effect of glucose on NGAL, MMP-9, and MMP-8 gene expression in the cultured neutrophils.

*Normal glucose (5mM), high glucose (25mM), and presence of insulin (25mM+INS) after (A) 1, (B) 2, and (C) 6 hours of incubation. Values are corrected for the expression of 36B4 as housekeeper and expressed as fold change from control. Data are Mean ± SEM.*P<0.05 vs Normal glucose.*

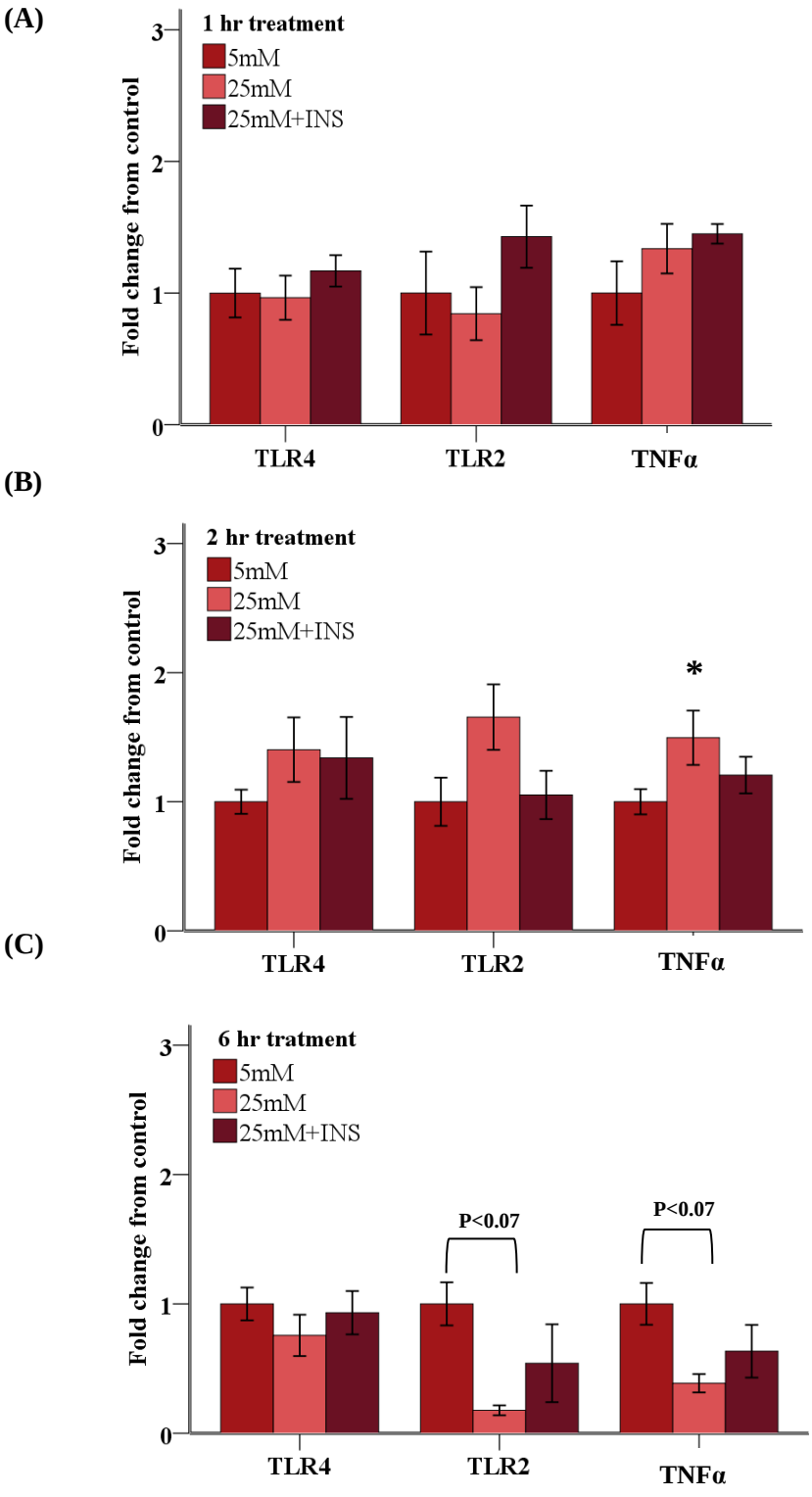

Figure 3-35: The effect of glucose on TLR4, TLR2, and TNFα gene expression in cultured neutrophils.

*Normal glucose (5mM), high glucose (25mM), and presence of insulin (25mM+INS) after (A) 1, (B) 2, and (C) 6 hours of incubation. Values are corrected for the expression of 36B4 as housekeeper and expressed as fold change from control. Data are Mean ± SEM.*P<0.05 vs Normal glucose.*

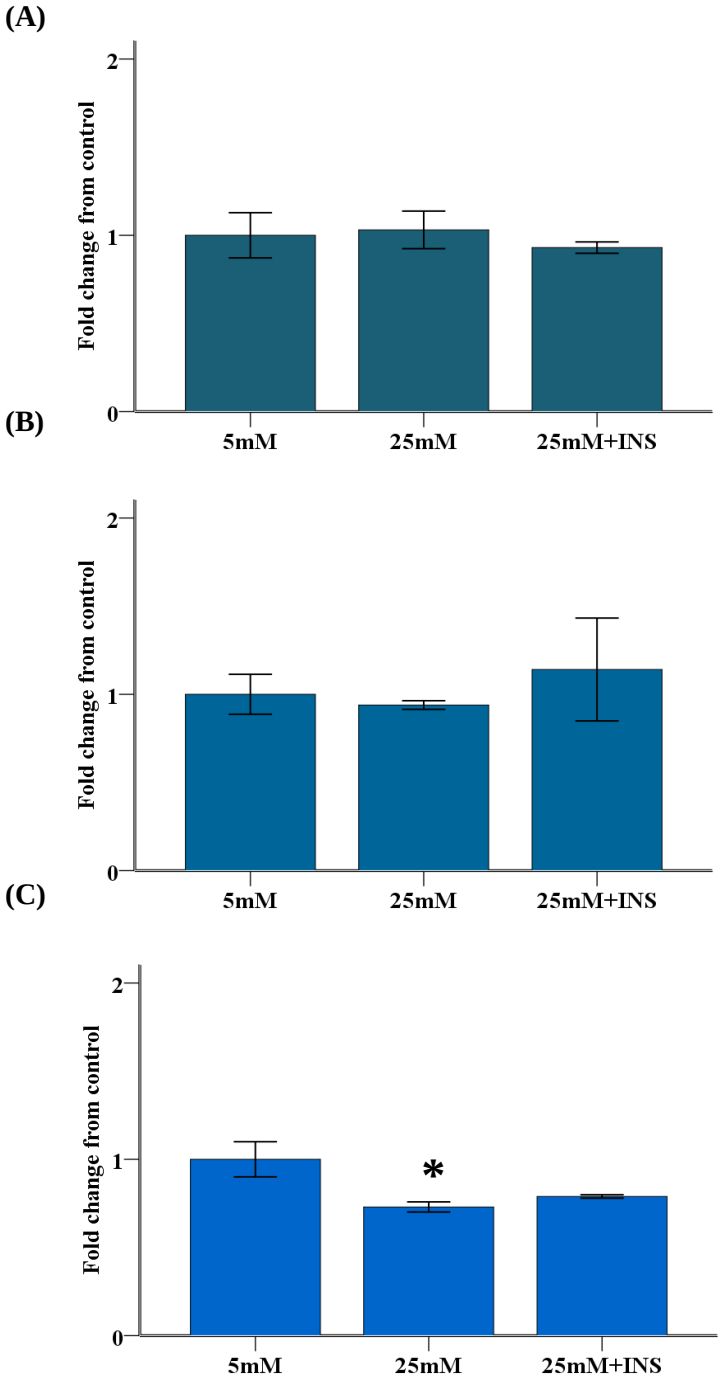

Figure 3-36: The effect of glucose on MCL-1 gene expression in cultured neutrophils. *Normal glucose (5mM), high glucose (25mM), and presence of insulin (25mM+INS) after (A) 1, (B) 2, and (C) 6 hours of incubation. Values are corrected for the expression of 36B4 as housekeeper and expressed as fold change from control. Data are Mean ± SEM.*P<0.05 vs Normal glucose.*

3.4 **DISCUSSION**

Delayed wound healing represents a major health problem and is a common experience for persons with diabetes (Brem & Tomic-Canic, 2007). Whilst intensive research effort has focussed on some of the complications of diabetes, the pathophysiology of poor wound healing has received less attention. In chronic wounds including wounds in diabetes, poor healing is associated with persistent inflammation as shown by an increase in inflammatory cells (Raffetto, 2013). The increased number of inflammatory cells in the wound is important as they secrete cytokines, MMPs and other proteinases which can further activate MMPs and proinflammatory cytokines (Martins et al., 2013). Together, these changes may contribute to the pattern of increased MMPs, frequently observed in chronic wounds (Liu et al., 2009; Lobmann et al., 2006). To date most studies have measured MMP expression in wound tissue and fluids and apart from the persistence of inflammatory cells the mechanism for the increased MMP activities is poorly understood. We have utilized two different rodent wound models to show diabetes related changes in wound and peripheral blood neutrophil and monocyte number. The wound sponge model has the advantage that the interstitial fluid and inflammatory cells within the defined sponge space can be harvested for analysis. Additionally, it uses a relatively inert material, so the site has minimal inflammation. At later time points (>2 weeks) the implants can also accumulate granulation tissue with a gradient of less well formed tissue in the center of the sponge to the most mature at the periphery, but this aspect was not studied in this work. Our study has focused on the neutrophil, and shown that in diabetic animals circulating neutrophils are increased in association with an increase in neutrophil numbers in the wound. The expression of MMP-9 and its regulator NGAL are also affected by diabetes and

the pattern of expression is different in the circulation and in the wound tissue. Further insulin treatment from the onset of diabetes can ameliorate these changes, suggesting that glycaemic control is important.

There is conflicting data regarding the effect of diabetes on neutrophil function and the effect of diabetes on wound tissue neutrophils has not been extensively studied. In peripheral neutrophils from rodents and humans some authors have reported alterations in cytokine production (Alba-Loureiro et al., 2007), respiratory burst (Osar et al., 2004) and bacterial killing (Kewcharoenwong et al., 2013) but others have failed to detect such changes (Balasoiu et al., 1997). In this study using acute wound models in diabetic animals healing is delayed, and inflammatory cell numbers are increased. In addition to change in neutrophil number by MPO staining, the increased neutrophil number in the sponge implant model was supported by the observation of increased mRNA levels of both MMP-8 and MMP-9 by neutrophils. This inflammatory cell profile in the wound tissue and implant is consistent with other studies in excisional wounds (Casqueiro et al., 2012; McLennan et al., 2008) as well as studies of subcutaneous wounds and foreign body implant models, which have described persistence or increased neutrophil numbers (Socarras et al., 2014; Thomson et al., 2010). The observed change in wound neutrophil number may occur as a result of increased circulating numbers as reported in this study. This increased neutrophil number, either in the wound or in the circulation, can also occur as a result of alteration in function e.g. increased apoptosis or decreased clearance. Data presented here show effects of diabetes or high glucose on apoptosis. Additionally the transient nature of the change and the dysregulation of markers of activation suggest a diabetes related functional change. The increase in wound neutrophil

number was unlikely to be due to increased infection in the diabetic wound tissue as TLR4 and TLR2 gene expression which are activated by ligands of microbial origin (Kawasaki & Kawai, 2014) were not altered in the wound fluid and wounded skin of diabetic animals.

Previous studies in chronic wounds and in poorly healing wounds from diabetic patients have shown high levels of MMP-9. In this study we used zymography to examine the various forms of MMP-9 in wound fluids and in plasma. Using this technique we showed a transient increase in the sponge fluid MMP-9 from diabetic animals at day 6. This was due largely to an increase in the biologically active 82kDa form of MMP-9 and increased formation of NGAL/MMP-9 complex. This change in MMP-9 activities appears to be a dynamic process, with increased levels of active MMP-9 preceding the formation of the NGAL/MMP-9 complex, which then persists. The alteration in MMP-9 activities was not accompanied by an increase in MMP-9 gene expression suggesting that the observed effect of diabetes on MMP-9 activities is not mediated by an overall increase in inflammatory cell activation and occurs via posttranslational mechanisms. This concept is supported by our observation increased formation of the NGAL/MMP-9 complex in the absence of change in expression of TNFα. The increased formation of the NGAL/MMP-9 heterodimer can stabilise MMP-9 to prevent it from degradation as well as support allosteric activation (Tschesche et al., 2001; L. Yan et al., 2001) together these changes suggest that in diabetic wounds increased NGAL can act to provide a reservoir of MMP-9 which would then be available for later release.

At the cellular level the subpopulation of peripheral neutrophils, which showed peri-nuclear staining for both MMP-9 and NGAL was more abundant in the diabetic animals. This increase has potential to deliver more MMP-9 and NGAL rich neutrophils to the wound and by this mechanism provide a source of MMP-9, which can be released on neutrophil activation and degranulation. There were other similarities between the circulating neutrophils cells and the inflammatory cells obtained from the sponge implant. At day 6, the time when the most prominent diabetes related changes in sponge inflammatory cell neutrophil markers (NGAL and MMP-8) were observed, their expression NGAL, MMP-9 and MMP-8 in peripheral blood neutrophils was also increased. There were also some differences, for example at day 6 in the peripheral cells but not the wound cells, the gene expression of MMP-9 as well as the inflammatory markers TNFα, TLR2 and TLR4 was increased. Why these differences in pattern occur is uncertain. The differences in cellular composition i.e. neutrophils in the peripheral blood versus a mixture of neutrophils and monocytes in the sponge implant model is one possible explanation. However, it is unlikely as in the implant model neutrophils are two times more abundant than the macrophages. Another possibility is that at day 6 there is an up-regulation of generalised inflammation in the diabetic animal, which leads to increased activation of circulating neutrophils. Evidence for this is suggested by the increased expression of inflammatory markers (TLR2, TLR4 and TNFα). These changes may cause degranulation of neutrophils in the circulation, but how this is translated to unaltered change in expression of MMP-9 in the wound cells is not clear. The hyperglycemia of diabetes is known to increase monocyte TLR expression (Dasu et al., 2012) but little is known regarding its effect on neutrophil TLRs. Further, the transient nature of the observed increase and its association with

increased expression of inflammatory markers such as NGAL and MMP-9 suggests an alteration in neutrophil phenotype in response to wounding at this time point.

Insulin is known to play a critical role in regulation of wound healing and has been shown to have a role in insulin deficiency as well as at the level of signalling (Lima et al., 2012). Insulin treatment has been shown to accelerate wound healing rate by increased collagen deposition (Madibally et al., 2003) and increased proliferation and migration of keratinocytes (Brem & Tomic-Canic, 2007). In this study we have shown that insulin treatment to a level which partially improved blood glucose level and body weight in the diabetic animals was able to improve wound closure and prevented diabetes associated changes in wound and circulating neutrophils. Similar to other reports in this study insulin treatment was able to decrease inflammatory cell number in the wound. This effect was likely mediated impart by prevention of activation of circulating inflammatory cells. Insulin has been shown to have a direct effect on neutrophil metabolism and function, even with no significant change in glycemia (Alba-Loureiro et al., 2006). It has previously been shown that insulin has a strong regulating effect on the functional activities of immune cells (Walrand et al., 2006). Altered neutrophil functions of diabetic subjects could be restored by controlling hyperglycemia with insulin. The mechanisms of this uncertain as neutrophils do not require insulin for uptake glucose. Nevertheless, glucose use and glycogen metabolism by neutrophils are both insulin dependent (Walrand et al., 2006).

In conclusion, the main findings of this work show diabetes related differences in circulating and wound tissue neutrophil profiles in particular in relation to the

expression and activities of MMP-9. As neutrophils are critical for the initiation and progression of wound repair a better understanding of the effect of diabetes on their function at the various phases of wound repair is required. Additionally, these studies examining the relationship of MMP-9 and NGAL in diabetic wounds have shown for the first time the persistence of the NGAL/MMP-9 complex. Whether these changes are exacerbated clinically in wounds in people with diabetes where healing is often more prominently delayed and infection risk is high remains to be studied.

We next investigated the effect of doxycycline as a known medicine for inhibition of MMPs on the wound closure and expression of activated neutrophil markers. Some studies have shown the positive effect of doxycycline on wound healing with improved adhesion and scar formation in treated rats (Olmarker, 2010). Doxycycline also significantly reduces inflammation and elevates levels of pro-inflammatory cytokines, which degrade the ECM, and stimulate increased numbers of inflammatory cytokines (Mast & Schultz, 1996). However, the effect of doxycycline on neutrophil activity during wound healing in not well known and will be discussed in more detail in following section.

CHAPTER 4: Effect of Doxycycline on Wound Healing and Neutrophil Activation in Diabetes

4.1 **INTRODUCTION**

The studies described in the previous chapter showed increased active MMP-9 in wound tissue and wound fluids from diabetic animals. The expression of NGAL a protein secreted by neutrophils, known to regulate MMP-9 activity as well as neutrophil activation was also increased. Improved blood glucose control prevented these changes and also improved wound healing rate. Together these results suggest a role for increased MMP-9 activity and altered neutrophil activation in poor wound healing in diabetes. In the studies described in this chapter the effect of doxycycline a semisynthetic, chemically modified tetracycline compound with known ability to inhibit MMP expression and activities was examined. Whether doxycycline could improve wound healing via effects on NGAL and neutrophil activation was also investigated.

Doxycycline is widely used to treat infections caused by both gram-negative and gram-positive microorganisms (Wilcox et al., 2012). It has also been shown to inhibit the activity of MMPs by a number of mechanisms including by inhibition of activated MMPs, inhibition of pro-MMP-9 activation and inhibition of MMP expression (Gerald et al., 2001). In several animal studies, oral treatment with doxycycline or analogs of tetracycline have improved wound healing rate but mechanism of this effect in particular its effect on wound inflammatory cell MMP production is not known. Therefore, in this study we investigated the mechanism of the effect of doxycycline on MMP-9 expression and activity in inflammatory cells in two different diabetic wound models of wounds.

4.2 MATERIALS AND METHODS

The models used for these studies were described in detail previously (Chapter 2.2.2). As described diabetes was induced by STZ (65mg/kg) in the Sprague Dawley rats and confirmed by tail vein blood glucose level > 11mmol/L, three days after injection. The experiments were commenced six weeks later using the experimental design shown in Figure 4-1. At the time of surgery control and diabetic animals were divided as follows:-

1) Control (CON) animals that did not receive any treatment.
2) Control animals treated with 100 mg/kg of doxycycline (CON+HDOX).
3) Control animals treated with 20 mg/kg of doxycycline (CON+LDOX).
4) Diabetic (DM) animals that did not receive any treatment.
5) Diabetic animals treated with 100 mg/kg of doxycycline (DM+HDOX).
6) Diabetic animals treated with 20 mg/kg of doxycycline (DM+LDOX).

Doxycycline was administrated orally by gavage daily from the time of wounding or sponge implant and animals were terminated 6 days later. In the excisional model wound closure was measured and calculated as described previously (Chapter 2-2-3). Skin wounds and sponge implants were removed and inflammatory cells were separated from the sponges as previously described (Chapter 2.2.4). Blood was also collected and the neutrophils were isolated as described (Chapter 2.3). Neutrophil specific genes NGAL, MMP-8, MMP-9 and other pro-inflammatory markers TNFα and TLR4 and TLR2 were measured in the sponge implant inflammatory cell population as well as the circulating neutrophils. The NGAL/MMP-9 complex and

pro- and active forms of MMP-9 in the wound fluid, and plasma were quantified by zymography as described in detail in Chapter 2.4.4.

Due to the loss of the excisional skin wound samples (freezer break down), we were unable to undertake the analysis of skin wounds by gene expression and immuno fluorescence for the animals treated with 20mg/kg of doxycycline (LDOX).

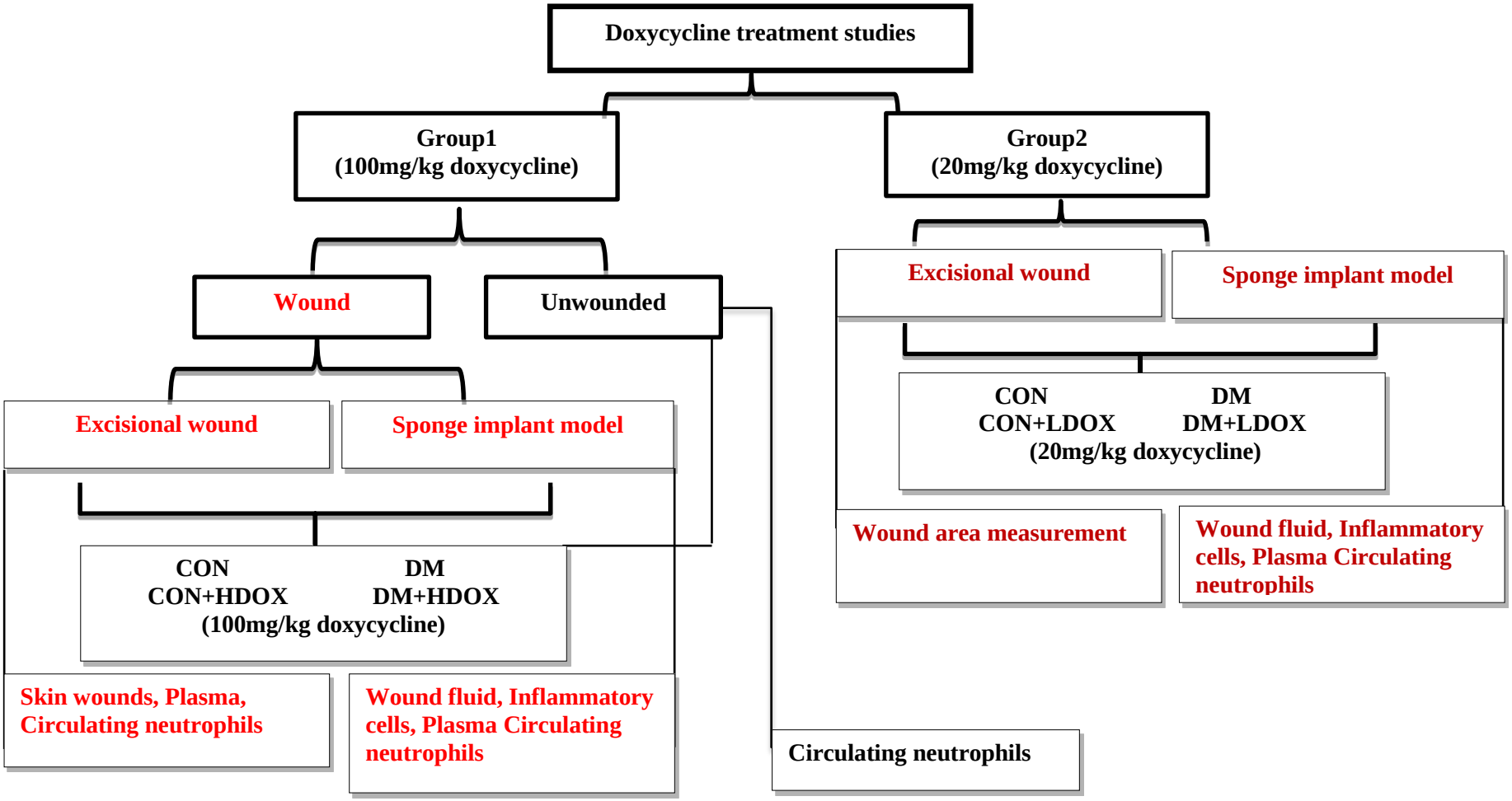

Figure 4-1: Experimental design for doxycycline treatment studies

4.3 **RESULTS**

4.3.1 **Animals characteristics**

Body weight and non-fasting blood glucose levels were measured weekly from the induction of diabetes in all experimental animals.

As expected the diabetic rats had a significantly higher blood glucose level and decreased body weight when compared to the control and control treated animals ($P<0.05$). The oral administration of doxycycline (HDOX: 100mg/kg) to the diabetic animals had no effect on blood glucose level or body weight (Table 4-1).

Table 4-1: Blood glucose level and body weight in the high doxycycline (HDOX) experimental groups.

	Start weight (g)	Termination weight (g)	Non fasting BGL (mmol/L)
Groups	Week1	Week 6	Week 6
CON	338.5±7.5	488.3± 10	5.0±0.1
DM	334.8±6.5	373.8±10.3*	21.2±1.4*
CON+HDOX	339.0±11.2	516.7±0.9	4.1±0.1
DM+HDOX	346.9±10.3	335.1±10.1*	23.4± 1.3*

*Results are expressed as Mean ± SEM,*P<0.05 different from Control (CON).*

The data for blood glucose level and body weight for all animals treated with 20mg/kg doxycycline (LDOX) are shown in Table 4-2. Similar to the HDOX study the diabetic animals had higher blood glucose levels (non-fasting) and considerably lower body weight than control animals.

Table 4-2: Body weight and blood glucose level in the low doxycycline (LDOX) experimental group.

Groups	Start weight (g)	Termination weight (g)	Blood glucose (mmol/L)
CON	296.5 ± 5.6	428.4 ± 14.8	4.2 ± 0.3
DM	294.5 ± 7.0	356.4 ± 37.4*	17.7 ± 3.2*
CON+LDOX	302.1 ± 20.1	429.2 ± 36.5	4.6 ± 0.3
DM+LDOX	303.7 ± 14.8	383.2 ± 44.4*	17.1 + 2.9*

*Results are expressed as Mean ± SEM,*P<0.05 different from Control.*

4.3.2 **Effect of doxycycline treatment on wound closure**

Wound area was measured in all skin wounds and the percentage wound closure was calculated 3 and 6 days after wound creation as described in Chapter 2.2.3.

Shown in the Figure 4-2-A at day 6 the wounds in control animals were significantly smaller than those in the diabetic group ($P<0.05$). As shown in Figure 4-2-B doxycycline at 100 mg/kg had no effect on wound closure in diabetic animals and it was significantly slower at day 3 and 6 compared to control (Figure 4-2-C) and control doxycycline -treated animals (Figure 4-2-D).

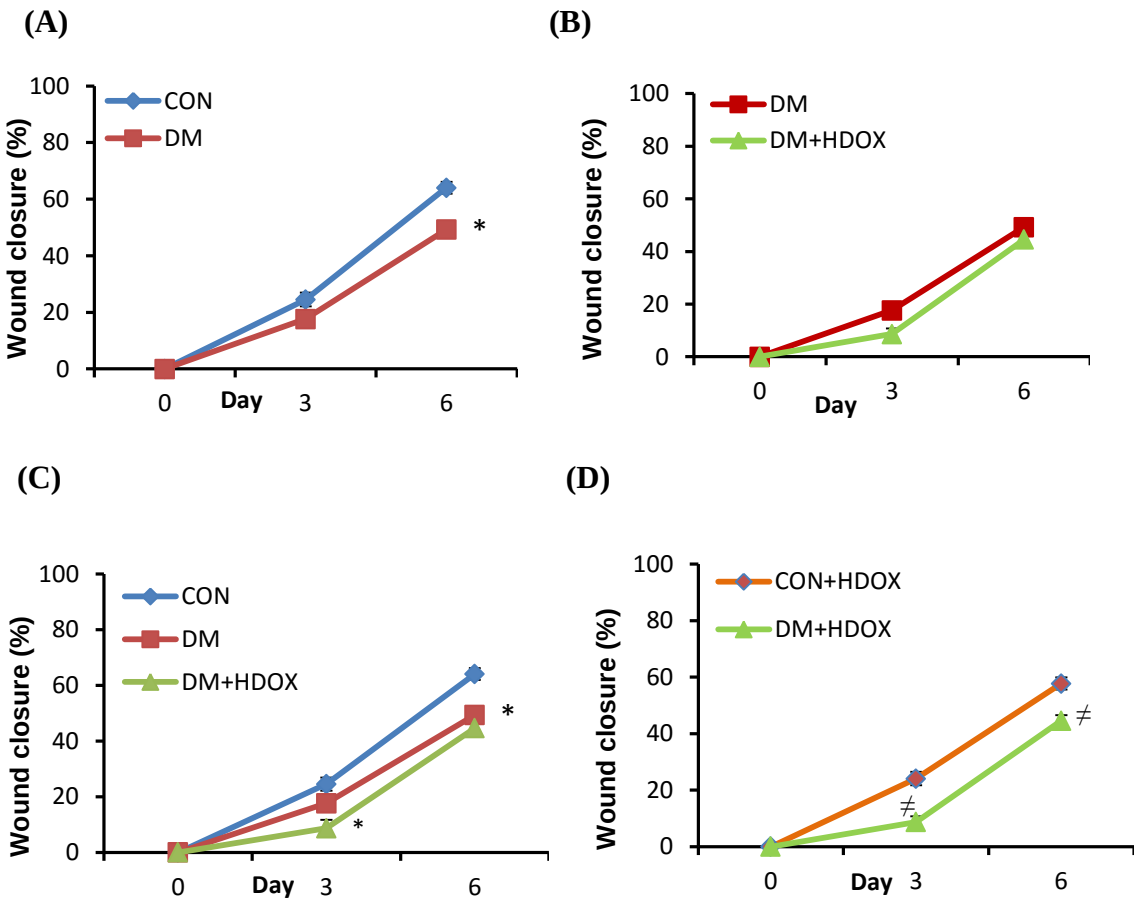

Figure 4-2: Effect of oral treatment with doxycycline (100mg/kg) on wound closure. *Wound area was measured at day 3 and day 6 and the results were expressed as percentage of the intial wound area i.e. precentage wound closure. Control (CON), diabetic (DM), control doxycycline-treated (CON+HDOX) and diabetic doxycycline-treated (DM+HDOX) animals.Data are Mean ± SEM.*P<0.05 vs control. #P<0.05 vs (CON+DOX)*

In a parallel study the effect of a lower dose of doxycycline on wound healing in diabetes was examined. Similar to the previous studies diabetes significantly delayed wound closure (Figure 4-3). In contrast to the effect of high dose doxycycline low dose of doxycycline (20mg/kg) partially ameliorated the delay (Figure 4-3-B), such that there was no difference in percentage wound closure when the LDOX treated diabetic animals were compared to control (Figure 4-3-C) and control LDOX treated (Figure 4-3-D) animals.

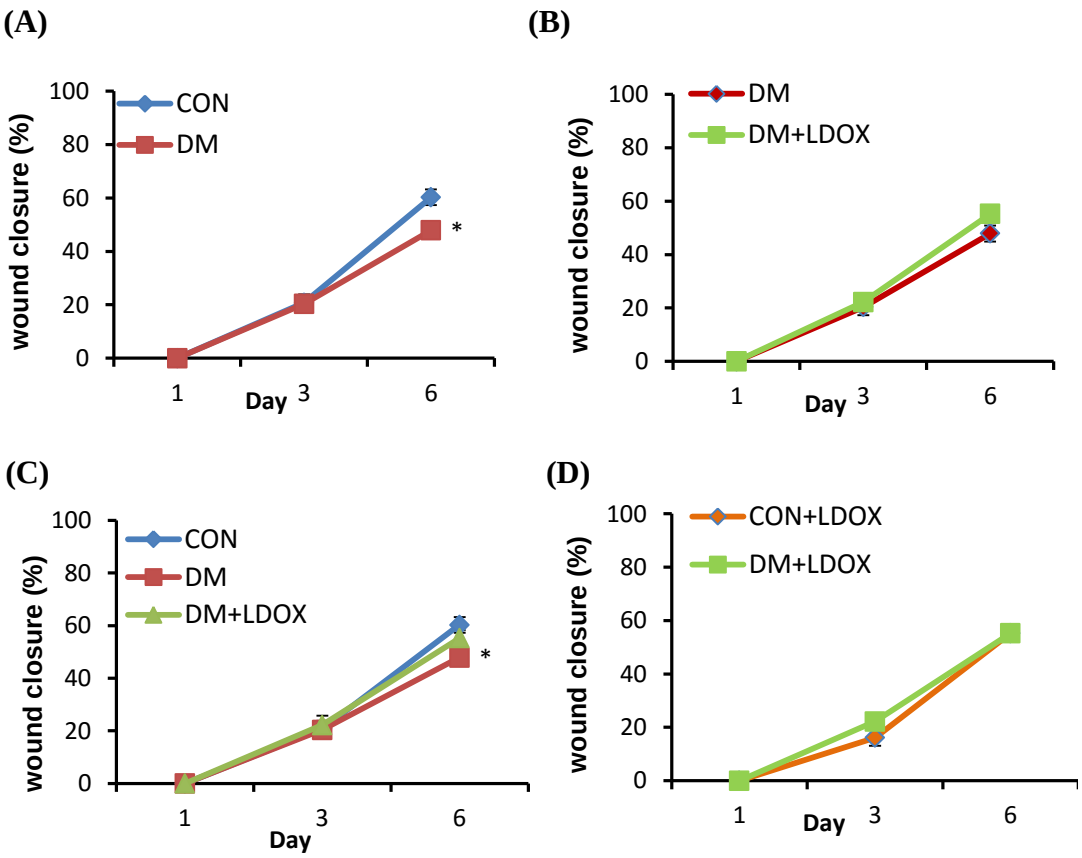

Figure 4-3: Effect of oral doxycycline (20mg/kg) on wound closure.
Wound area was measured at day 3 and day 6 and the results were expressed as percentage of the intial wound area i.e. precentage wound closure. Control (CON), diabetic (DM), control doxycycline-treated (CON+LDOX) and diabetic doxycycline-treated (DM+LDOX) animals. Data are Mean ± SEM. *P<0.05 vs Control.

4.3.3 **Effect of diabetes on skin wounds**

Skin wound morphology was only examined in tissue from animals treated with HDOX (100 mg/kg Doxycycline). As previously described, the granulation tissue was examined by H&E and PSR staining. Representative photos of the H&E stains (Figure 4-4) show diabetic wounds contained more inflammatory cells and the extracellular matrix was less well formed with more gaps than seen in control animals. Interestingly, by H&E the wound tissue from the doxycycline-treated diabetic animals also showed a less dense extracellular matrix, less vessel formation and less fibroblasts compared to non-diabetic control animals.

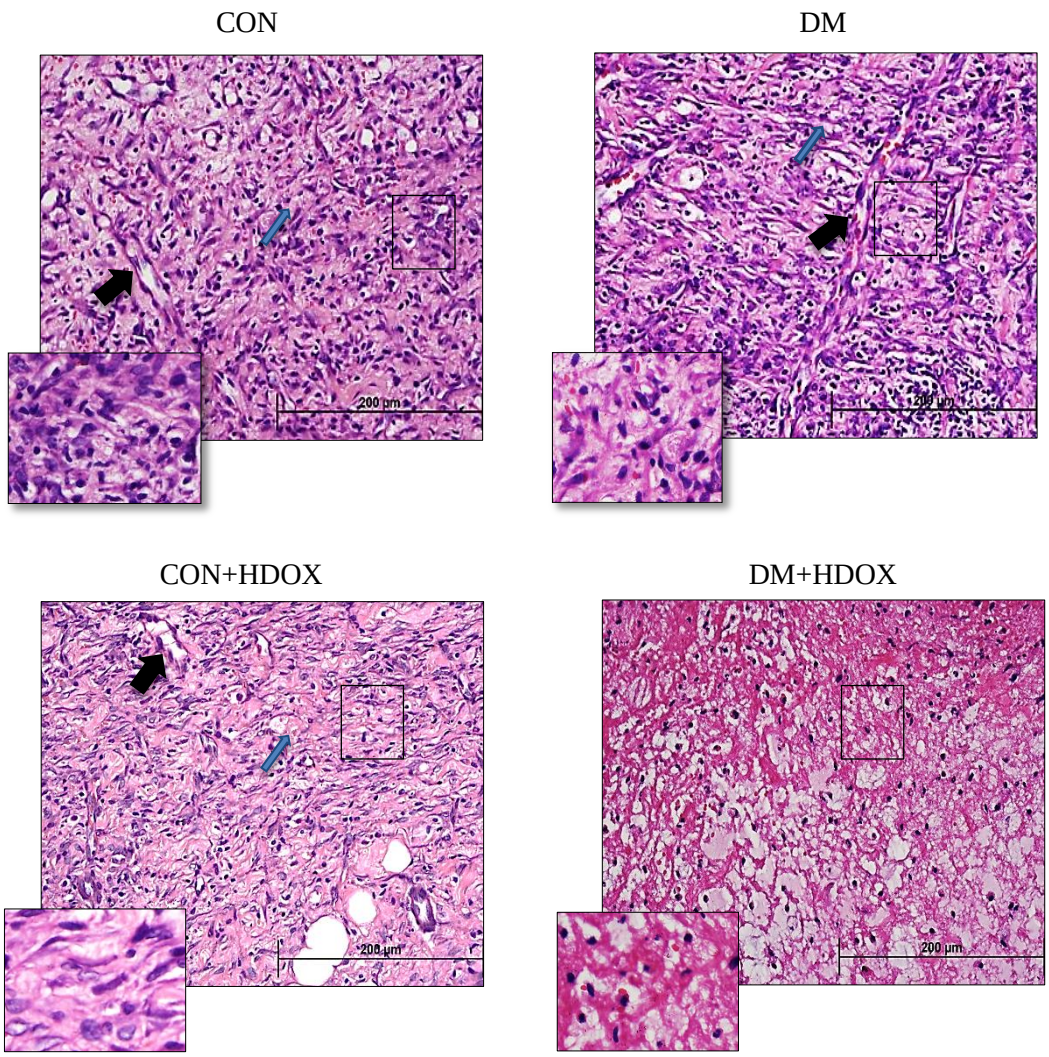

Figure 4-4: Representative Hematoxylin and Eosin images showing the effect of treatment with doxycycline (100mg/kg) on skin wound morphology.
H&E stain of control (CON), diabetic (DM), control doxycycline-treated (CON+HDOX) and diabetic doxycycline-treated (DM+HDOX) wounds at 6 days post wounding. Black arrows show vessels and blue arrows shows fibroblasts. Image magnification ×400.

Shown in Figure 4-5 are representative images of the wounds after PSR staining. The intensity of the staining was assessed by two independent persons blinded to the source of the tissue. The wound scores were then analysed using a Chi-Square Test. The wound collagen deposition was scored based on the staining isotype control from light (0) to intense (3) staining. As shown in the Table 4-3 the staining was more intense in wounds from control and control animals treated with HDOX than the untreated and HDOX treated diabetic animals. In addition there was a distinctly different pattern of collagen in the granulation tissue with fewer bundles of collagen in wound tissue from diabetic and HDOX treated diabetic animals.

Table 4-3: Chi-Square tests for analysis of PSR staining in the skin wounds.

	Dermis	
Groups	**Scores < 2**	**Scores > 2**
CON (n=4)	1	3
DM (n=3)	3	0
CON+HDOX (n=3)	1	2
DM+HDOX (n=3)	2	1
Asymp. Sig. (2-sided)	**0.2**	

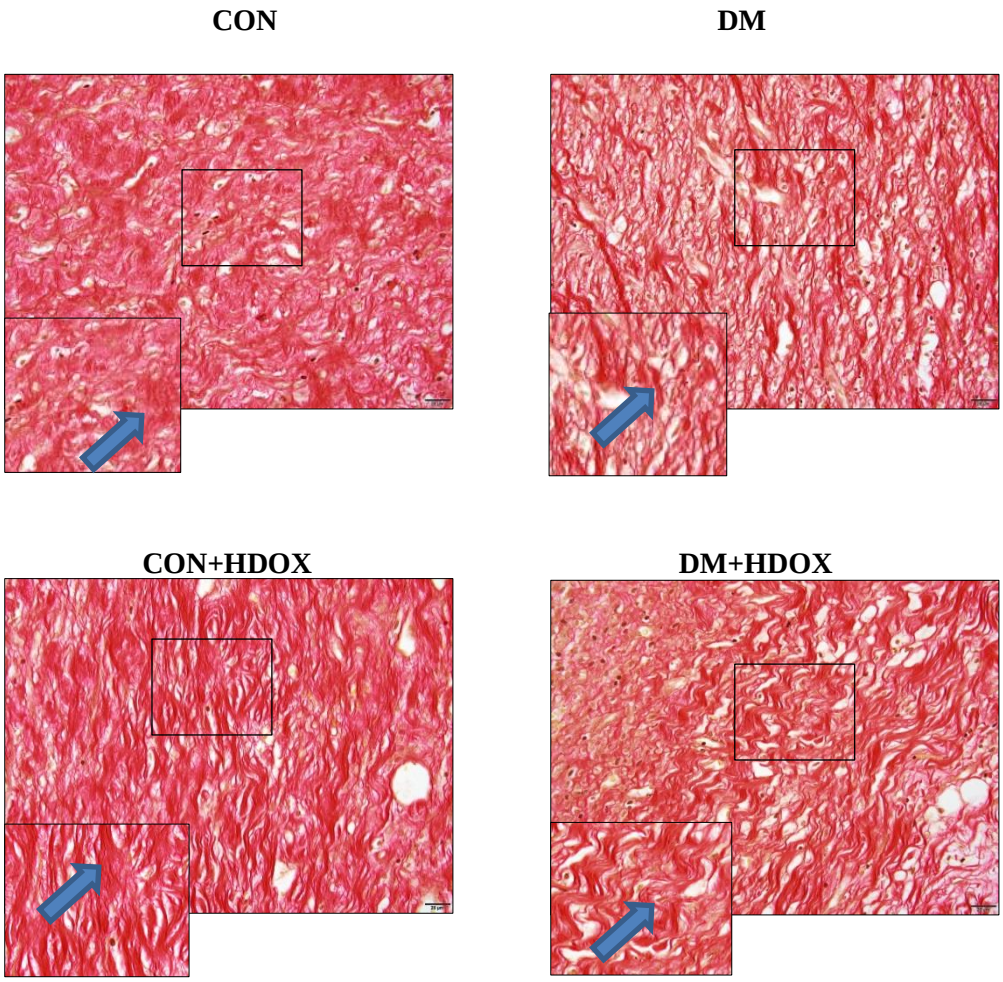

Figure 4-5: Representative images showing the effect of treatment with doxycycline (100mg/kg) on skin wound morphology after staining with PSR.
PSR stain of Control (CON), diabetic (DM), control doxycycline-treated (CON+HDOX) and diabetic doxycycline-treated (DM+HDOX) wounds at day 6 post wounding. Arrow shows collagen bundles in the skin wound. Image magnification×400.

4.3.4 **Effect of doxycycline (HDOX) treatment on wound tissue NGAL and MMP-9 expression**

NGAL in the skin wounds was studied by IHC and representative images are shown in Figure 4-6. As previously described, the NGAL expression was examined in the epidermis, dermis, and hypodermis. The intensity of the stain was scored from none (0) to intense (3) based on the isotype control as 0. The average scores from each area were calculated and categorised into scores less and greater than 2 for Chi-Square analysis. The most intense staining was observed in the dermis where the number of animals with a score greater than 2 was higher in the diabetic animals compared to control (Table 4-4). These changes were partially improved by doxycycline treatment.

Table 4-4: Chi-Square tests for analysis of NGAL staining in the skin wounds.

Groups	Epidermis		Dermis		Hypodermis	
	Scores< 2	**Scores ≥ 2**	**Scores < 2**	**Scores ≥ 2**	**Scores< 2**	**Scores ≥ 2**
CON	4	1	5	0	1	4
DM	5	0	1	4	0	5
CON+HDOX	3	0	2	1	0	3
DM+HDOX	3	0	1	2	0	3

	Asymp. Sig. (2-sided)
Epidermis	.50
Dermis	**.06**
Hypodermis	.50
Overall	.218

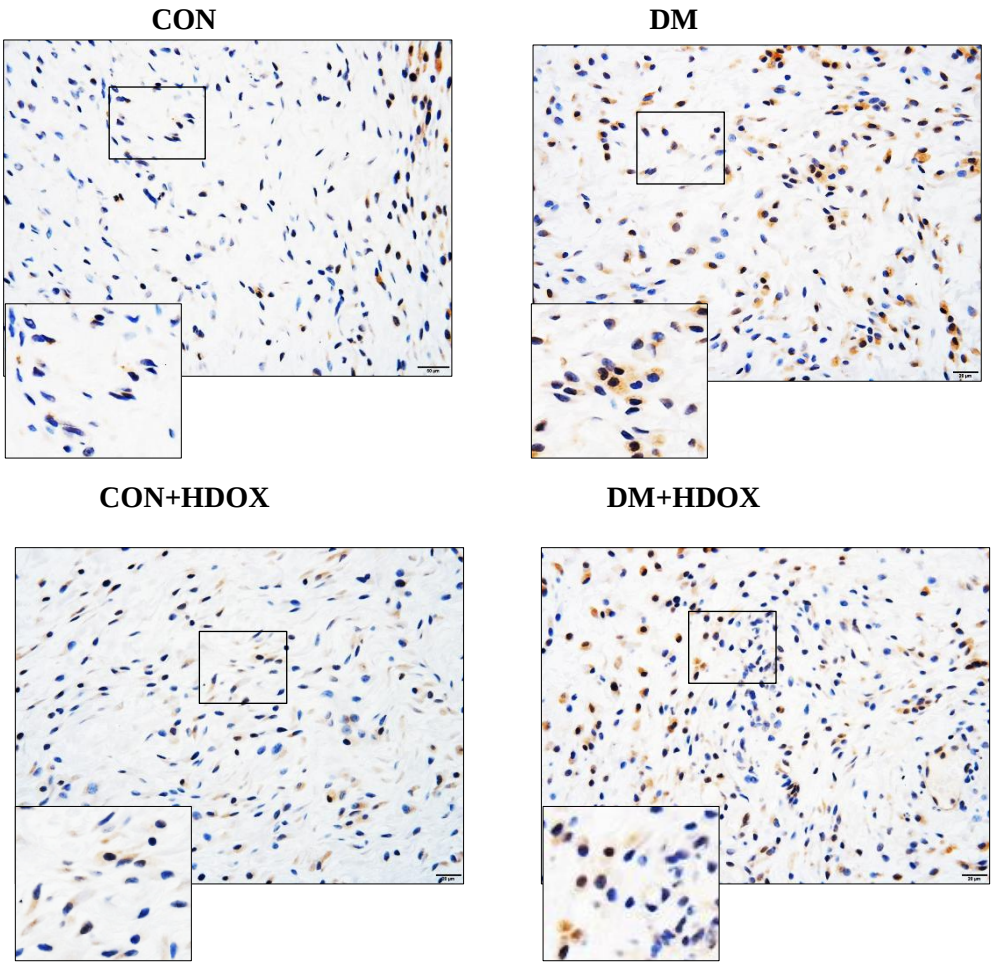

Figure 4-6: Representative images showing the effect of treatment with doxycycline (100mg/kg) on skin wound NGAL expression.
Control (CON), diabetic (DM), control doxycycline-treated (CON+HDOX) and diabetic doxycycline-treated (DM+HDOX) wounds at day 6 post wounding. Image magnification×400.

MMP-9 expression was also examined by IHC and representative images are shown in Figure 4-7-A. The MMP-9 positive cells were calculated using Image J and the results were analysed using ANOVA. Compared to controls, diabetes tended to increase skin wound MMP-9 (by 1.7 fold). Doxycycline treatment of diabetic animals at 100mg/kg appeared to be without effect and surprisingly in control doxycycline-treated animals increased compared to untreated controls (Figure 4-7-B).

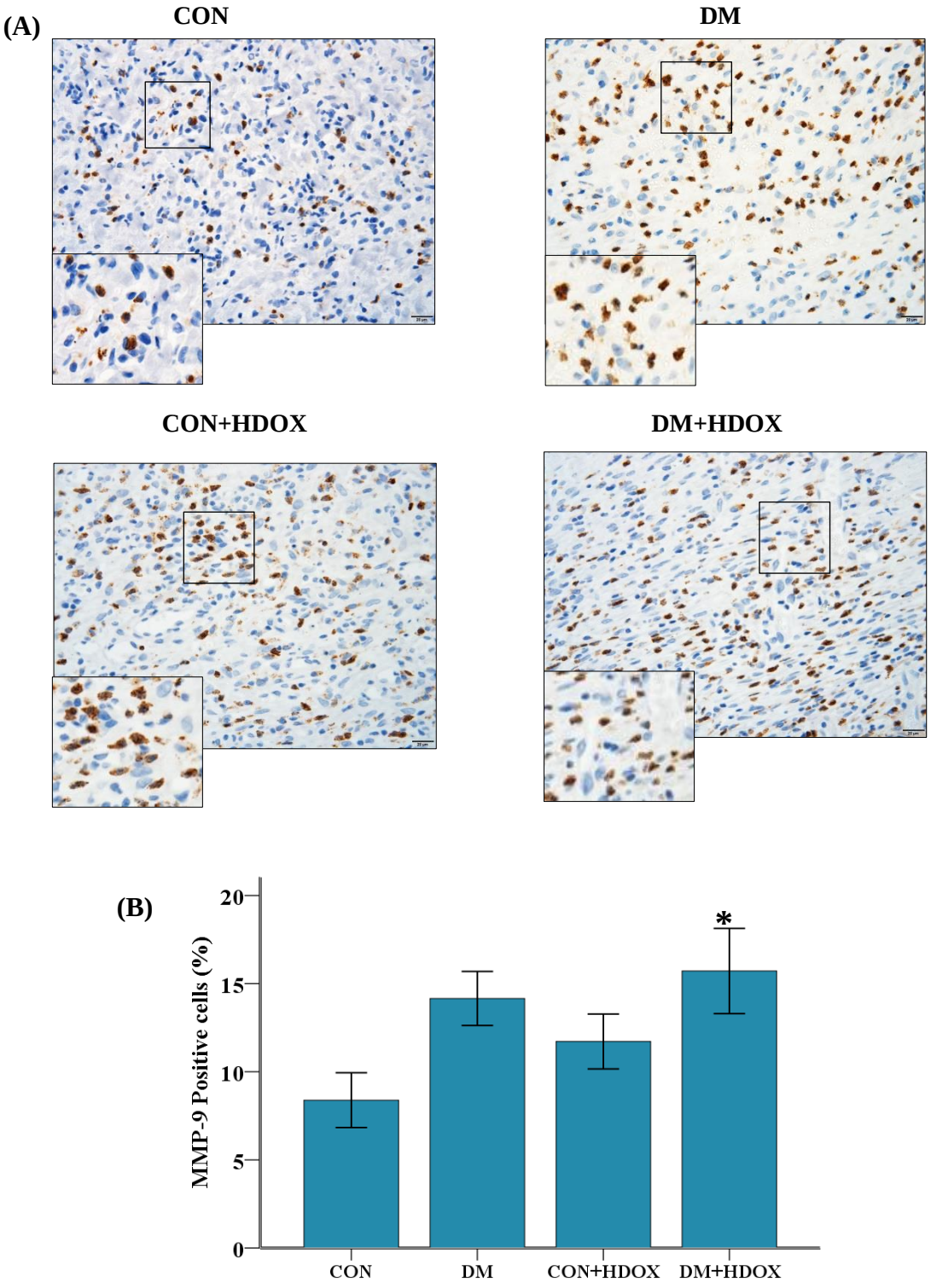

Figure 4-7: Representative images and quantitative data of skin wounds showing MMP-9 positive cells.

*Showing (A) MMP-9 stained cells by IHC staining and (B) percentage of MMP-9 positive cells in wounds from control (CON, n=4), diabetic (DM, n=4), control doxycycline-treated (CON+HDOX n=3,) and diabetic doxycycline-treated (DM+HDOX, n=3) animals at day 6 post wounding. Image magnification×400. Results are Mean ± SEM *P<0.05 vs. control.*

4.3.4.1 **Effect of doxycycline treatment on wound inflammatory markers**

The gene expression of neutrophil markers as well as TLR4 and TLR2 in the skin wounds was measured in extracted RNA from homogenised skin wounds from the 100 mg/kg doxycycline treatment study as described (Chapter 2.5). As shown in Figure 4-8 the expression of NGAL, MMP-9, and MMP-8 tended to be increased in the diabetic animals but this failed to reach significance. Oral doxycycline (100mg/kg) significantly increased MMP-8 but had no effect on NGAL and MMP-9 mRNA. With regard to TLR2 and TLR4 in both groups, doxycycline treatment tended to increase their expression and this increase reached significance for the diabetic animals.

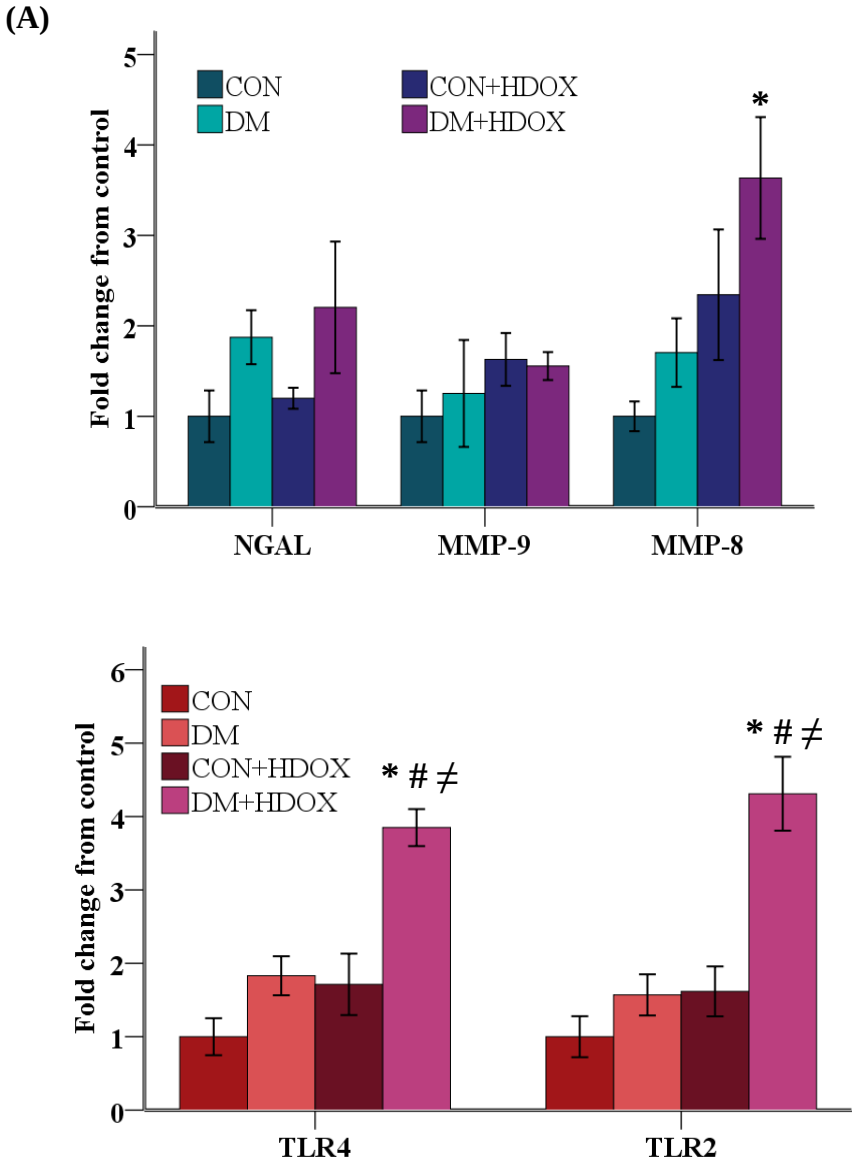

Figure 4-8: Effect of oral doxycycline (100mg/kg) on gene expression of (A) NGAL, MMP-9, and MMP-8 and (B) TLR4 and TLR2 in skin wounds.
*Wounds tissue from control (CON, n=5), diabetic (DM n=5,), control doxycycline-treated (CON+HDOX, n=6) and diabetic doxycycline-treated (DM+HDOX, n=4) animals at 6 days post wounding. Values are corrected for expression of 36B4 as a housekeeper and expressed as fold change from control. Group data are Mean ± SEM. *P<0.05 vs. control. #P<0.05 vs. diabetic. ≠P<0.05 vs. CON+HDOX.*

4.3.5 **Effect of Doxycycline Treatment on Sponge Implant Inflammatory Cells**

To determine the effect of diabetes on wound inflammatory cell MMP-9 and NGAL expression and neutrophil activity, the cells and fluids were extracted from the sponge implant at day 6 post wounding. As described previously, the cellular fraction was analysed by qRT-PCR. As before the pro-inflammatory mediators TLR4, TLR2, and TNFα were also measured.

4.3.5.1 **Effect of doxycycline treatment on sponge implant MMP-9, neutrophil activation and expression of inflammatory markers**

As shown in Figure 4-9 compared to controls, the gene expression of NGAL and MMP-8 were significantly up-regulated in the cell infiltrates from sponges implanted in the diabetic animals ($P < 0.05$). The diabetes associated increase in NGAL was improved by doxycycline treatment (100mg/kg) but the gene expression of MMP-9 was unaffected and MMP-8 expression up-regulated by doxycycline. The expression of TLR4 and TLR2 was not altered by diabetes or doxycycline.

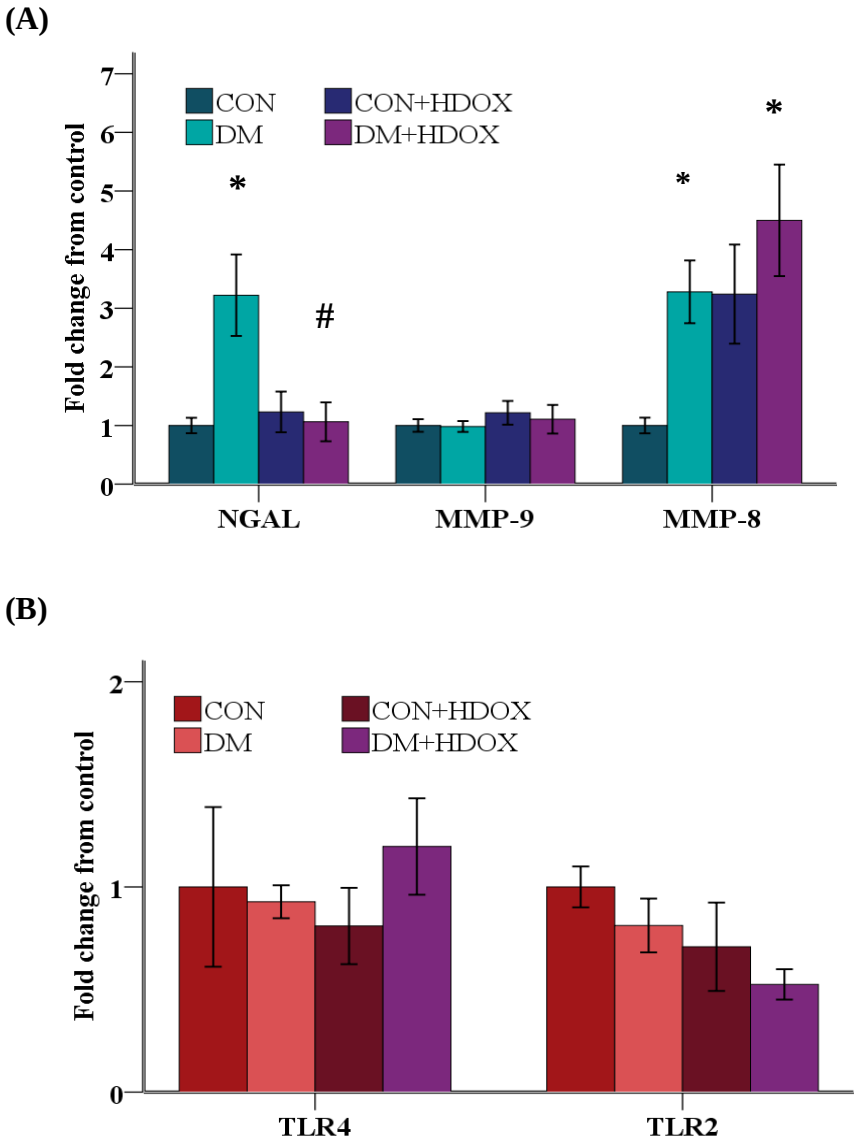

Figure 4-9: Effect of oral doxycycline (100mg/kg) on gene expression of (A) NGAL, MMP-9, MMP-8, (B) TLR4, and TLR2 in the sponge implant inflammatory cells.
*Control (CON), diabetic (DM), control doxycycline-treated (CON+HDOX) and diabetic doxycycline-treated (DM+HDOX) inflammatory cells from animals 6 days after wounding. Values are corrected for expression of 36B4 as a housekeeper and expressed as fold change from control. Data are Mean ± SEM. *P<0.05 vs. control. #P<0.05 vs. diabetic.*

In the sponge inflammatory cells and similar to the pattern seen with the high dose of doxycycline a lower dosage of doxycycline (Figure 4-10), up-regulated the expression of NGAL and MMP-8 in diabetic animals compared to control ($P \leq 0.05$). The increased expression of NGAL was partially prevented by doxycycline treatment and different to the high dose doxycycline, the lower dose of doxycycline (20mg/kg) partially prevented the increase in MMP-8 gene expression in the diabetic animals. The low dose of doxycycline had no effect on the gene expression of MMP-9, or the TLRs and TNFα.

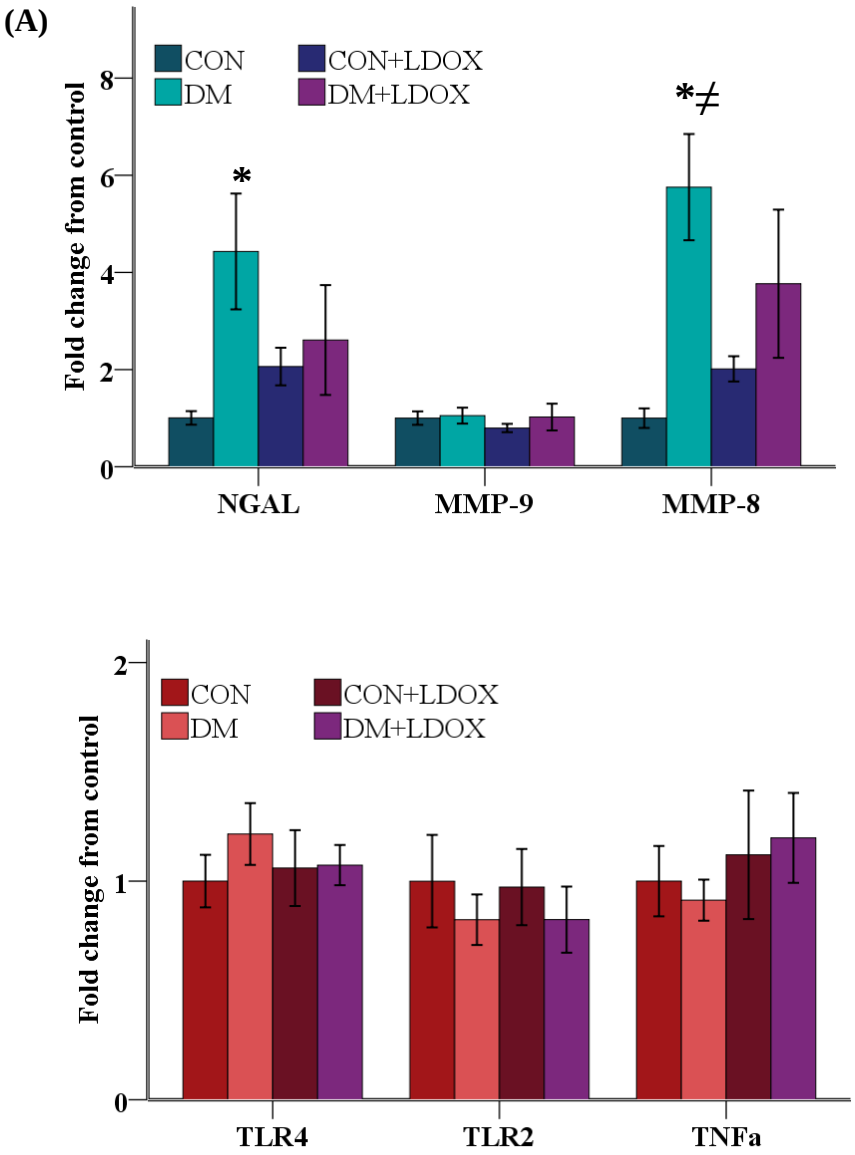

Figure 4-10: Effect of oral doxycycline (20mg/kg) on gene expression of (A) NGAL, MMP-9, MMP-8, (B) TLR4, TLR2 and TNFα in the sponge implant inflammatory cells. *Control (CON), diabetic (DM), control doxycycline-treated (CON+LDOX) and diabetic doxycycline-treated (DM+LDOX) inflammatory cells at 6 days post implant. Values are corrected for expression of 36B4 as house keeper and expressed as fold change from control. Data are Mean ± SEM. *P<0.05 vs. CON. ≠P <0.05 vs. CON+LDOX.*

4.3.5.2 **Cell expression of apoptosis markers in the implant inflammatory cells**

The effect of doxycycline on the expression of Bcl-2 an anti-apoptotic protein was then measured in implant inflammatory cells by qRT-PCR. As shown in Figure 4-11 diabetes or doxycycline treatment had no effect on inflammatory cell Bcl-2 gene expression.

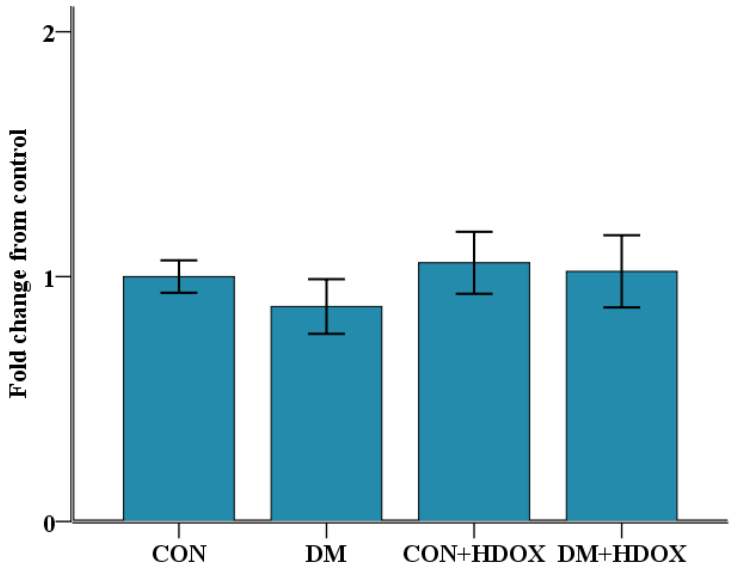

Figure 4-11: Gene expression of Bcl-2 in the sponge implant inflammatory cells.
Control (CON), diabetic (DM), control doxycycline-treated (CON+LDOX) and diabetic doxycycline-treated (DM+LDOX) inflammatory cells 6 days post implant. Values are corrected for expression of 36B4 as housekeeper and expressed as fold change from control. Data are Mean ± SEM.

4.3.5.3 **Effect of doxycycline treatment on wound fluid MMP-9 and NGAL**

To examine whether doxycycline treatment could alter wound fluid MMP-9 activity as well as its ability to form a complex with NGAL, the various forms of MMP-9 and its complex with NGAL was measured by zymography. At day 6 AMMP-9, TMMP-9, and the NGAL/MMP-9 complex were all significantly increased in the diabetic animals compared to control animals ($P<0.05$). Similar to the pattern seen in Chapter 3 the increase in TMMP-9 level was mainly due to the increase in AMMP-9 (Figure 4-12). In diabetic animals doxycycline treatment (100mg/kg) partially prevented the increased AMMP-9 and had no effect on the NGAL/MMP-9 complex.

The concentration of soluble NGAL in the wound fluid samples was not altered by diabetes or doxycycline treatment (Figure 4-12-B).

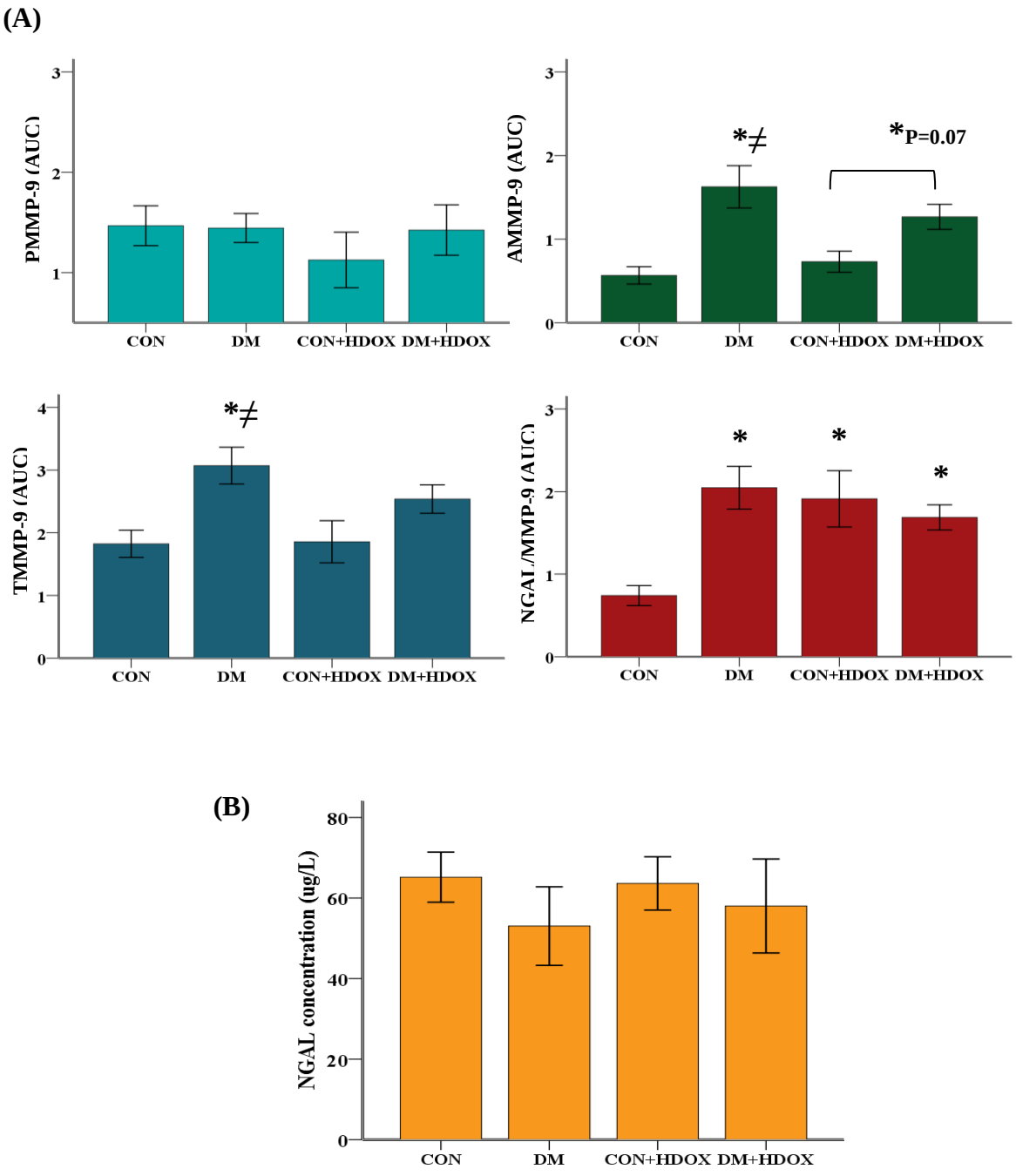

Figure 4-12: The effect of diabetes on MMP-9 activity and NGAL level in wound fluid of high-dose doxycycline groups.

*Enzymatic activity of MMP-9 in pro and active forms as well as activity level of NGAL/MMP-9 complex in control (CON, n=19), diabetic (DM, n=22) control doxycycline-treated (CON+HDOX, n=12) and diabetic doxycycline-treated (DM+HDOX, n=18) sponge inflammatory cells at 6 days post wounding in wound fluid by (A)zymography. (B)ELISA for soluble NGAL. Data are Mean ± SEM.*P <0.05 vs. control. #P<0.05 vs. diabetic.*

As shown in (Figure 4-13) and seen previously in the studies described in Chapter 3, diabetes was associated with significantly increased wound fluid AMMP-9, TMMP-9, and NGAL/MMP-9 levels but PMMP-9 was unaltered. Different to the effect of the higher dose doxycycline, the lower dose of doxycycline increased AMMP-9 and TMMP-9 in both control and diabetic rats compared to untreated control animals reaching significance for the diabetic rats ($P<0.05$). However, doxycycline treatment partially prevented the diabetes related increase in formation of NGAL/MMP-9 complex.

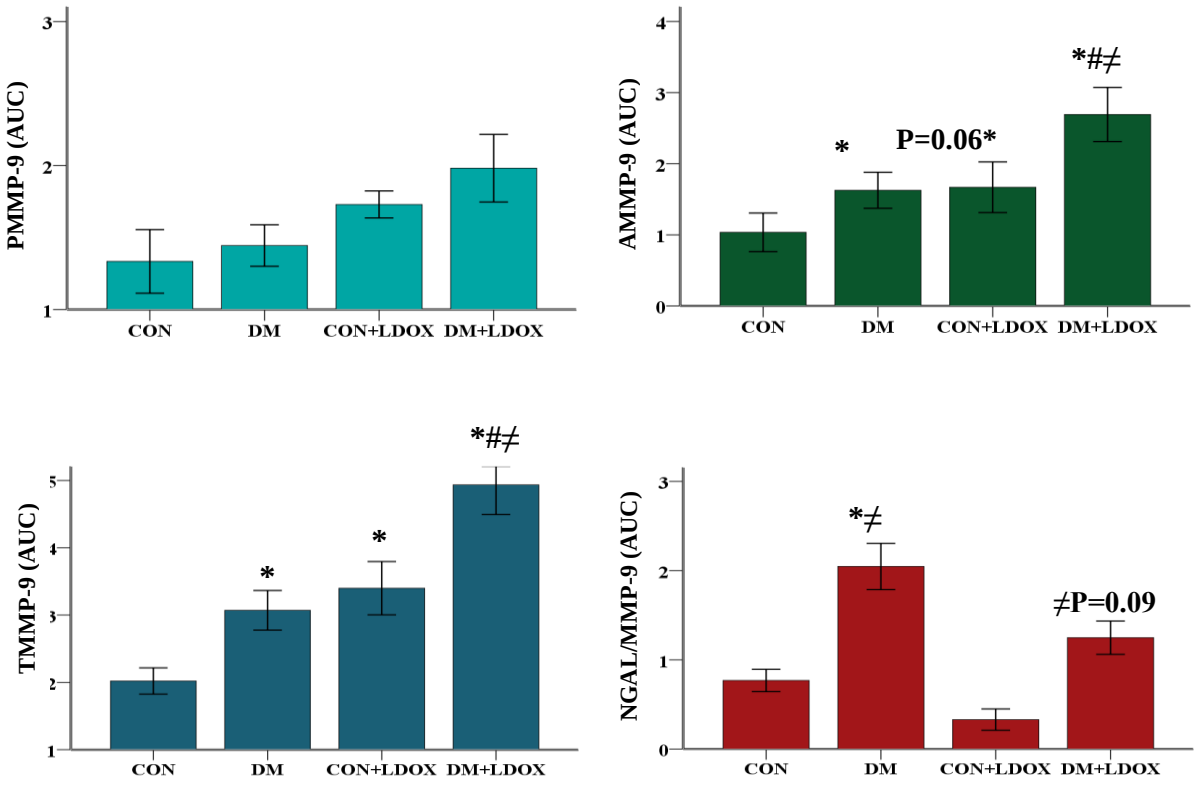

Figure 4-13: The effect of diabetes on MMP-9 activity and NGAL level in wound fluid of low-dose doxycycline groups.

*Wound fluid enzymatic activity of MMP-9 in pro and active forms as well as the level of NGAL/MMP-9 complex in wound fluids obtained 6 days post wounding from control (CON, n=14), diabetic (DM, n=22), control doxycycline treated (CON+LDOX, n=10) and diabetic doxycycline-treated (DM+LDOX, n=10) animals. Data are Mean ± SEM.*P<0.05 vs. control. #P<0.05 vs. diabetic. ≠P<0.05 vs. CON+LDOX.*

4.3.5.4 **Effect of doxycycline treatment on wound neutrophil number**

The effect of doxycycline treatment on sponge implant neutrophil activity was then examined at 6 days after implant. Only cells from the animals treated with 20mg/kg doxycycline were examined and results were compared with untreated control and diabetic animals.

Shown in Figure 4-14-A and similar to the previous study (Chapter 3) diabetes increased the percentage of NGAL and MPO positive cells as well as NGAL/MPO co-localisation reaching significance for the MPO positive and NGAL/MPO co-localised cells. Doxycycline treatment prevented these changes.

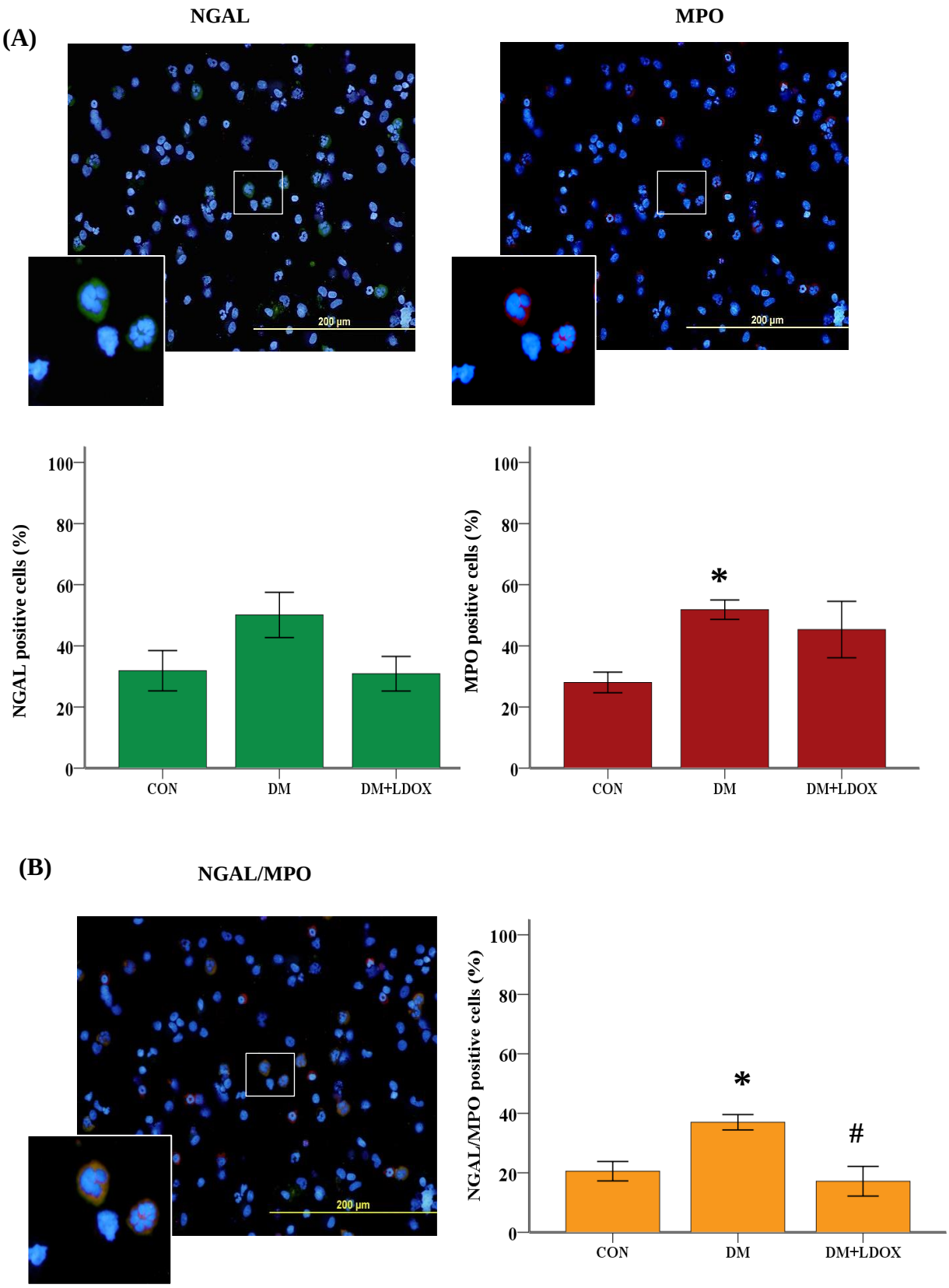

Figure 4-14: Representative images and quantitative group data from (A) NGAL (green), MPO (red) staining and (B) NGAL/MPO co-localisation (yellow) in sponge implant inflammatory cells by immunofluorescence.
*Control (CON), diabetic (DM), control doxycycline-treated (CON+LDOX) and diabetic doxycycline-treated (DM+LDOX) animals. Data are Mean ± SEM. *P <0.05 vs. control. #P<0.05 vs. diabetic.*

4.3.6 **Effect of Diabetes and Doxycycline Treatment on Circulating Neutrophils**

Results presented in Chapter 3 suggested that the circulating neutrophil number was increased in wounded diabetic animals compared to similarly treated controls. The effect of wounding on circulating neutrophil numbers as well as the expression of neutrophil activation markers, TLRs 2 and -4 and TNFα were quantified in animals treated with 100 mg/kg doxycycline. The effect of 20mg/kg doxycycline on circulating neutrophils was examined only in the sponge implant model.

4.3.6.1 **Effect of diabetes and doxycycline on circulating neutrophil markers in unwounded animals**

Shown in Figure 4-15, in unwounded animals diabetes increased circulating neutrophil expression of NGAL, MMP-9 and MMP-8, but this only reached significance for MMP-9. Doxycycline treatment at 100mg/kg further increased NGAL gene expression in the diabetic and MMP-9 in both diabetic and control animals. In diabetic animals expression of TLR4 tended to increase but TLR2 was unaltered. The tendency toward increased TNFα gene expression in diabetic animals was further amplified by doxycycline treatment. These effects of doxycycline on MMP-9 and TNFα were also seen in the doxycycline-treated control animals.

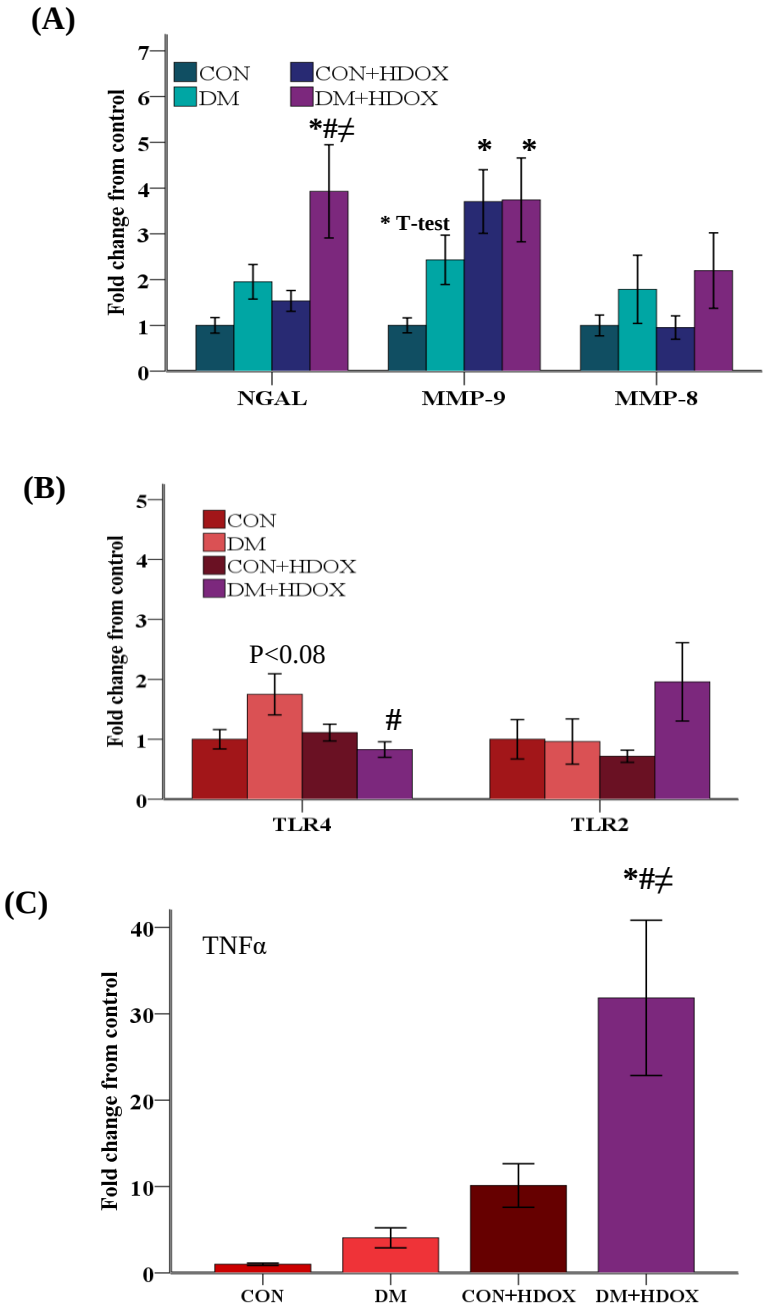

Figure 4-15: Effect of diabetes and doxycycline treatment (100 mg/kg) on circulating neutrophil gene expression (unwounded animals).
*Shown are results for A) NGAL, MMP-9, MMP-8, B) TLR4, and TLR2 and C) TNFa gene expression in neutrophils isolated from control (CON, n=6), diabetic (DM, n=6), control doxycycline-treated (CON+HDOX, n=6) and diabetic doxycycline-treated (DM+HDOX, n=4) animals. Values are corrected from expression of 36B4 as housekeeper and expressed as fold change from control. Data are Mean ± SEM.*P<0.05 vs. control. $^{\#}$P<0.05 vs. diabetic. $^{\neq}$P<0.05 vs. CON+HDOX.*

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4.3.6.2 **Effect of diabetes and wounding on circulating neutrophils in the excisional wound model**

The next series of experiments examined expression of neutrophil markers in circulating neutrophils obtained from animals that received the excisional wounds. Results in Figure 4-16 showed the gene expression of NGAL, MMP-9, and MMP-8 was increased in wounded diabetic animals but did not reach to a significant level. Doxycycline treatment had no effect on TLR4 and attenuated the increase in TLR2. The expression of TNFα was increased by doxycycline. This increase in TNFα was similar to the effect observed in similarly treatment-unwounded animals.

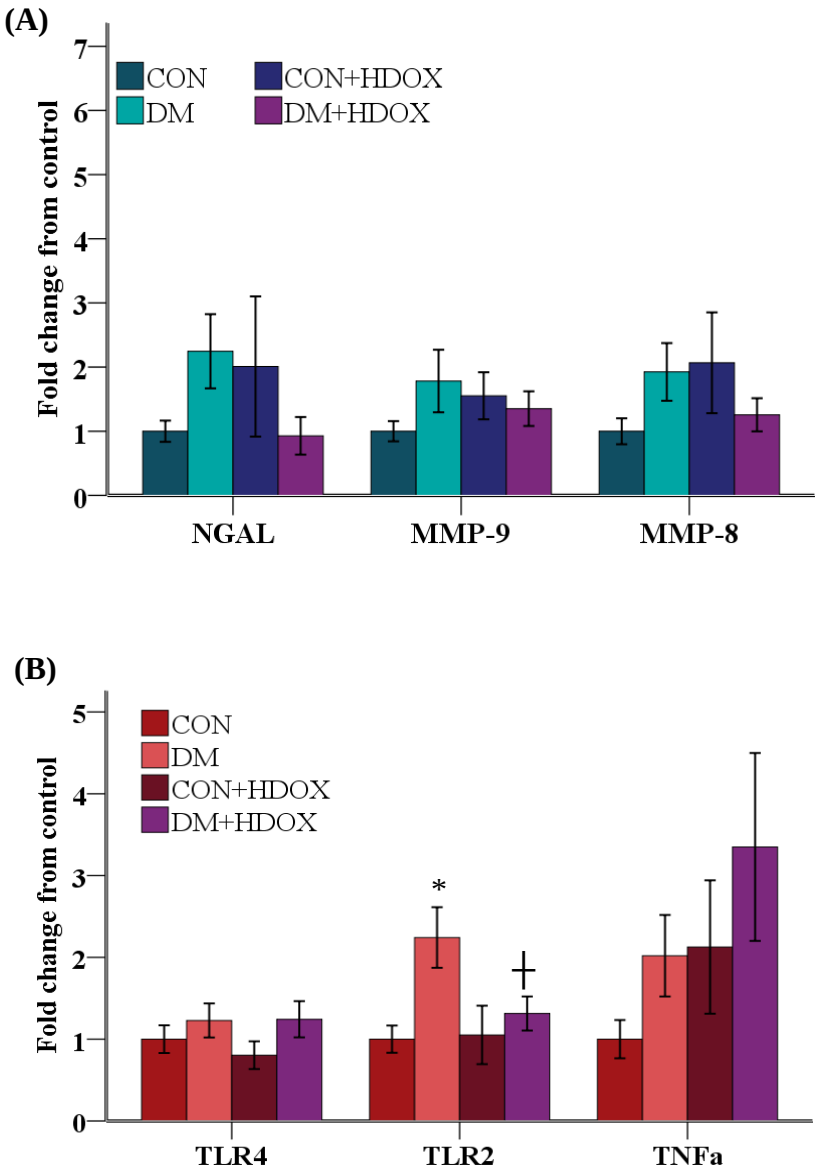


Figure 4-16: Effect of diabetes and doxycycline treatment (100 mg/kg) on circulating neutrophil gene expression (excisional wound model).
*Shown are results for A) NGAL, MMP-9, MMP-8, B) TLR4, and TLR2 and TNFa gene expression in neutrophils isolated from control (CON, n=7), diabetic (DM, n=9), control doxycycline-treated (CON+HDOX, n=6) and diabetic doxycycline-treated (DM+HDOX, n=5) animals. Values are corrected for expression of 36B4 as house keeper and expressed as fold change from control. Data are Mean ± SEM. *P<0.05 vs. control. #P<0.05 vs. diabetic. †P<0.05 vs diabetic by T-test.*

4.3.6.3 **Effect of diabetes and doxycycline on circulating neutrophils in the sponge implant inflammatory model**

Gene expression of activated neutrophil markers and pro-inflammatory markers were also measured in circulating neutrophils from animals which received the sponge implants. Shown in Figure 4-17 at 6 days after implant of sponges the circulating neutrophil expression of NGAL, MMP-9 and MMP-8 as well as TLRs 2 and 4 and TNFα were all significantly increased in diabetic animals compared with controls. Doxycycline (100mg/kg) attenuated the changed NGAL, TLR2 and TNFα but had no effect on MMP9, MMP-8 and TLR4. As before doxycycline treatment of control animals tended to increase MMP-9, MMP-8 and TLR4 expression.

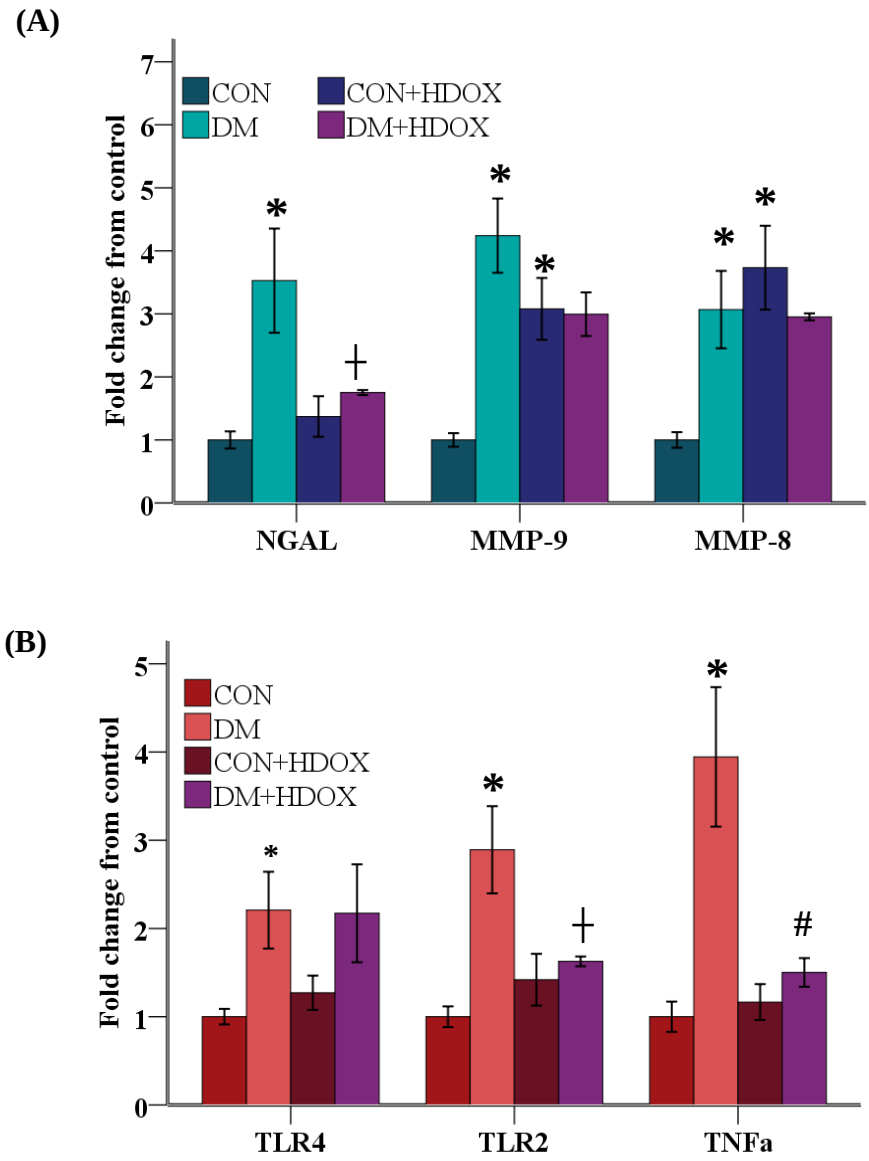


Figure 4-17: Effect of diabetes and doxycycline treatment (100 mg/kg) on circulating neutrophil gene expression (Sponge implant wound model).
*Shown are results for A) NGAL, MMP-9, MMP-8, B) TLR4, and TLR2 and TNFa gene expression in neutrophils isolated from control (CON, n=9), diabetic (DM, n=10), control doxycycline-treated (CON+HDOX, n=6) and diabetic doxycycline-treated (DM+HDOX, n=3) animals. Values are corrected for 36B4 expression as housekeeper and expressed as fold change from control. Group data are Mean ± SEM. *P<0.05 vs. control. #P<0.05 vs. diabetic. †P<0.05 vs diabetic by T-test*

The effect of 20 mg/kg doxycycline on circulating neutrophils was also studied in animals wounded by implantation of the sponge. Figure 4-18 shows, as before diabetes significantly increased circulating neutrophil expression of NGAL and MMP-9, MMP-8 gene expression tended to increase but it was not significantly different from control. The expression of TLR4, TLR2, and TNFα also tended to be increased but this was only significant for TNFα. In contrast to the effect of the higher dose of doxycycline in this study the lower dose of doxycycline (20mg/kg) prevented the diabetes related changes and had no effect on the control neutrophils.

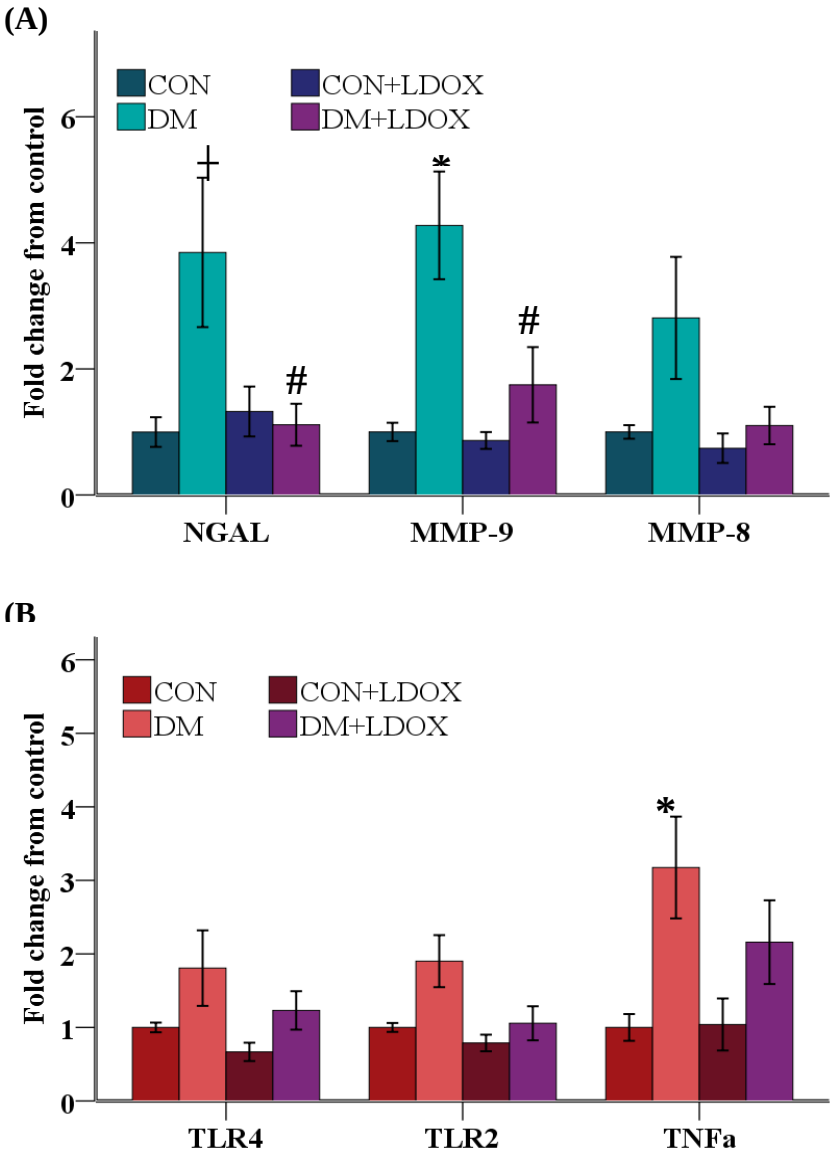


Figure 4-18: Effect of diabetes and doxycycline treatment (20 mg/kg) on circulating neutrophil gene expression (Sponge implant wound model).
*Shown are results for A) NGAL, MMP-9, MMP-8, B) TLR4, and TLR2 and TNFa gene expression in neutrophils isolated from control (CON, n=5), diabetic (DM, n=7), control doxycycline-treated (CON+LDOX, n=6) and diabetic doxycycline-treated (DM+LDOX, n=6) animals. Values are corrected for expression of 36B4 as housekeeper and expressed as fold change from control. Group data are Mean ± SEM. *P<0.05 vs. control. #P<0.05 vs. diabetic. P<0.05 vs diabetic by T-test.*

4.3.6.4 **Effect of diabetes and doxycycline treatment on protein expression in the circulating neutrophils by immunofluorescence**

Immunofluorescence was then used to semiquantitatively examine NGAL and MMP-9 protein level and co-localisation in neutrophils. As shown (Figure 4-19-A & B & C) a significantly higher percentage of neutrophils from diabetic animals expressed either NGAL or MMP-9 than controls (29% vs 10 % for NGAL and 47% vs 26% for MMP-9). Doxycycline partially prevented the increase in NGAL expression and significantly increased MMP-9 expression in both control and diabetic animals.

The percentage of neutrophils which expressed MMP-9 was higher than those expressing NGAL (26% vs 10% respectively for controls and 47% vs 29% respectively for diabetic animals) and was significantly higher in the diabetic animals compared to control animals. This effect was partially prevented by doxycycline treatment. Consistent with our previous staining results when NGAL was co-localised with MMP-9 the co-localisation occurred in the peri nuclear region and this was not altered by diabetes (Figure 4-19).

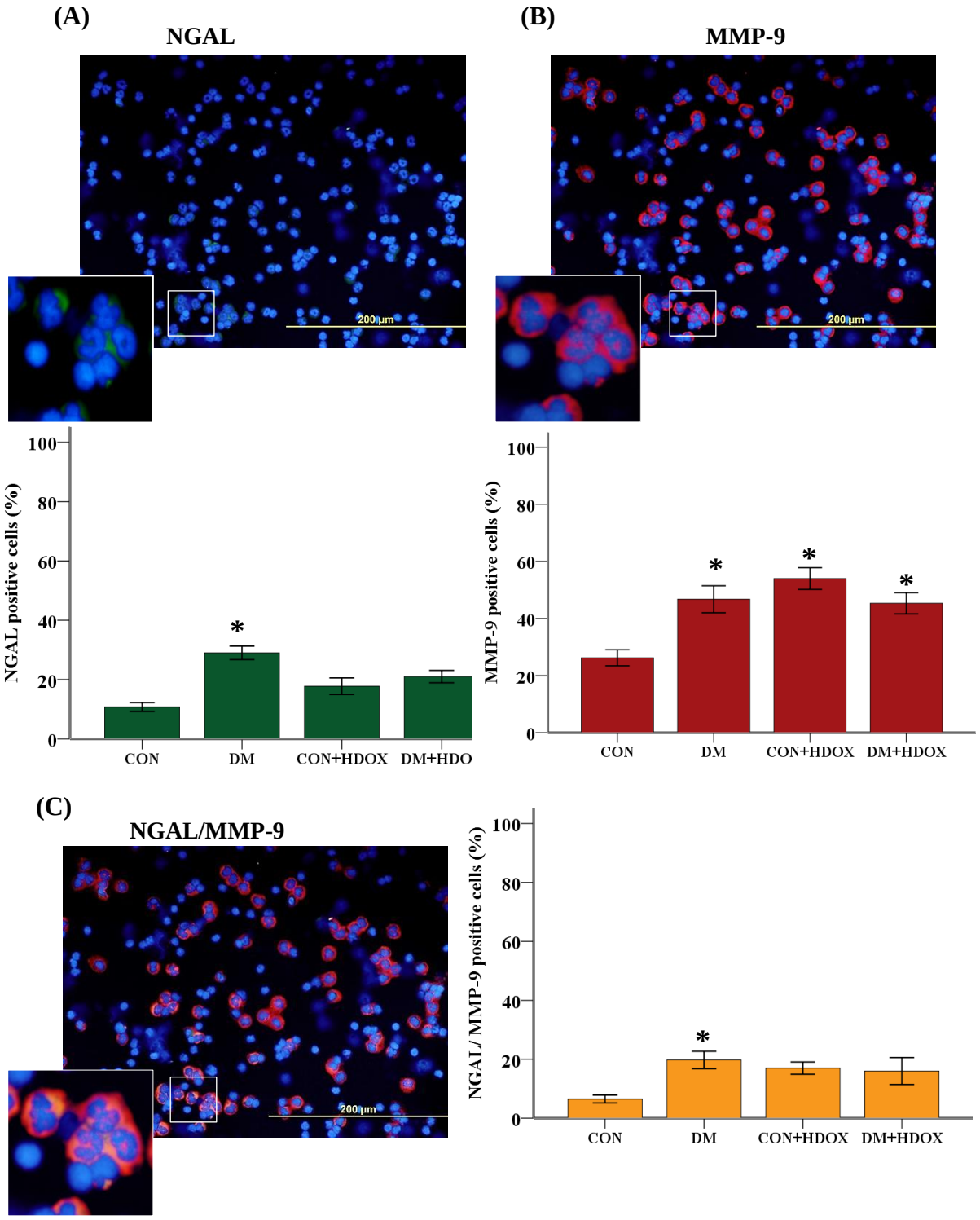


Figure 4-19: Effect of doxycycline (100mg/kg) treatment on neutrophil NGAL and MMP-9.

*Representative images and quantitative data of (A) NGAL (green), MMP-9 (red) and (B) co-localisation of NGAL and MMP-9 (yellow) in circulating neutrophils.Control (CON), diabetic (DM), control doxycycline-treated (CON+HDOX) and diabetic doxycycline-treated (DM+HDOX) animals. Group data are Mean ± SEM.*P<0.05 vs. control.*

4.3.6.5 **Effect of diabetes and doxycycline treatment on neutrophil apoptosis**

We have shown (Chapter 3) that circulating neutrophil apoptosis is delayed in the sponge implanted diabetic animals, whether doxycycline could prevent this change was investigated in this study. In diabetic animals which were either unwounded or wounded with an excisional wound no change in neutrophil MCL-1 gene expression was observed. As before MCL-1 gene expression was significantly increased in circulating neutrophils from diabetic animals in the sponge implant group; this increase was prevented by treatment with doxycycline (Figure 4-20).

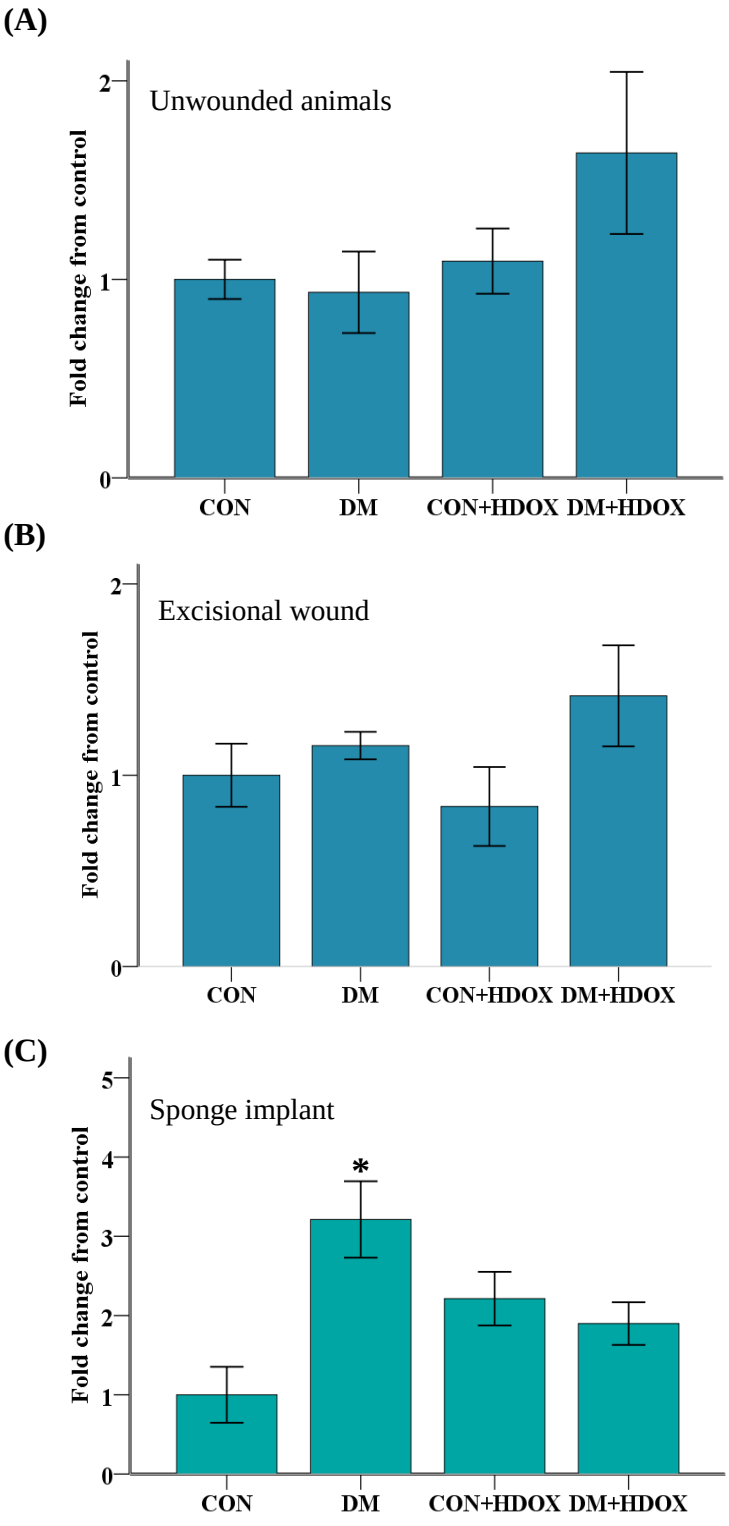


Figure 4-20: MCL-1 gene expression in the circulating neutrophils from doxycycline treated animals (100 mg/kg) which were either (A) unwounded, or wounded with (B) an excisional wound or (C) by sponge implant.
*In control (CON, n=5), diabetic (DM, n=5), control doxycycline-treated (CON+HDOX, n=5), and diabetic doxycycline-treated (DM+HDOX, n=5) animals. Values are corrected for expression of 34B4 as housekeeper and presented as fold change from control. Data are Mean ± SEM. *P<0.05 vs. control.*

4.3.6.6 **Effect of diabetes and doxycycline on plasma MMP-9 activity and NGAL concentration**

The activity of MMP-9 was measured by zymography in plasma from rats treated with high dose doxycycline. As shown in Figure 4-21-A at day 6 post wounding diabetes had no effect on the plasma MMP-9 levels including the PMMP-9 and AMMP-9 forms. In non-diabetic rats and similar to the effect of doxycycline on neutrophil activities in the wound and in the circulation, high dose doxycycline significantly increased plasma PMMP-9 and TMMP-9 and NGAL/MMP-9 complex (Figure 4-21-A). By ELISA the soluble form of NGAL was not affected by diabetes or doxycycline treatment (Figure 4-21-B).

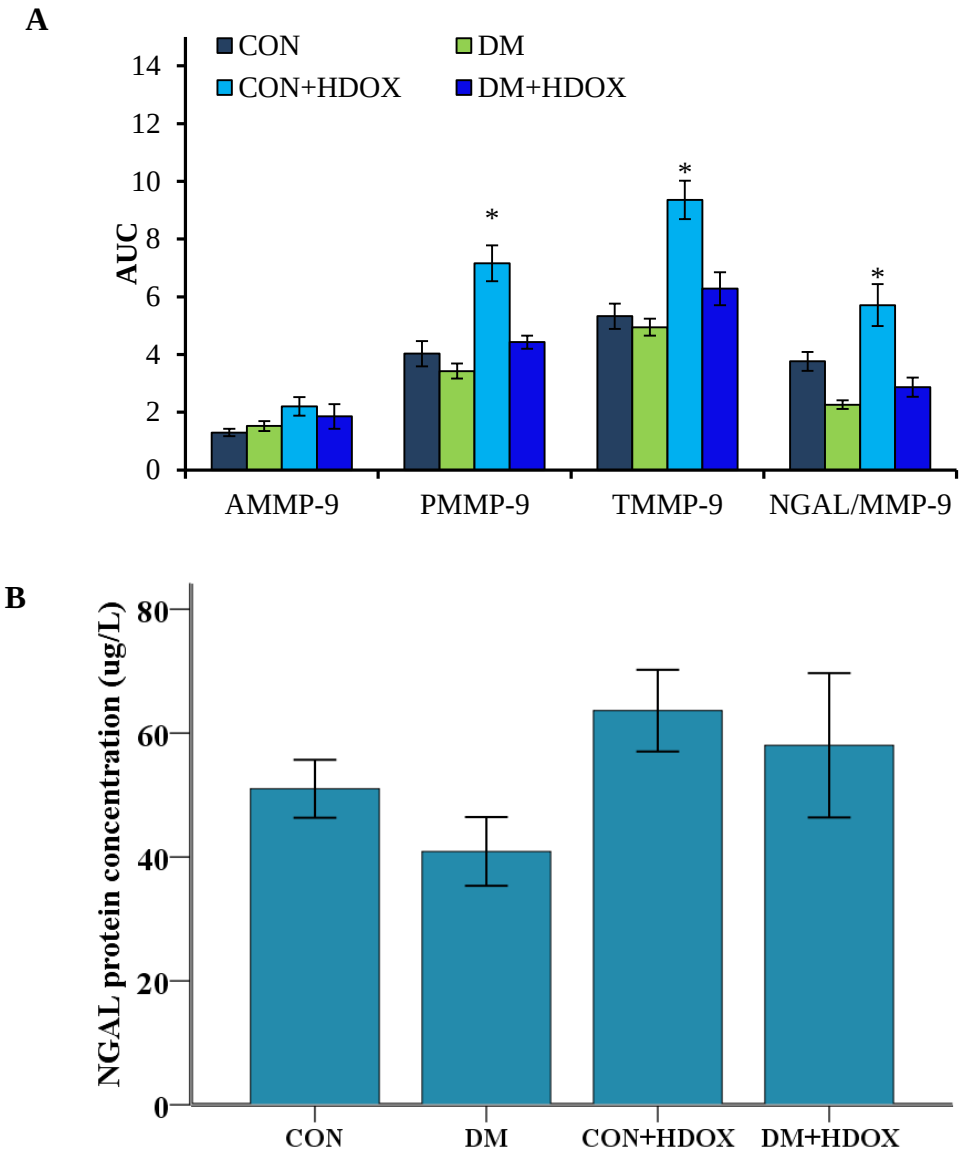


Figure 4-21: The effect of diabetes and high dose doxycycline (100 mg/kg) on plasma MMP-9 and NGAL.

*Plasma enzymatic activity of (A) MMP-9 in pro (PMMP-9), and active (AMMP-9) forms and total MMP-9 (TMMP-9) as well the NGAL/MMP-9 complex 6 days after wounding and (B) soluble form of NGAL measured by ELISA in control (CON, n=15), diabetic (DM, n=16), control doxycycline-treated control (CON+HDOX, n=16) and doxycycline-treated diabetic (DM+HDOX, n=16) animals. Group data are Mean ± SEM. *P <0.05 vs. control.*

A similar series of studies was performed on plasma samples of animals in the low doxycycline treatment study. As shown in Figure 4-22 and similar to the high dose doxycycline (100mg/kg) study diabetes had no effect on MMP-9 activity in any form. Interestingly, in this study low dose doxycycline (20mg/kg) treatment of control animals had no effect on PMMP-9 or TMMP-9 but the slight increase in the AMMP-9 and PMMP-9 in the doxycycline-treated diabetic animals resulted in a significant increase in TMMP-9 in this group. There was no significant difference in the concentration of NGAL/MMP-9 complex in control and diabetic animals. However, the low dose doxycycline decreased NGAL/MMP-9 complex formation in the both control and diabetic animals.

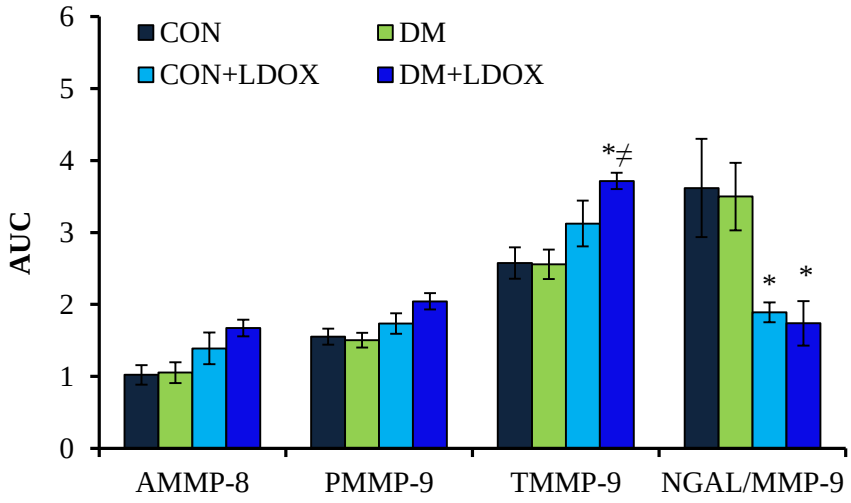


Figure 4-22: The effect of diabetes on MMP-9 activity and NGAL level in plasma.
*Plasma enzymatic activity of MMP-9 in pro and active forms as well as activity level of NGAL/MMP-9 complex in plasma in control (CON, n=6), diabetic (DM, n=5), control doxycycline-treated (CON+LDOX, n=7) and diabetic doxycycline-treated (DM+LDOX, n=6) animals. Data are Mean ± SEM. *P<0.05 vs. control. ≠P<0.05 vs. CON+LDOX.*

4.4 DISCUSSION

The MMPs are key regulators of wound healing and inhibition of their activity may improve wound healing. Doxycycline a known weak antibiotic can inhibit MMP-9 activity through the chelation of calcium and zinc ions. In some studies treatment with doxycycline has been shown to improve wound closure (Wilcox et al., 2012). In this study we used treatment with doxycycline to attempt to regulate MMP-9 activity to examine the importance of MMP-9 in wound healing in our diabetic rodent wound models. Further, whether doxycycline treatment could regulate neutrophil activity and NGAL was also examined.

In these studies two different dosages of doxycycline were used. We used 100 mg/kg as high dose of doxycycline and 20 mg/kg as a low dose. Administration of a 20 mg/kg dose of doxycycline daily is the only drug therapy approved by the United States Food and Drug Administration (FDA) for adult periodontitis as a collagenase inhibitor (Lee et al., 2004). However, the 100 mg/kg/day dose has been shown to be effective for reduction of MMP-9 expression in the mice brain tissue (Lee et al., 2006). Additionally, Chin and colleagues (2003) found that doxycycline at 50, 500, and 5,000 mM significantly reduced protease activity by 44%, 75%, and 89% respectively, in diabetic foot ulcers (Chin et al., 2003).

In this work we have shown that doxycycline at a dosage of 100 mg/kg was not able to improve the wound closure and did not substantially alter wound MMP-9 activity. In addition, in both control and diabetic animals this dose of doxycycline negatively impacted on neutrophil function as evidenced by increased neutrophil number and activation in this group. These results were supported by increased gene expression

of NGAL, MMP-9 and MMP-8 in skin wounds as well as MMP-8 gene expression in the sponge inflammatory cells. On the other hand, we showed that low dose (20 mg/kg) doxycycline improved wound closure in diabetic animals. The results from these studies also showed the number of neutrophils as well as gene expression of NGAL and MMP-8 was decreased in the sponge implant inflammatory cells in doxycycline-treated diabetic animals. This dose of doxycycline had no effect on increased MMP-9 level in the wound fluid of diabetic treated animals. Together these studies suggest that oral doxycycline treatment at a low dose may be able to prevent increased neutrophil activity and may have some utility in improvement of wound healing in diabetes.

In this work we further investigated the effect of wounding on circulating neutrophil activation and apoptosis. In diabetes, wounding increased the circulating neutrophil expression of NGAL, MMP-9 and MMP-8 as well as TLRs -4 and -2 and TNFα. Moreover, the level of increase with the invasiveness of the surgery with the greatest increases occurring in the sponge implant model. Like the effect of the different doses of doxycycline on wound healing rate the dose of doxycycline also had substantially different effects on neutrophil activation markers. For example, treatment with doxycycline (100 mg/kg) attenuated the increased expression of NGAL in both models but had no effect on MMP-8 and MMP-9 gene expression. In contrast doxycycline at 20mg/kg significantly prevented the diabetes associated increase in expression markers of neutrophil activity NGAL, MMP-8, and MMP-9. The expression of pro-inflammatory markers TLR4, TLR2, and TNFα were also partially improved by low dose of doxycycline. Several studies in animals have reported that treatment with doxycycline or other tetracycline analogues improved

healing parameters. For example, systemic treatment of rats with doxycycline increased tensile strength of rat intestinal anastomoses 3 days after surgery (Pasternak et al., 2008). Topically applied chemically modified tetracycline enhances wound healing in streptozotocin diabetic rat skin (Ramamurthy et al., 1998).

The mechanism of this effect of doxycycline on neutrophil activation is not clear. Other workers have also shown that some members of the doxycycline family can decrease NGAL and MMP-9 (Serra, Buffone, et al., 2013) and TNFα (Cazalis et al., 2009) by suppressing superoxide generation, degranulation and migration of neutrophils. We have shown that doxycycline can prevent the increase in TNFα. TNFα is an important inflammatory mediator that regulates neutrophil activity (Cazalis et al., 2008). TNFα is also a potent inducer of MMP-9 expression and activity. It is possible that the prevention of increased TNFα by doxycycline treatment can improve neutrophil activity and decrease their expression of MMP-9.

A novel new finding of this study was the observation that diabetes and wounding can increase neutrophil longevity. The expression of markers of activated neutrophil was also increased as was MMP-9. Others have also shown that activation of neutrophils in response to inflammation increases their longevity (Hanses et al., 2011; Kolaczkowska & Kubes, 2013). Further, the presence of primed neutrophils at the site of inflammation can also contribute to cell injury by activation of neutrophil gene expression and function (Kolaczkowska & Kubes, 2013). This prolonged activation can produce cytokines such as interleukins and TNFα (Cowburn et al., 2005; Schultz et al., 2005) and in normal wounds has been shown

to inhibit re-epithelialisation (Dovi et al., 2004). We have shown that diabetes can further increase this process. These results suggest in diabetes neutrophil activation is prolonged as is their longevity. Together these changes can contribute to the increased inflammation in diabetes.

In conclusion, treatment of diabetic animals with oral low dose doxycycline improved wound healing. The apparent beneficial effect of low dose of doxycycline treatment on the wound healing of our diabetic animal models was probably not due to the antibiotic action of doxycycline, since the expression of TLR4, and TLR2 as markers for bacterial infection did not alter in the wound infiltrated cells. The results obtained from the circulating cells suggest that low dose doxycycline treatment can also prevent the diabetes and wounding associated increase in neutrophil activation and longevity as well as a reduction in MMP-9 activities.

Thus far, the results obtained from rodent studies suggest diabetes and wounding related increase in neutrophil activity. Whether these changes also occur in people with diabetes and whether neutrophil activities and apoptosis are altered in association with wound healing is not known. For this reason the effect of diabetes on inflammatory cells and expression of activated neutrophil markers NGAL, MMP-8 as well as MMP-9 was investigated in the white blood cells of diabetic patients.

CHAPTER 5: Effect of Diabetes on Circulating Neutrophil Markers in Human

5.1 **INTRODUCTION**

Many studies have described the association in diabetic patients between increased severity of infection and neutrophil function (Alba-Loureiro et al., 2007). Neutrophils can recognise and kill invading microorganism and to stimulate other immune cells to effectively eliminate threats of infection (Kumar & Sharma, 2010). NGAL is produced from neutrophils to participate in the antibacterial iron depletion strategy of the innate immune system (Goetz et al., 2002). There has been a report of persistent NGAL protein levels in the wound fluid (Pukstad et al., 2010) and plasma (Serra et al., 2013) from persons with non-healing chronic venous leg ulcers. In addition, recent studies have focused on NGAL as a marker of early renal tubular damage and its association with renal pathophysiology. For example, elevated NGAL expression in the kidney, as well as elevated urinary NGAL levels have been observed in acute kidney injury (Mishra et al., 2003)

The results from previous chapters of this thesis have shown that in rodents, diabetes impairs neutrophil function and activation in wound fluids with increased wound inflammatory cell expression of NGAL and MMP-8 and activity of MMP-9 being observed. Whether these changes are also observed in people with diabetes is not as yet clear. Therefore, pilot studies were performed on two independent cohorts of diabetic patients and the expression of NGAL, MMP-8, as specific markers of activated neutrophils, and MMP-9 was also measured in the blood white cells.

5.1 STUDY PROCEDURES

As mentioned, pilot studies were performed investigating circulating levels of NGAL, MMP-8, and MMP-9 in two different cohorts of diabetic patients. In the first study whether the expression of these markers was altered in association with the presence or absence of diabetic complications was examined. In the second study, the expression of NGAL, MMP-8, and MMP-9 was measured at various time points in association with wound healing in patients attending the Foot Clinic of the Diabetes Centre of RPA Hospital. All patients regardless of the study group attended the Diabetes Centre of RPA Hospital. Controls were members of the Diabetes Centre or Laboratory staff. The ethics for the studies were approved by the Ethics Review Committee of the RPAH hospital and were carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

The details of each of the studies are as follows:-

Study 1: Seventy three individuals including control (n=22), and diabetic (n=51) persons participated in this study. All people with diabetes had duration of the disease for greater than 10 years. Based on the absence or presence of complications (micro or macro vascular), patients were divided into two groups: diabetic patients with no complications (DM+NC) and diabetic patients with complications (DM+C). Diabetes complications included neuropathy, retinopathy and nephropathy.

In all patients the presence or absence of retinopathy was confirmed by fundal examination and/or photography. The presence or absence of diabetic nephropathy was confirmed by normal serum creatinine and urinary albumin/creatinine ratio (U

Alb/Cr) <2.5 mg/mmol for males and <3.5 mg/mmol for females. The characteristics of subjects in this group are shown in Table 5.1.

Table 5-1: Clinical parameters of control and diabetic subjects with or without complications.

Clinical Parameters	**Control** n=22	**Diabetic** n=51	**Diabetic**	
			DM +NC n=24	**DM+C** n=27
Age (yrs)	47.4±2.8	62.7±1.6*	64.1±1.7*	61.6±2.6*
Duration (yrs)	NA	19.5±7.5	17.8±6.7	21.8±8.1
Weight (kg)	75.7±4.1	80.7±2.0	80.6±2.9	80.7±2.9
BMI (kg/m^2)	28.5±7.8	28.7±4.9	29.1±5.1	28.2±4.7
HbA_{1c} (%)	NA	7.7±1.1	7.7±0.6	8.2±1.2#
Serum Creatinine (µmol/L)	69.5±3.1	83.7±3.1*	75.9±3.6	91.2±4.5*#
U Alb/Cr Ratio (mg/mmol)	0.8±0.1	2.03±2.02*	1.1±0.2*	3.2±0.7*#
eGFR (ml/min)	91.4±4	81.8±3.5*	87.3±4.9	75.8 ±4.8
HDL cholesterol (mmol/L)	0.8±0.1	1.3±0.1*	1.3±0.1*	1.2±0.1*
LDL cholesterol (mmol/L)	2.9±0.2	2.1±0.1	2.2±0.2	2.1±0.2

*Data are expressed as Mean ± SD, *P<0.05 different from control (CON). #P<0.05 different from diabetic without complications (DM+NC).*

Blood was obtained from all patients and the plasma and white blood cells were isolated (as described below). The gene expression of NGAL, MMP-9, and MMP-8 was measured in white blood cells and NGAL, MMP-9, NGAL/MMP-9 complex and TNFα were measured in the plasma by ELISA and zymography.

Study 2: The characteristics of the participants in the second study are shown in Table 5-2 . In this study all patients were diabetic and had a foot ulcer and were attending the Foot Clinic of the Diabetes Centre of RPA Hospital. After consent blood samples were obtained at the initial (Visit 1) and the last visit (Visit 2: week 8 or week 12) and wound area was also measured at each visit. All participants had type 2 diabetes and the duration of diabetes ranged between 3 to 24 years.

Table 5-2: Clinical parameters of diabetic subjects with foot ulcers investigated in this study

Diabetic patient's characteristics	
Age (yrs)	60.4 ±6.6
Sex (male)	9:0
Diabetes duration (yrs)	3-24
DM (T1:T2)	0:9
HbA1c (%) at entry	8.1± 1.3
HbA1c (%) at healing	7.3± 1.0
Triglycerides (mmol/L)	1.2±0.5
HDL cholesterol (mmol/L)	1.3±0.3
LDL cholesterol (mmol/L)	2.6±0.9
Ischemia present (yes: no)	2:7
Infection present at entry (yes: no)	4:5

Data are mean ± SEM

The wound severity was classified based on Texas stage as follows: I: grade 0; pre or post ulcerative lesion completely epithelised II: grade 1; superficial wound, not involving tendon, capsule or bone, III: grade 3; wound penetrating to bone or joint. All wounds were either grade 1or 2 at entry and grade 0 or 1 at the last visit. Of the 9 patients enrolled, 5 had wounds with no evidence of clinically significant bacterial infection and 4 wounds were infected with the bacteria identified as Staphylococcus Aureus and Enterococcus Faecalis.

5.1.1 **Isolation of White Cells and Plasma from Whole Blood**

For the both studies white cells were isolated from heparinised blood samples as follows; samples were centrifuged at 2000 rpm for 15 minutes. The plasma was removed aliquoted and stored for later measurement of proteins of interest. To separate the white cells from the buffy coat, the buffy coat layer was resuspended in red blood cell lysis buffer and the mixture was centrifuged at 2000 rpm for 10 min. The pellet was resuspended in PBS (1ml) and then divided and stored in i) Tri-reagent for later RNA extraction ii) OCT for IHC studies and iii) RIPA buffer for protein analysis.

5.1.2 **Measurement of Soluble NGAL and NGAL/MMP-9 Complex by ELISA**

The NGAL concentration was determined in the plasma samples using an ELISA kit which detected only Lipocalin-2/NGAL (R&D Systems). Another ELISA was used to measure plasma NGAL/MMP-9 complex (R&D ELISA kit, DM9L20). Each assay was performed according to the manufacturers' instructions and all samples were assayed in duplicate. The optical density of each well was determined within 30 min of colour development, using a microplate reader set to 450 nm. The optical

density of 570 nm was used for correction for plate optical densities. The concentrations of NGAL or NGAL/MMP-9 complex were then quantified by reading from a calibration curve, which used either recombinant, human Lipocalin-2 or an NGAL/MMP-9 complex as the respective standard. A representative standard curve for Lipocalin-2 is shown in Figure 5-1.

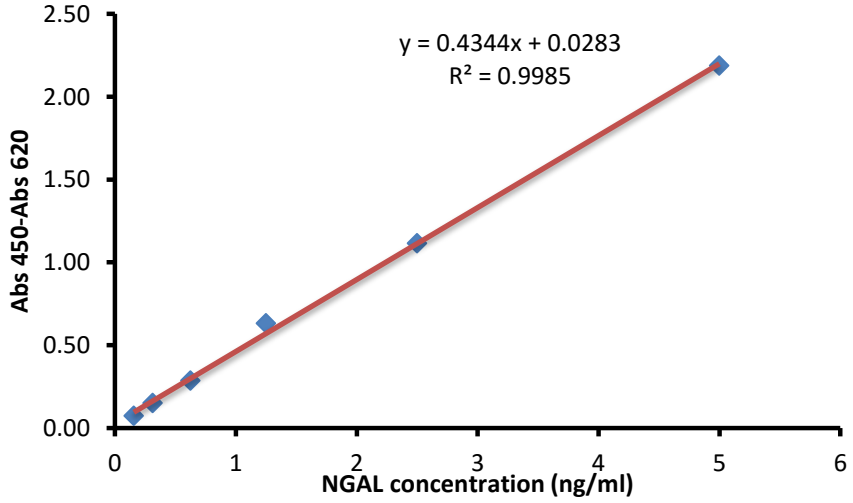


Figure 5-1: A representative standard curve for NGAL ELISA

5.1.3 **Extraction of RNA and Quantification of Gene of Interest by qRT-PCR**

5.1.3.1 **Primer design**

All primers for the human study (Table 5-3) were designed using primer-blast at Blast (Basic Local Alignment Search Tool).NIH http://www.ncbi.nih.gov. For each product there was a single peak of the melt curve at the expected temperature. Serial dilutions were performed to ensure amplification at an RNA concentration in the linear range and for all primers the no template control failed to amplify amplicons.

Table 5-3: Primer sequence of gene of interest

Target gene (human)	Forward primer (5-3)	Reverse primer (5-3)
NGAL	GGACGGAGGTGACATTGTAGCT	GGCAGGGAATGCAATTCTCA
MMP-9	CTGCCCCAGCGAGAGACTCTAC	GCTGTCAAAGTTCGAGGTGGTA
MMP-8	GACCAACACCTCCGCAAATTACA	GGGTCACAGGGTTTGGGTGTGC

5.1.3.2 **RNA extraction, production of cDNA and amplification from cells**

RNA was extracted from isolated cells and reverse transcribed to cDNA as previously described in Chapter 2.5.3 using oligo dt primers and Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturers' instructions. Quantitative PCR was performed using a Rotor-Gene 6000 and SensiMix SYBR Hi-ROX kit and primers specific for NGAL, MMP-8, and MMP-9 as described previously (Table 5-3). B2M was used as a housekeeper and all PCR amplicons were shown to amplify in parallel with B2M.

5.2 **RESULTS STUDY 1**

5.2.1 **Clinical characteristics**

As shown the control subjects were younger than the diabetic group (average 47.5 vs 62.5 respectively). The diabetic patients had a long duration of diabetes with an average of 19.5±7.5 years. As expected the HbA1c level was higher than non-diabetic controls in those with diabetes. In the group with complications the HbA1c was significantly higher in those without complications compared to those without ($P<0.05$). Compared with non-diabetic controls the serum creatinine and urinary albumin /creatinine ratio were significantly increased by diabetes and both were significantly higher in the diabetic group with complications compared to those without complications ($P<0.05$). Shown in Figure 5-2 the estimated glomerular

filtration rate (eGFR) was also significantly decreased in the diabetic group compared with controls and in the diabetic group had a negative but not significant correlation with age. Additionally, as expected eGFR negatively correlated with serum creatinine ($P < 0.0001$).

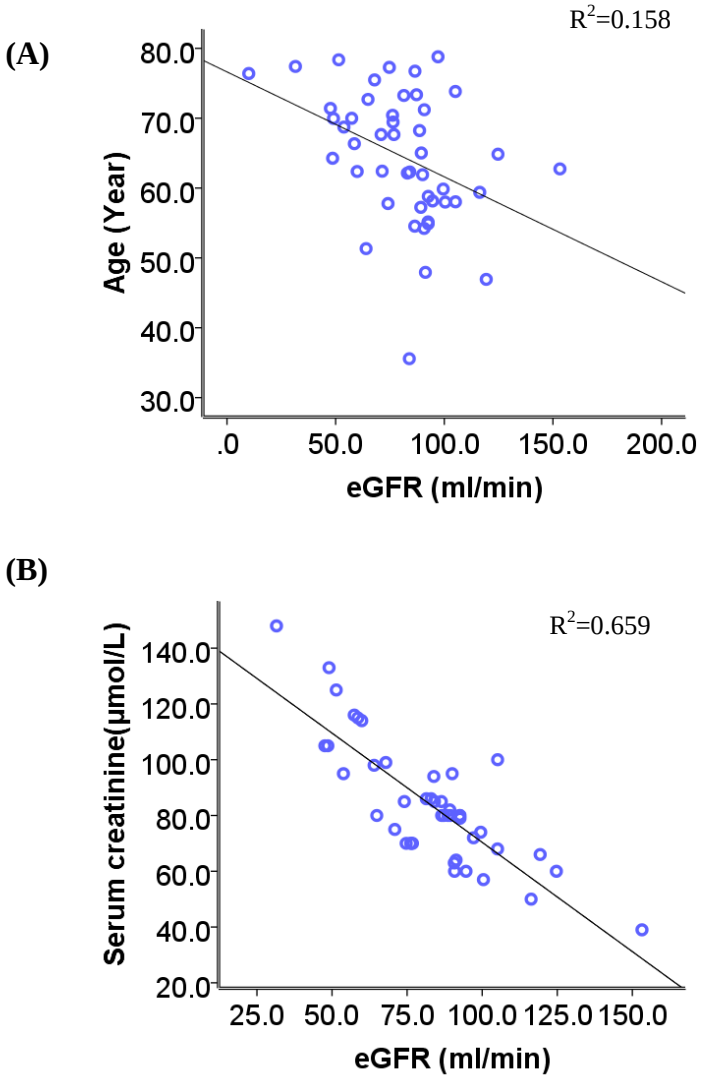


Figure 5-2: Linear correlations between (A) age and estimated glomerular filtration rate (eGFR) and (B) eGFR with serum creatinine in diabetic patients P<0.0001. n=50.

5.2.2 **Effect of diabetes on gene expression of NGAL, MMP-8, and MMP-9 in circulating inflammatory cells**

The gene expression of markers of activated neutrophils was measured in the white blood cells of normal (n=8-12) and diabetic subjects (n=38). As shown (Figure 5-3) compared to controls the gene expression of NGAL, MMP-9 and MMP-8, were significantly increased in those with diabetes (all P <0.05).

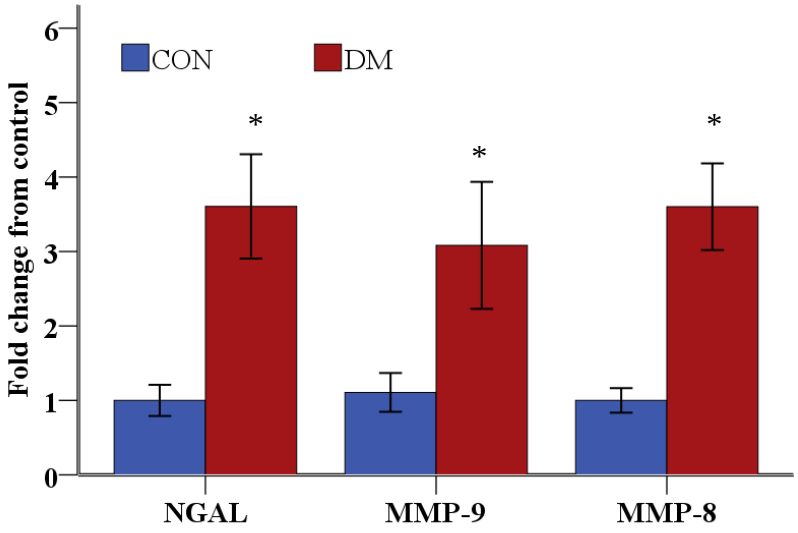


Figure 5-3: Effect of diabetes on NGAL, MMP-9, and MMP-8 gene expression in circulating inflammatory cells.
*Control (CON, n=9), diabetes (DM, n=37). Values are corrected for expression of B2M as a housekeeper and expressed as fold change from control. Group data are Mean ± SEM. *P<0.05 vs. control by Students T-test*

The pattern of increased expression of NGAL, MMP-9 and MMP-8 in diabetes was apparent irrespective of the presence or absence of complications (Figure 5-4).

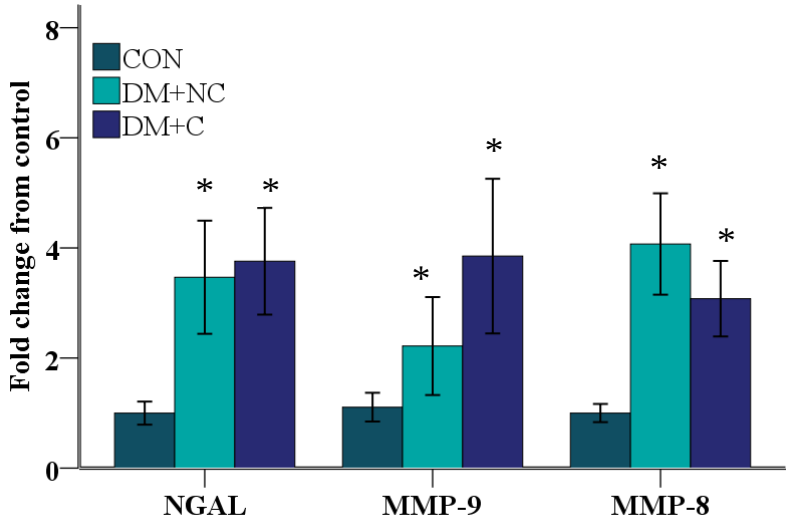


Figure 5-4: Effect of diabetic complications presence on NGAL, MMP-9, and MMP-8 gene expression in circulating inflammatory cells
*Control (CON, n=9), diabetic without complications (DM+NC, n=22) and diabetic with complications (DM+C, n=15). Values are corrected for expression of B2M as a housekeeper and expressed as fold change from control. Data are Mean ± SEM. *P<0.05 vs. control.*

5.2.3 **Effect of diabetes on MMP-9 activity and NGAL levels in plasma**

The plasma activity of MMP-9 and NGAL/MMP-9 complex was examined by zymography. The pro but not active forms of MMP-9 were detected. The NGAL/MMP-9 complex was detected in most samples but a few samples from each group did not have this complex. As shown in Figure 5-5-A the level of PMMP-9 was significantly increased in the diabetic patients compared to non-diabetic controls. The level of NGAL/MMP-9 complex did not alter across the groups (Figure 5-5-B). In the diabetic group the circulating level of MMP-9 and NGAL/MMP-9 complex were not affected by the presence or absence of complications Figure 5-6.

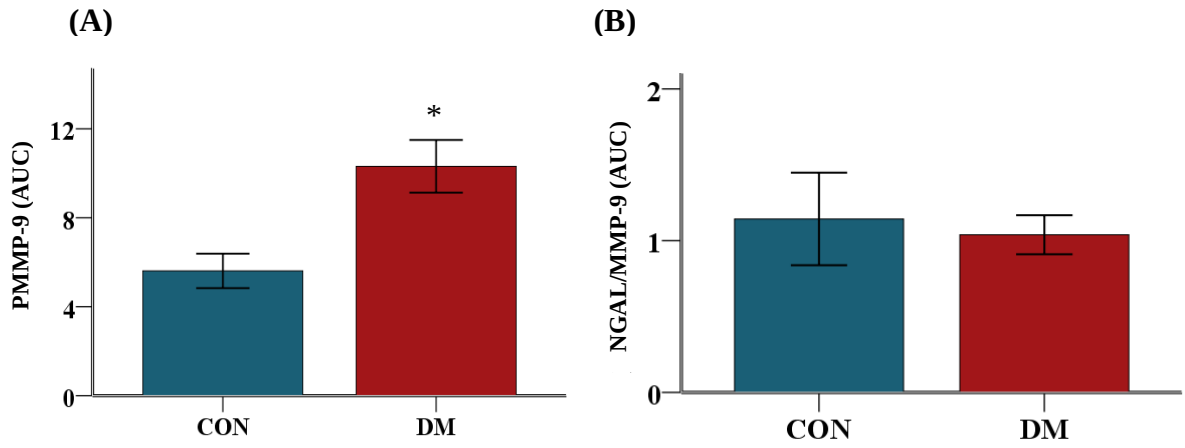


Figure 5-5: Effect of diabetes on MMP-9 and NGAL/MMP-9 complex by zymography in human plasma.
*Plasma level of (A) PMMP-9, and (B) NGAL/MMP-9 complex measured by zymography. Control (CON, n=19), diabetic (DM. n=38) patients. Data are Mean ± SEM. *P<0.05 compared to control by Student Ttest.*

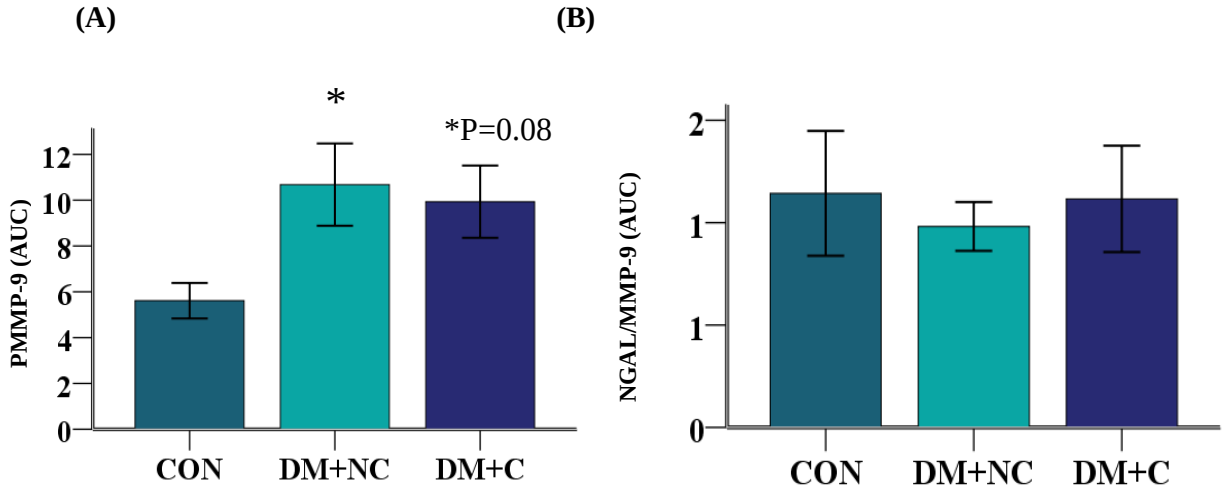


Figure 5-6: Effect of present or absence of diabetic complications on MMP-9 and NGAL/MMP-9 complex by zymography in human plasma.
*Plasma level of (A) PMMP-9, and (B) NGAL/MMP-9 complex measured by zymography. Control (CON, n=19), diabetic without complications (DM+NC, n=21) and diabetic with complication (DM+C, n=17) patients. Data are Mean ± SEM. *P<0.05 compared to control by Students T-test.*

5.2.4 **Effect of diabetes on NGAL, NGAL/MMP-9, MMP-9 and TNFα levels in plasma**

The plasma levels of NGAL and NGAL/MMP-9 complex were measured using an ELISA. Plasma NGAL was increased in diabetic patients compared with non-diabetic controls and similar to the zymography results plasma NGAL/MMP-9 complex was not different to control (Figure 5-7-A and B respectively).

The plasma level of total MMP-9 (PMMP-9, AMMP-9), and TNFα were also measured and found by ELISA to be not different to control (Figure 5-7-C and D respectively).

As shown in (Figure 5-8-A) when the diabetic group was divided into those with and without complications the increase in NGAL concentration was due to an increase in its level in the group with complications. Interestingly, in those with complications TNFα was significantly decreased compared to those without complications (Figure 5-8-D). The level of MMP-9 and the NGAL/MMP-9 complex were unaffected.

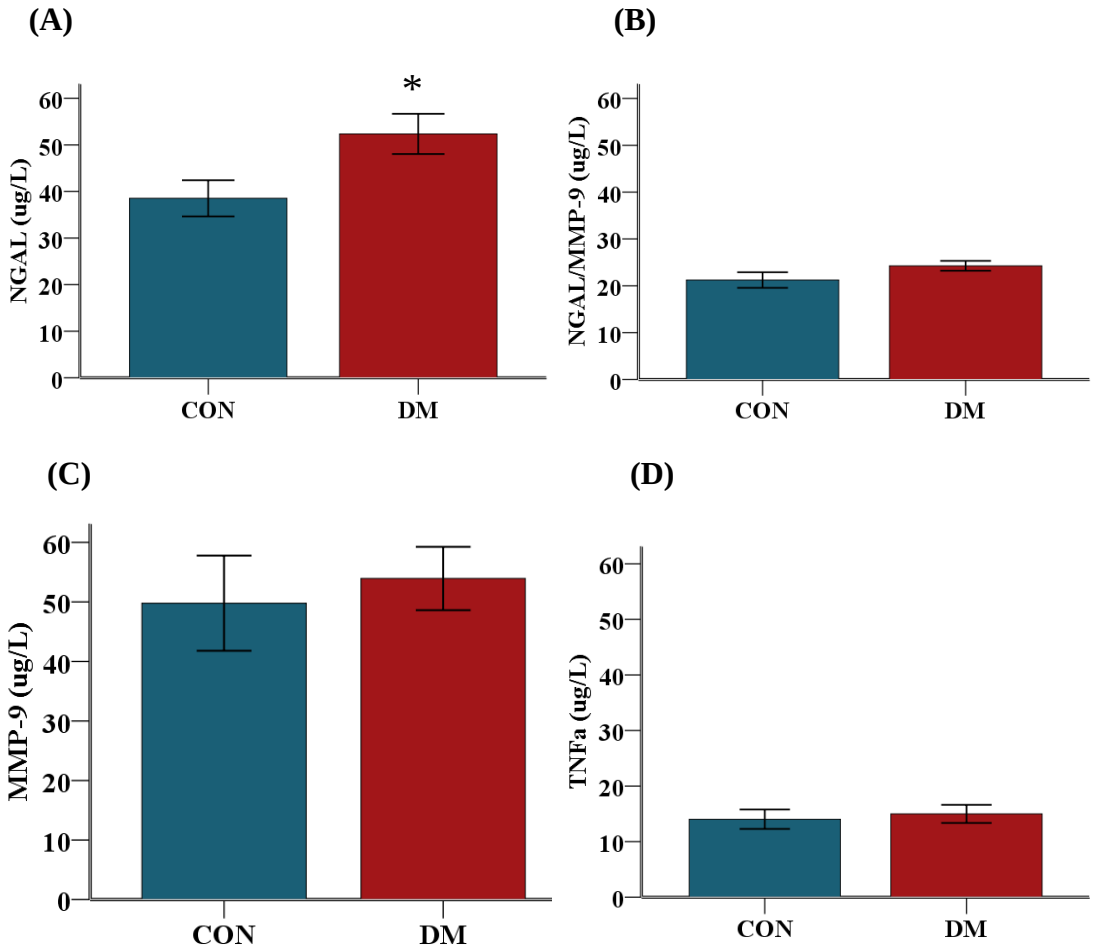


Figure 5-7: Effect of diabetes on (A) NGAL, (B) NGAL/MMP-9 complex, (C) MMP-9, and (D) TNFα concentration in plasma.
*Measured by ELISA in control (CON), diabetic (DM). Data are Mean ± SEM. *P<0.05 compared to control by Students T-test.*

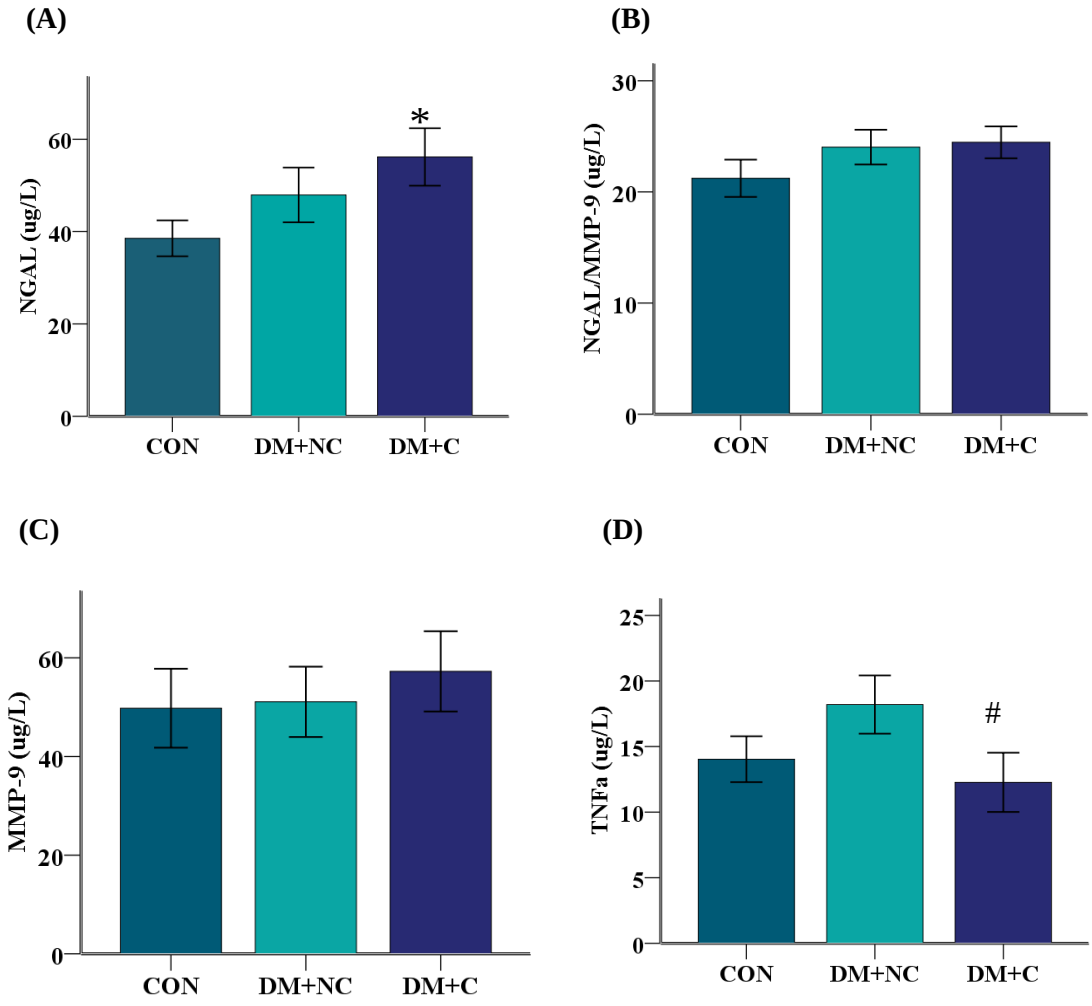


Figure 5-8: Relationship between presence or absence of diabetic complications on (A) NGAL, (B) NGAL/MMP-9 complex, (C) MMP-9, and (D) TNFα concentration in plasma. *Plasma concentration of soluble form of NGAL, NGAL/MMP-9 complex, MMP-9, and TNFα measured by ELISA in control (CON), diabetic without complications (DM+NC) and diabetic with complication (DM+C) patients. Data are Mean ± SEM. *P<0.05 compared to control and #P<0.05 compared to diabetic by Students T-test.*

5.2.5 **Associations between NGAL and MMP-8**

To investigate the relationship between neutrophil markers and neutrophil activation the correlation between NGAL and MMP-8 mRNA levels in the diabetic white blood cells was calculated. As shown in Figure 5-9 there was significant positive correlation between NGAL and MMP-8 gene expression. No correlation between these markers and expression of TNFα were observed.

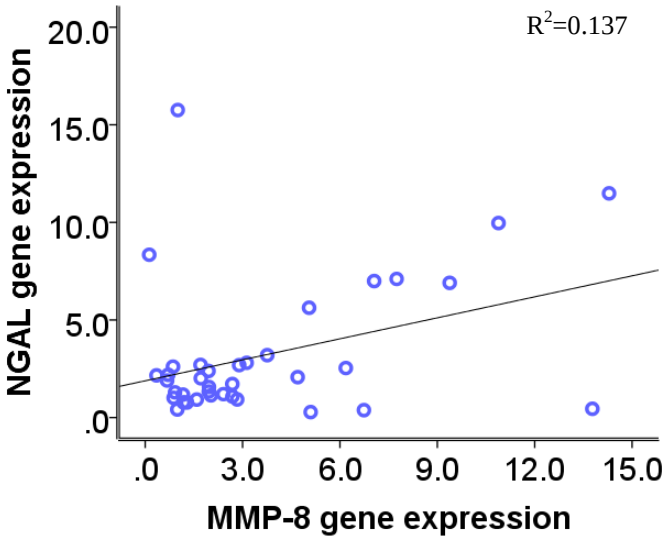


Figure 5-9: Linear correlation between NGAL and MMP-8 gene expression in diabetic patients: P < 0.02.

5.2.6 **Correlations between markers of neutrophil activation and clinical variables**

To determine whether there was any association between plasma NGAL and clinical variables, the correlation between plasma NGAL, eGFR and serum creatinine and diabetes duration was examined. As shown no significant correlation was found between NGAL and HbA1c and only weak associations were observed for NGAL and duration of diabetes (Figure 5-10-A and B respectively). There was a significant negative correlation between the level of NGAL and eGFR and positive correlation between NGAL and serum creatinine in the diabetic patients (Figure 5-10-C and D respectively). No association between MMP-8 and clinical variables were observed.

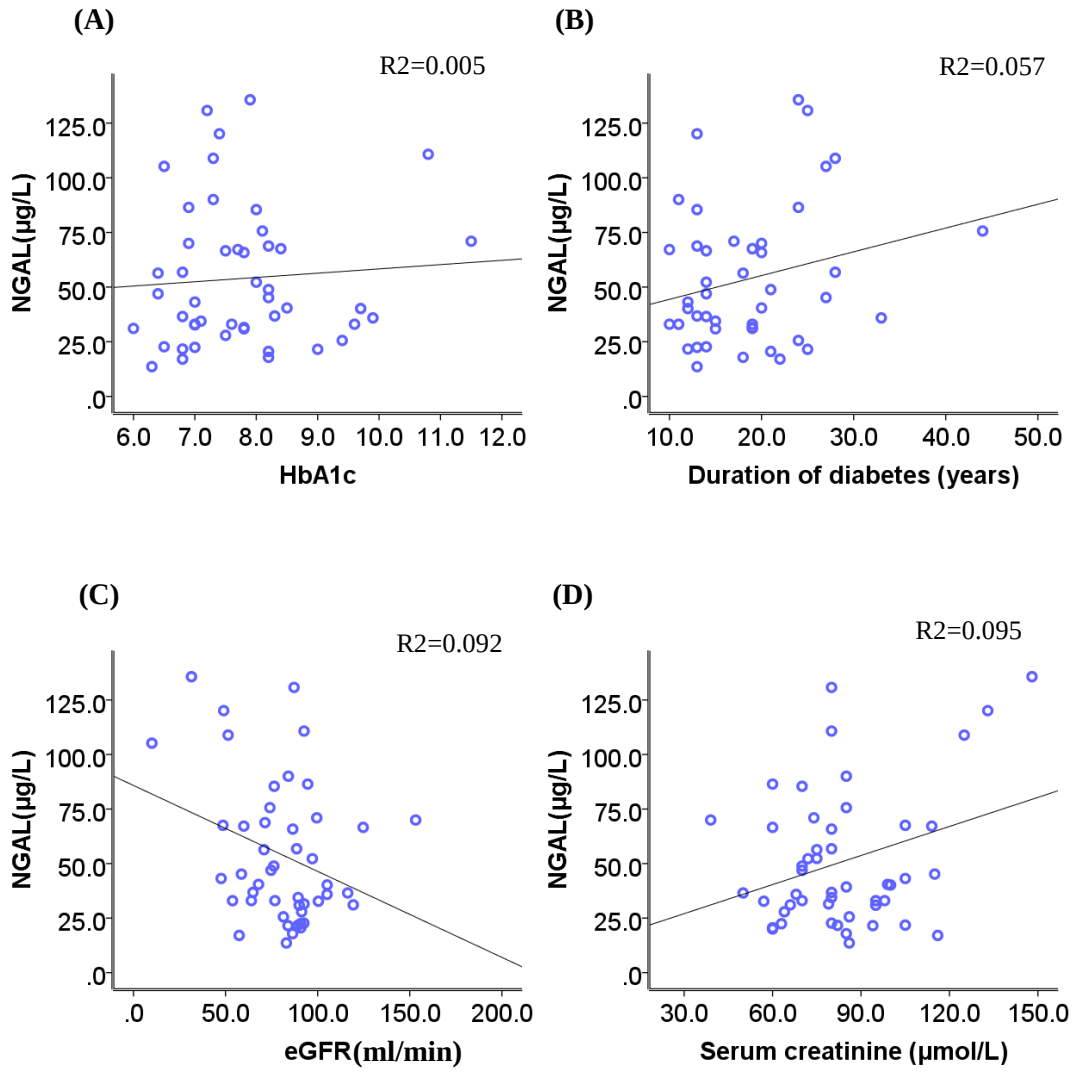


Figure 5-10: Linear correlations between plasma NGAL and (A) HbA1c, (B) duration of diabetes (C) eGFR (P<0.05) and (D) serum creatinine (P<0.05) levels in diabetic patients

5.2.7 **Correlation between PMMP-9 and NGAL/MMP-9 complex**

As described the level of pro-MMP-9 and NGAL/MMP-9 complex was measured by zymography. As shown in Figure 5-11 there was a significant positive correlation (R^2 = 0.124, P < 0.05) between plasma NGAL and NGAL/MMP-9 complex in diabetic subjects.

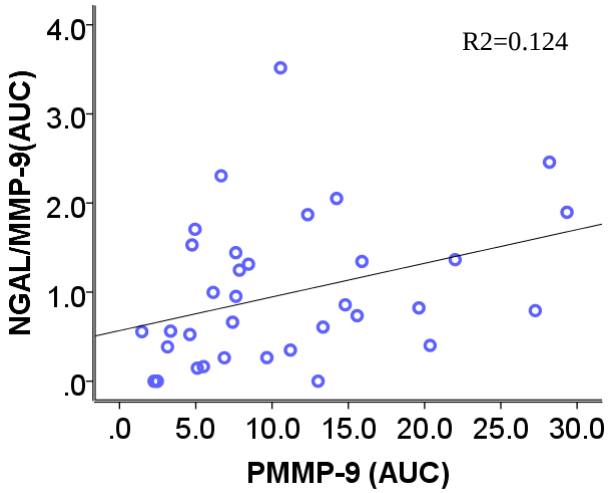


Figure 5-11: Linear correlation between plasma NGAL/MMP-9 complex levels and PMMP-9 measured by zymography in diabetic patients: P < 0.05.

No associations between MMP-9 or NGAL/MMP-9 complex and HbA1c, eGFR and serum creatinine were observed. However, the NGAL/MMP-9 complex formation correlated weakly with diabetes duration (data not shown).

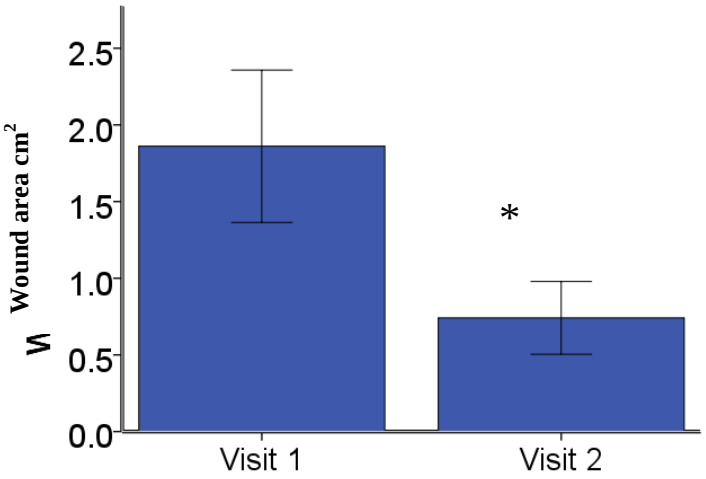
5.3 **RESULTS STUDY 2**

In the second study, we examined circulating markers of neutrophil activation in patients with foot ulcers attending the High Risk Foot Clinic of the Diabetes Centre of RPA Hospital. As for study 1 the expression of NGAL, MMP-8 and MMP-9 in circulating white cells were measured and their associations with wound healing rate were examined.

5.3.1 **Wound area and its correlation with wound grade**

Wound area was measured at the first visit (visit 1) and each visit until the wound healed. The mean wound area at visit 1 (1.8 ± 0.5 cm^2) and the area at the last visit in this study called (visit 2) was used for calculation of wound healing rate and wound closure. The wound-healing rate for the whole group was 0.02 ± 0.009 cm per day. However, there was variability across the group of the nine patients studied sequentially; only two wounds healed completely, and in two patients there was no change in wound area. The data for the whole group is shown in Figure 5-12-A and individually in Figure 5-12-B.

(A)



(B)

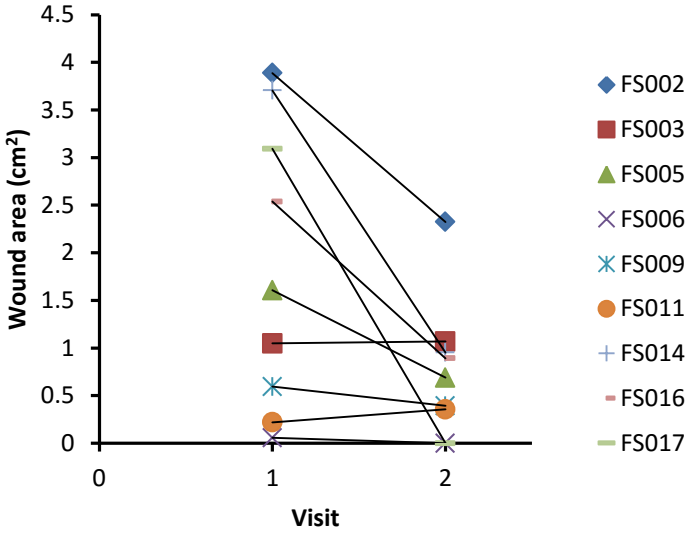


Figure 5-12: Wound area in diabetic patients with foot ulcer.
*Wound area measured in the entry time visit 1 and in the visit 2 (A) whole group (B) individual patients. Data are Mean ± SEM.*P<0.05 compared to visit 1 at entry to the study.*

Wound severity was graded using the Texas scale of 0, 1, 2, or 3 as described in Section 5.1. At the entry visit two wounds penetrated to tendon or capsule and were graded as Texas grade 2 and the remainders were superficial wounds, which did not involve tendon, capsule or bone (i.e. Texas grade 1). Some wounds at entry had Staphylococcus Aureus and Enterococcus faecalis infections but the wounds, which were healed at end of study, did not have bacterial infection. At the final visit, 2/7 stage 1 wounds had healed completely and the remaining 7/9 wounds were level 1. There was no trend for wound grade and wound area. However, as shown in Figure 5-13 at Visit 2 those with a higher wound area had a greater wound severity.

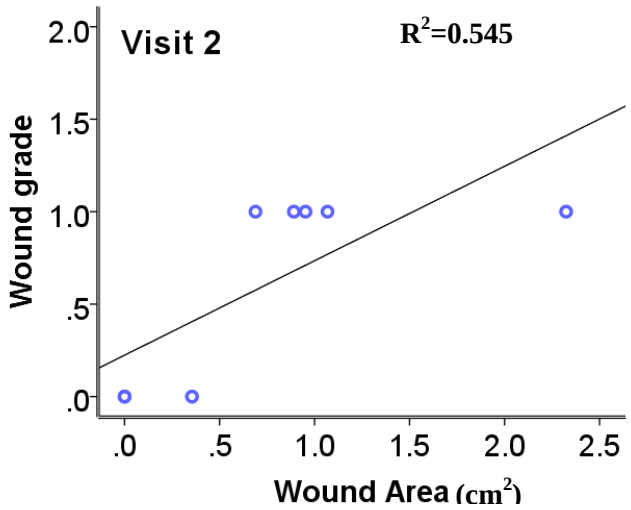


Figure 5-13: Correlation between wound grade and wound area in visit 2 in diabetic foot ulcers.

5.3.2 **Change in expression of neutrophil markers and MMP-9 with healing**

The gene expression of NGAL and MMP-8 as specific markers of activated neutrophils as well as MMP-9 was measured in the inflammatory cells isolated from blood at visit 1 and visit 2. A sample of normal patients (WBC cDNA) was included for use as the internal control. As shown in the group data the overall expression of these markers did not change between the visits (Figure 5-14). Our data showed that at each visit NGAL expression was considerably higher than MMP-9 expression (7.5 fold and 9.5 fold, visit 1 and visit 2 respectively). Likewise, MMP-8 expression at visit 1 was 4 fold and at visit 2 was 8 fold higher than MMP-9.

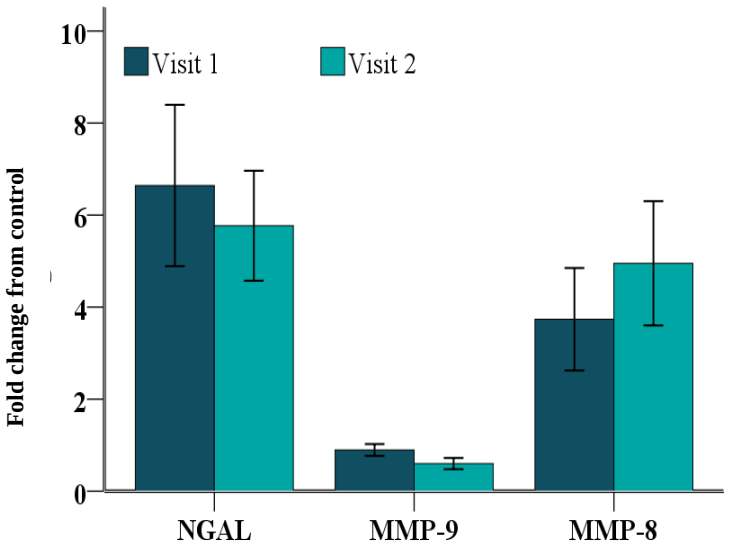


Figure 5-14: Effect of diabetes on NGAL, MMP-8, and MMP-9 gene expression in circulating inflammatory cells.
Diabetic foot ulcer patients in visit 1 and visit 2. Group data for NGAL, MMP-9, and MMP-8. The data was corrected for expression of B2M as the housekeeper and are expressed as fold change from internal control. Data are Mean ± SEM.

The data for the individual patients also showed that the NGAL changes were variable whilst MMP-8 expression increased in most of the patients between the first and final visits (7/9 patients). Interestingly, the gene expression of MMP-9 was decreased and this reduction was associated with increased wound closure (8/9 patients) (Figure 5-15-A-C). These changes were apparent regardless of the presence or absence of infection.

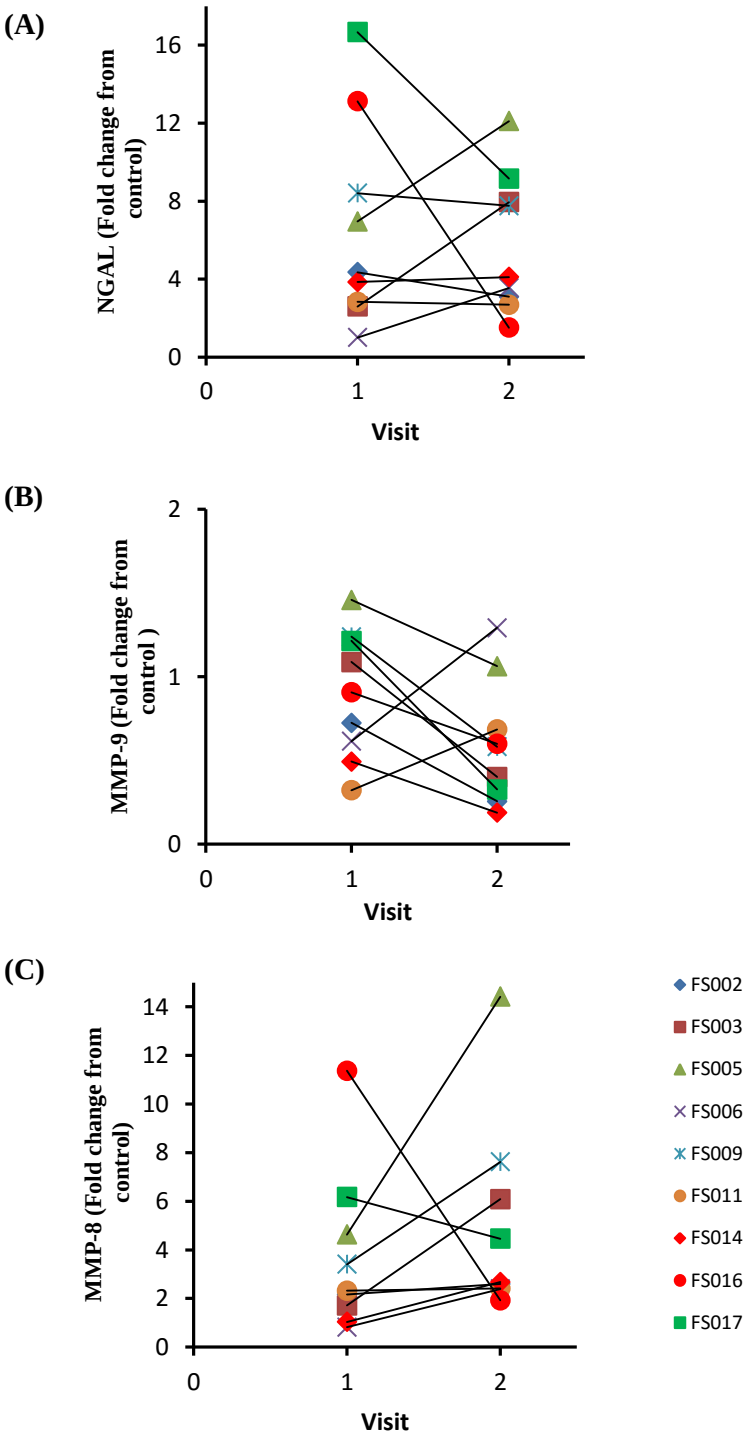


Figure 5-15: Effect of diabetes on NGAL, MMP-8, and MMP-9 gene expression in circulating inflammatory cells.

Diabetic foot ulcer patients in visit 1 and visit 2. Individual patient data for (A) NGAL, (B) MMP-9, and (C) MMP-8. The data was corrected for expression of B2M as the housekeeper and are expressed as fold change from internal control. Data are Mean ± SEM.

5.3.3 **Wound healing rate and its correlation with gene expression**

To examine whether baseline gene expression of NGAL or MMP-8 as specific markers of activated neutrophils and MMP-9 are associated with future wound healing rate, the baseline gene expression of each of these markers was correlated with the wound healing rate calculated from visit 1 to visit 2. Although the numbers are small the initial visit white cell NGAL and MMP-8 mRNA correlated positively with wound healing rate. The strongest correlation was observed for NGAL ($P<0.02$) with a lesser correlation for MMP-8. White cell MMP-9 did not correlate with wound healing rate (Figure 5-16).

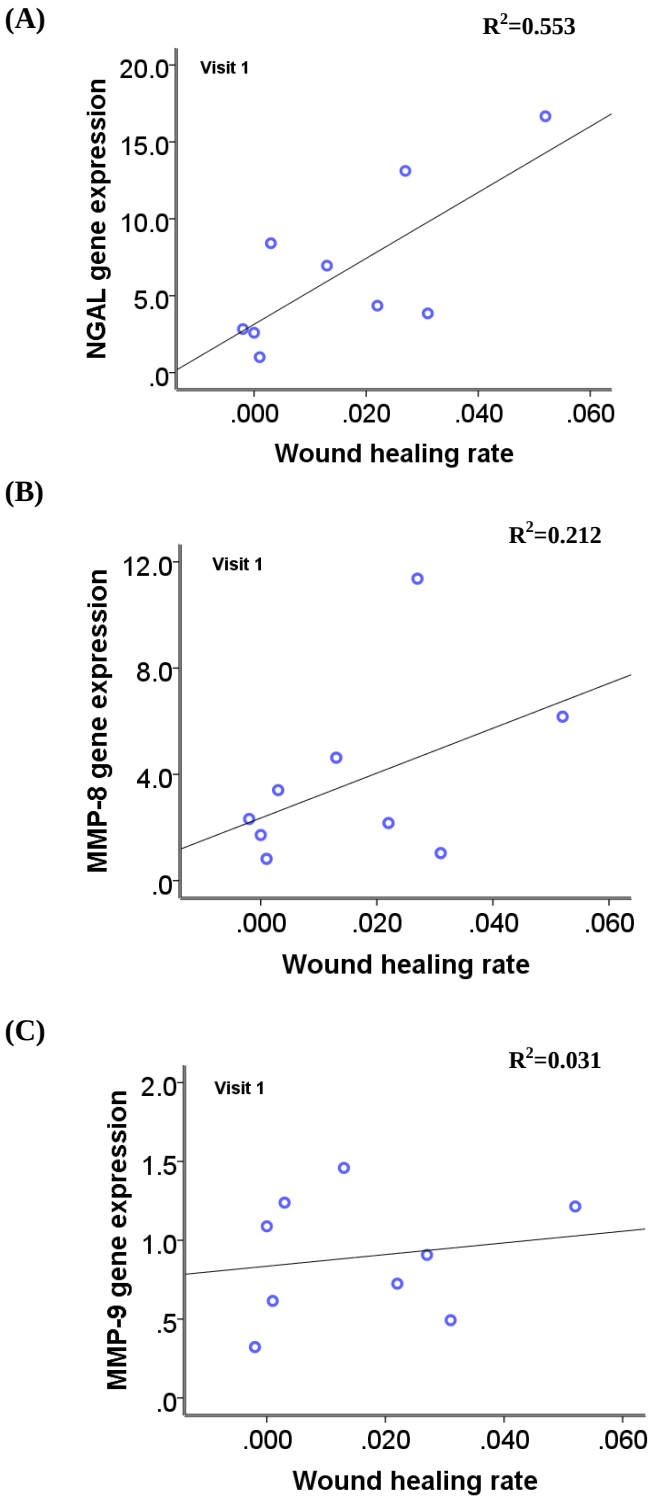


Figure 5-16: Correlation between wound healing rate and circulating inflammatory cells gene expression of (A) NGAL, (B) MMP-8, and (C) MMP-9 at entry time (visit 1) in diabetic foot ulcers.

Significant correlation between NGAL gene expression and wound healing rate, P<0.02.

5.4 **DISCUSSION**

In the presented pilot studies the white cell mRNA levels of markers of neutrophil activation NGAL and MMP-8 as well as MMP-9 were studied in two different diabetic populations. The first study those with long-term diabetes and the second study a group of diabetic patients with foot ulcers. These studies were performed to provide information regarding the effect of diabetes alone and diabetes and wound healing on neutrophil activation.

The results of study 1, the long-term diabetes study, albeit in a small population showed a significant increase in gene expression of NGAL and MMP-8 as specific markers of activated neutrophils in diabetic patients. Increased expression of NGAL was positively associated with increased MMP-8 gene expression suggesting that increased activation of circulating neutrophils in diabetes. Moreover, the correlation analysis highlighted the relationship between NGAL and some clinical parameters. We have also shown that the circulating NGAL concentration is increased in diabetes and is not affected by complications status. Other studies have also shown that plasma NGAL concentration correlates with parameters of kidney disease. For example, increased NGAL is associated with GFR in children with chronic kidney disease (Mitsnefes et al., 2007) and in patients with polycystic kidney disease (Bolignano et al., 2007; Bolignano et al., 2009). In this study NGAL was negatively correlated with eGFR and positively correlated with serum creatinine confirming the results of other work which have shown an association between plasma NGAL and decline in renal function (Bolignano et al., 2009).

Many studies suggest that inflammatory processes and immune cells are involved in the development and progression of diabetic nephropathy (Chow et al., 2004). In particular neutrophils have been shown to play a role in the development and progression of diabetic nephropathy (Galkina & Ley, 2006). In persons with diabetic nephropathy neutrophil dysfunction has been described; they have for example been shown to have rapid exocytosis of neutrophil primary granules as well as to contribute to increased vascular damage in these patients (Fardon et al., 2002). We found some changes in circulating factors in diabetes, but whether these changes are due to neutrophil dysfunction is not possible to ascertain. We have also shown in diabetes an increase in circulating pro-MMP-9 activity that was positively related to NGAL/MMP-9 complex. NGAL by formation of a complex with MMP-9 can prevent MMP-9 degradation. In addition, it can activate the MMP-9 precursor directly, and counteract the inhibiting effect of TIMP-1 (Coles et al., 1999). Therefore, it is likely that both increased plasma NGAL and the increase in MMP-9/NGAL complex can preserve the enzymatic activity of MMP-9.

In study 2 we performed a pilot study in a small group of diabetic subjects attending the Diabetes Center of RPA Hospital for treatment of a foot ulcer. It is well known that diabetic patients are at high risk of infection, and the infection if it occurs is often more serious and more prolonged than a similar infection in non-diabetic persons. It has been suggested that the impaired function of neutrophils accounts for the increased susceptibility to infection observed in diabetic patients (Alba-Loureiro et al., 2007).

In this pilot study we focused on the expression of circulating NGAL, MMP-8 as specific markers of activated neutrophils and MMP-9 as one of the key regulators of wound healing. The white cell expression of NGAL at the first visit was not different to the level in white blood cell mRNA at visit 2. The white cell mRNA NGAL level at the first visit correlated positively with wound healing rate. The same pattern was observed for MMP-8 expression. Together, these data suggest that neutrophil activation is measureable in circulating neutrophils and increased activation is associated with wound healing rate. Studies by others have reported that innate immune responses contribute to chronic inflammation in non-healing venous ulcers and wound fluid NGAL is increased as a reflection of this contribution (Pukstad et al., 2010).

Together, these two small pilot studies showed increased circulating neutrophil activity which was confirmed with high expression of specific activated neutrophil markers in the circulation. Additionally, in study 2 the relationship between NGAL and wound closure indicate an association between decreased NGAL expression and increased wound healing regardless of the treatment. Overall and despite the small sample size in each of these pilot studies in diabetic patients the data were found to be consistent with our rodent studies.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION

Multiple physiological factors contribute to wound healing deficiencies in diabetes. These include decreased and impaired cytokine production and activation, altered growth factor production and degradation (Galkowska et al., 2006), delayed and impaired neovascularisation (Galiano et al., 2004), impaired keratinocyte and fibroblast migration and proliferation (Lerman et al., 2003) , insufficient function of epidermal nerves (Gibran et al., 2002), as well as macrophage (Khanna et al., 2010) and neutrophil dysfunction (Dovi et al., 2003). Disruption of any of these factors ultimately leads to delayed healing which in some patients can result in amputation (Reiber et al., 1999). Despite current treatments and excellent care, patients with diabetes are at increased risk for developing foot ulcerations. The reason why this occurs is uncertain but the consequences of persistent and poorly controlled hyperglycemia contribute to neuropathic and vascular abnormalities that can cause foot deformities and ulceration (Singh et al., 2005) and result in significant morbidity and mortality (Wu et al., 2007). The pathogenesis of foot ulceration in diabetes is complex and poorly understood. As discussed in Chapter 1 there are several factors which are known to contribute including peripheral neuropathy, including ischemia, accidental trauma, limited joint mobility, and consequences of cardiovascular and cerebrovascular disease (Jeffcoate & Harding, 2003).

The molecular environment of chronic wounds including some diabetic foot ulcers contains abnormally high levels of pro-inflammatory cytokines such as TNFα, interleukins and MMPs (Grellner et al., 2000). As discussed in detail in the Chapter 2, the MMPs are a family of zinc-dependent enzymes with the ability to degrade all components of the extracellular matrix. They are produced by most cells involved in wound healing including keratinocytes, endothelial cells, fibroblasts, and

inflammatory cells such as neutrophils, macrophages, mast cells, and eosinophils (Nagase et al., 2006). The MMPs are essential for normal wound healing but in a chronic wound, abnormally high levels of MMPs contribute to the persistence of inflammation and maybe associated with the increase in cytokines and reactive oxygen species (Martins et al., 2013). Although it is well known that inflammation is increased in a non-healing wound, few studies have focused on the mechanism of the increased MMP-9. In particular the association between MMP-9 and altered inflammatory cell function. More specifically neutrophil activity and wound healing in diabetes has not been studied. Diabetes is known to impair neutrophil phagocytic function but whether neutrophil activation in response to wounding in diabetes is altered is not known. In this study we examined the effect of diabetes and wounding on the expression of NGAL, and MMP-8 as specific markers of activated neutrophils in two different wound models (an excisional model and a sponge implant model). The effect of diabetes and wounding on the expression and activities of MMP-9 and its complex with NGAL was also examined. The results were compared with those obtained in unwounded animals and in non-diabetic animals as control. Using the sponge implant model we were able to examine the activity of neutrophils in the circulation and in response to a stimuli in the same animals. The effect of interventions such as insulin treatment to improve diabetic control or doxycycline a regulator of MMPs, on NGAL and MMP-9 levels in diabetic wounds was also examined. *In vitro* studies examined the effect of high glucose concentration on neutrophil activity in neutrophils isolated from rat blood. Whether the changes seen in circulating markers in wounded rats are also observed in diabetic patients and alter with wound healing was also examined in pilot studies.

The new findings of these studies are summarised below and discussed in more detail in the following sections (Table 6-1).

Table 6-1: New findings of this research

Finding	Evidence
In the wound inflammatory cells diabetes increased neutrophil number, and activity	Increased neutrophil number by morphology and MPO staining Increased NGAL and MMP8 as specific markers of activated neutrophils
In unwounded animals, diabetes had no effect on circulating neutrophil number or activation	No change in circulating neutrophil number which was measured by flow cytometry No significant change in NGAL, or MMP-8 expression
In contrast, diabetes and wounding significantly increased circulating neutrophil number and activity	Increased circulating neutrophil number which was measured by flow cytometry Increased NGAL, MMP-8, and MMP-9 expression
Diabetes significantly increased MMP-9 activity	Increased expression of MMP-9 in circulating neutrophils in unwounded and wounded animals as well as increased MMP-9 activity in wound fluids
Circulating neutrophil apoptosis was increased by diabetes	Increased expression of AnnexinV as a marker of apoptosis
Diabetes and wounding increased circulating neutrophil longevity	Increased expression of MCl-1as a marker of anti-apoptosis

We have shown in our previous study (McLennan et al., 2008) and again in this work that diabetic rats have slower wound healing rate than control animals. We have also shown that neutrophil number is increased and this increase persists and is associated with increased neutrophil activity. This pattern of increased neutrophil numbers observed in the this study is consistent with our previous study (McLennan et al., 2008). Further detailed investigations in this study have shown an increase in activation of neutrophils by increased expression of NGAL and MMP-8, in skin excisional wounds as well as the infiltrated cells in the implant model. Immunohistochemical examination showed that diabetes increased staining for NGAL and MMP-9 in the inflammatory cells in skin excisional wounds. In the circulation, this increase in the staining was observed in diabetic neutrophils. In the implant model, an increased in NGAL expression was observed in neutrophils and macrophages with predominantly located to neutrophils. These results also indicate role of these markers in diabetic poor wound healing.

In the results described in Chapter 3, pro and active forms of MMP-9 in wound fluids and in plasma were measured by zymography. There was a transient increase in the sponge wound fluid MMP-9 from diabetic animals which was due to increased formation of the biologically active form of MMP-9 (82 kDa) as well as increased formation of the NGAL/MMP-9 complex. This increase was not associated with an increase in MMP-9 gene expression and suggests that the observed effect on MMP-9 activity occurs via post-translational mechanisms. MMP-9 activity can be regulated at multiple sides; for example pro-inflammatory cytokines can induce MMP-9 mRNA but this is unlikely as we showed no change in TNFα and no change in MMP-9 gene expression. MMP-9 activity can also be

regulated by TIMPs or by formation of a complex with NGAL. Others have described decreased TIMPs in diabetic wounds and in unpublished work we also show decreased TIMP expression suggesting that this may also play a role. In this work we focussed on NGAL and showed increased NGAL/MMP-9 complex formation. The increased formation of the NGAL/MMP-9 heterodimer can stabilise MMP-9 to prevent it from degradation (Tschesche et al., 2001; L. Yan et al., 2001), and may act as a mechanism to preserve higher gelatinolytic MMP-9 activity in diabetic wounds.

Our results show that neutrophils are the main source of NGAL in the circulating inflammatory cells and we have shown that NGAL and MMP-9 are localised in these cells. In rodents, presence of diabetes appears to have no effect on circulating neutrophil number, or activation. In contrast diabetes significantly increased circulating neutrophil apoptosis which was confirmed by high expression of Annexin-V. In diabetic animals wounding altered circulating neutrophil activation but in this case circulating neutrophil apoptosis was delayed. Compared to controls in wounded diabetic animals circulating neutrophil expression of MMP-9 and MMP-8 was transiently increased returning toward control at day 12. The expression of other markers of neutrophil activation (NGAL), and function i.e TLR4, TLR2, and TNFα were also increased at day 6 but had returned to normal at day 12. This abnormal response would increase in circulating cells in inflammation at the wound site. We have also measured expression of the anti-apoptotic marker MCL-1 and shown that this response appears to increase neutrophil life span, an effect which can and may contribute to the high pro-inflammatory environment. More specifically at day 6 post wounding, increased neutrophil number and decreased neutrophil

apoptosis in circulating neutrophil suggesting increased neutrophil longevity in diabetic compared to control rats. These findings are consistent with other studies which have shown neutrophil apoptosis was reduced in response to infection and inflammation was prolonged (Hanses et al., 2011). Whilst not specifically studied in this work it is likely that prolongation of neutrophil apoptosis leads to necrotic death and the release of granule proteins and pro-inflammatory cytokines to result in tissue damage.

A summary of effect of diabetes on gene expression of NGAL, MMP-8, and MMP-9 in three different tissues, i.e skin wounds, sponge implant inflammatory cells, and circulating neutrophils is shown in table below (Table 6-2).

Table 6-2: Effect of diabetes on specific markers of neutrophils NGAL, MMP-8, as well as MMP-9

	Skin wounds	Implant inflammatory cell			Circulating neutrophils		
Activated neutrophil markers	D6	D3	D6	D12	D3	D6	D12
NGAL	↑	↔	↑	↑	↔	↑	↔
MMP-8	↑	↔	↑	↔	↑	↑	↔
MMP-9	↔	↔	↔	↔	↑	↑	↑

Black arrow significant increase, red arrow tendency toward an increase but not significant, blue arrow no significant change at day 3, 6, and 12. All data was compared to control.

We and others have shown that drugs or other treatments such as Propolis (McLennan et al., 2008) growth factors (Wang et al., 2008) or bioengineered skin–

tissue substitutes (Pham et al., 2007) can improve wound healing rate. As shown in chapter 3 controlling blood glucose with insulin treatment was able to improve wound healing and prevent the changes caused by diabetes. Better blood glucose control also effectively reduced the expression of NGAL, MMP-8 as well as MMP-9 in the circulating neutrophils of wounded animals. The increased activity of MMP-9 which was observed in the wound fluid of diabetic animals was also significantly prevented by insulin. That insulin treatment from onset of diabetes can prevent these changes, suggests that good lifetime glycemic control is likely an important protective factor. Additionally, this data also supports the concept that the effect of diabetes on neutrophil function plays a role in the delay in healing. Zagon et al. (2006) presented that intensive insulin therapy has been shown to prevent delayed wound healing on the ocular surface by maintaining blood glucose levels at near normal levels in diabetic rats (Zagon et al., 2006). A study by Guo et al. (2013) showed that insulin can decrease MMP-2 and -9 activities after arterial injury with no change in expression of their tissue inhibitors (Guo et al., 2013). In this study we have shown that insulin therapy can prevent the change in neutrophil function which is induced by wounding in diabetic animals. Further, whether this treatment is acting directly on the neutrophil in the tissue or effects on neutrophil developing in the bone marrow or activation in the circulation was not examined in this study. In this study the intense insulin treatment was commenced from the onset of diabetes, whether improved diabetic control from the onset of wounding also has the same effect is not known.

Beside insulin therapy, whether a strategy to reduce the negative effects of enhanced MMP activity in impaired healing could improve healing was also investigated. For

this reason chemical inhibitors or regulators of MMPs are an attractive approach and may prove to be effective in initiating the progression of wound healing. Doxycycline, which belongs to tetracycline antibiotic class is a nonspecific inhibitor of MMPs (Wilcox et al., 2012). In this study we investigated the effect of two doses of doxycycline a low (20mg/kg) and a high (100mg/kg) dose on MMP-9 expression and activity in inflammatory cells in two different models of diabetic wounded animals. Whether this treatment also affected neutrophil activity was investigated.

Our results, explained in detail in Chapter 4 showed that the low dose of doxycycline is more effective than the high dose with improved MMP-9 and neutrophil activity. Low dose of doxycycline also improved wound closure in diabetic animals and decreased the gene expression of NGAL and MMP-8 in the sponge implant inflammatory cells. It significantly decreased expression of neutrophil activity markers NGAL, MMP-8, and MMP-9 in the circulating neutrophils. In contrast, the high dose of doxycycline did not improve the wound closure and increased neutrophil activity and numbers in the diabetic treated group. Similar treatment of control animals also had the same effect as evidenced by increased gene expression of NGAL, MMP-9 and MMP-8 in skin excisional wounds as well as MMP-8 gene expression in the sponge inflammatory cells. MMP-9 activity as well as NGAL/MMP-9 complex formation was partially improved by high dose of doxycycline.

In the circulating neutrophils the high dose of doxycycline also negatively impacted on neutrophil function as evidenced by increased neutrophil expression of NGAL, MMP-9, and MMP-8 in unwounded animals. The results suggest the high dose of

doxycycline used maybe exceed the standard inhibitory concentration required for its anti-inflammatory effect since daily administration of a 20 mg/kg dose of doxycycline, is the only drug therapy approved by the FDA as an MMP inhibitor in chronic periodontitis patients (Lee et al., 2004).

Doxycycline has been shown to inhibit the activity of MMPs by a number of mechanisms including by inhibition of activated MMPs, inhibition of pro-MMP-9 activation and decrease of MMP expression (Gerald et al., 2001). Inhibition of MMP activity by doxycycline and other chemically modified tetracycline occurs through the chelation of calcium and zinc ions. The chelation of calcium and zinc ions enable inhibition of already active MMPs, the inhibition of pro-MMP activation, or /and the down regulation of MMP expression (Griffin et al., 2010).

In this study we have also shown that low but not high dose of doxycycline can alter neutrophil activity, suggesting that pathologically elevated activities of neutrophils can be inhibited by low dosage of doxycycline. Other workers have shown that some members of the doxycycline family beside of their ability to decrease the level of NGAL, MMP-9 (Serra, Buffone, et al., 2013) and TNFα (Cazalis et al., 2008) can supress neutrophil function by suppressing superoxide generation, degranulation and migration of neutrophils. Doxycycline treatment has a profound but selective effect on vascular inflammation and reduces aortic wall neutrophil (Abdul-Hussien et al., 2009). TNFα is an important inflammatory mediator that regulates the activity of neutrophil therefore doxycycline by preventing expression of TNFα improves neutrophil activity and decrease expression of MMP-9 by neutrophils. Several animal studies reported that treatment with doxycycline or other tetracycline

analogues improved healing parameters. For example, systemic treatment of rats with doxycycline increased tensile strength of rat intestinal anastomoses 3 days after surgery (Pasternak et al., 2008). Chemically modified tetracyclines beside of their regulation activities of release of inflammatory cytokines (Cazalis et al., 2009), inhibit neutrophil elastase and MMPs, namely gelatinases (Tilakaratne & Soory, 2014).

The results of both intervention studies showed prevention of increase in circulating neutrophil activation by either insulin treatment or low dose doxycycline in association with improved wound closure. Whether it is necessary to prevent the increase in circulating neutrophil activation to improve healing was not specifically studied. However, studies using topical insulin treatment (Lima et al., 2012) and topical doxycycline (Chin et al., 2003) also showed improved healing. Whether the mechanism of this effect is via improvement of neutrophil function is not known and could be studied in future work.

Using an *in vitro* cell culture system we have also shown that neutrophil activity is increased by high glucose concentration. This was confirmed by significantly increased expression of NGAL, MMP-8, and MMP-9 after 2 hours incubation in high glucose concentration. The increased neutrophil activity in high glucose concentration was also associated with increased neutrophil apoptosis after 6 hours incubation. These findings are in line with the results from unwounded animals that showed diabetes increased neutrophil apoptosis. As neutrophil apoptosis leads to cell death future cell culture studies examining in detail the contribution of neutrophil apoptosis to cytokines expression would be valuable. Comparisons of the

effect of diabetes and the interventions would also enable their role to be better understood. Further culture of neutrophils from control and diabetic wounds in the presence or absence and activation of stimulators such as LPS or fMLP would determine whether neutrophil viability in diabetes is altered. The effect of these stimulators on MMP-9 activity, the expression of MMP-9 and its complex with NGAL can also be measured in the culture media. Specific therapies targeted at blocking NGAL may reduce the activity of MMP-9 and provide a novel treatment to improve healing of wounds.

Another aspect of this work which requires further investigation is the role of prolonged neutrophil life span. We have shown that wounding increased circulating neutrophil life span by delaying apoptosis. Whilst we were unable to specially examine this in neutrophils in the wound implant model, we showed that circulating neutrophil pro apoptotic marker MCL-1 was increased. Whether, these changes in circulating neutrophils reflect a similar pattern in wounds is not clear. Bcl-2 an apoptotic marker was not altered in diabetic wounds but other pathway members were not studied because we were unable to isolate enough neutrophils for examination. Further studies by immunohistochemistry may assist in answering this question. Manipulating of neutrophil apoptosis *ex-vivo* is difficult as their life span is short. Treatment with flavones has been shown to induce neutrophil apoptosis *in vitro* and *in vivo* in a zebrafish model (Lucas et al., 2013). We have previously used Propolis, a high flavone containing compound, to improve wound healing (McLennan et al., 2008). Whether it exerts some of its effects by increasing neutrophil apoptosis is interesting and requires investigation.

In these series of cell culture studies treatment of cultured neutrophils with insulin did not prevent increased expression of NGAL, MMP-8, or MMP-9. How the addition of insulin can cause this change is not known. Topical insulin has shown to be beneficial in stimulating re-epithelialisation, angiogenesis and in the recovery of both acute and chronic wounds *in vitro* and *in vivo* (Liu et al., 2009*)*. Other studies have demonstrated that topical insulin can reducing wound healing times, and increase both protein and DNA synthesis by wound tissue (Zhang et al., 2011). The application of topical insulin has also been reported to increase wound closure in full thickness wounds via suppression of neutrophil infiltration and decreased level of macrophage inflammatory protein-2 expression in mice (Chen et al., 2012).

As described in Chapter 3 and 4 diabetes impaired neutrophil function and activation in the rodents and in particular in response to wounding. Whether these changes are also observed in people with diabetes is not known. To attempt to address this we examined neutrophil activity in diabetic subjects with long duration of diabetes and in a second group in association with wound healing. The data were found to be consistent with our rodent studies and showed high gene expression of NGAL and MMP-8 in the both groups suggesting increased circulating neutrophil activity. In the diabetic subjects with long duration of diabetes expression of NGAL had positive relation with increased MMP-8 gene expression. Additionally, in this study plasma soluble NGAL was significantly increased in diabetic patients and showed a relationship with other indexes. In particular plasma NGAL concentration was negatively correlated with eGFR and positively correlated with serum creatinine suggests an association between plasma NGAL and decline in renal function. These observations are in accordance with those of a previous study that suggested

association of plasma NGAL concentration with kidney injury (Bolignano et al., 2008; Mitsnefes et al., 2007).

In the second study we examined the association between neutrophil activities and wound healing. In this study, albeit in small numbers of patients a high level of NGAL was positively associated with increased wound area. A similar pattern of increase was observed for MMP-8 expression. Together, these results suggest maintenance of neutrophil activation as the wound heals. Whether neutrophil activation alters with healing is as yet not clear and requires further study in larger numbers of patients.

The studies described in this thesis investigated the effect of diabetes on neutrophil activity and the expression and activities of MMP-9 and its regulator, NGAL. In these studies we showed that the increased activities of MMP-9 in diabetic wounds are associated with induction of inflammatory cell NGAL and NGAL/MMP-9 concentrations. However, whether these changes are markers or mediators of impaired healing are not yet clear. To understand the activation mechanism completely more research is required. Increased NGAL has been demonstrated to form a complex with MMP-9 and may play a role in the maintenance of MMP-9 activity. To show this conclusively studies in models where NGAL is decreased such as in knocking down of NGAL knock-out mice or by topical application of an NGAL antibody are required. In addition, we have shown regulation of NGAL and MMP-9 by insulin and doxycycline. Therefore, studies using combinations of with good diabetic control with topical agents that reduce inflammation and protease

activities such as doxycycline might also be an effective adjunctive therapy for wound healing.

In conclusion, in these uninfected wound models, we propose as shown schematically in Figure 6-1 that increased wound MMP-9 activities in diabetic animals is associated with increased circulating and wound neutrophil activity, as well as increased neutrophil NGAL expression and the formation and persistence of NGAL/MMP-9 complex. These changes may be due to the presence in the circulation of higher numbers of neutrophils with increased expression of NGAL and MMP-9. Therefore, therapies targeting NGAL and MMP-9 may have utility in diabetic wound healing.

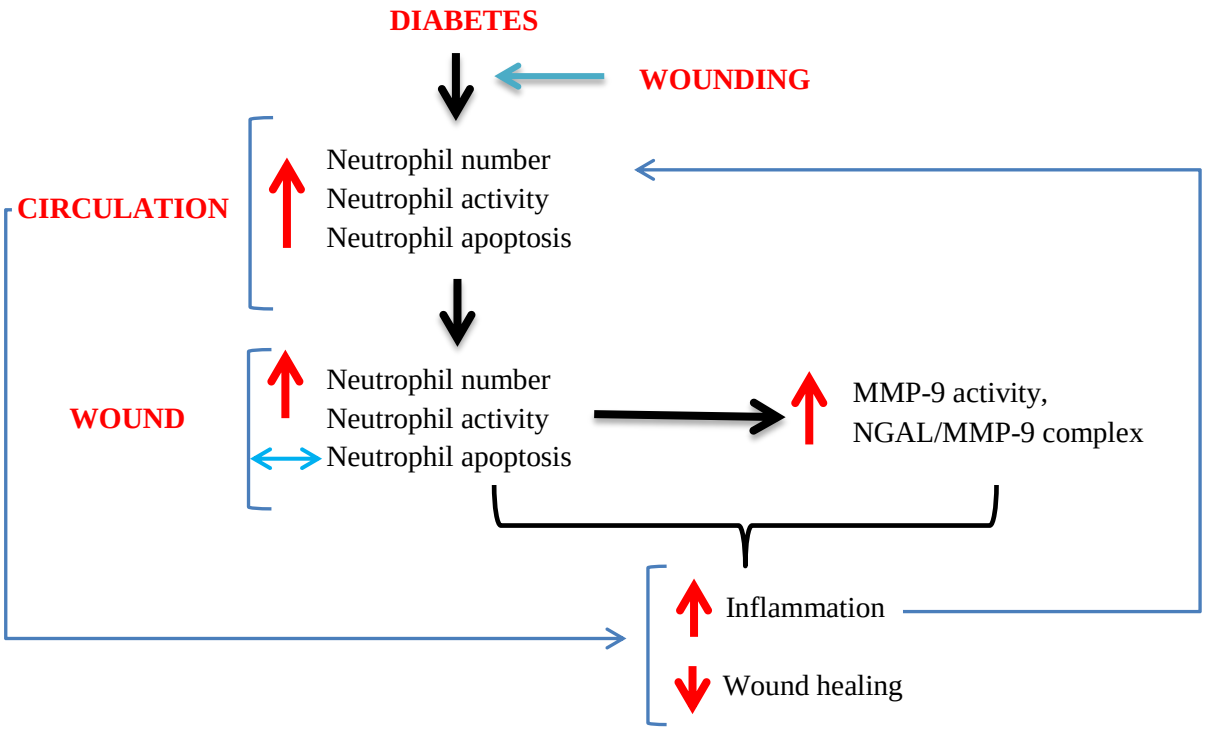


Figure 6-1: schematic diagram showing the effect of diabetes on neutrophils in the circulation and the implication for these changes in increased MMP-9 and poor wound healing.

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APPENDIX

Buffers

- RIPA buffer — Tris ; 65mM
  NaCl; 150mM; (Add dH20 make up to ~195mL, stir until clear and adjust to pH 7.4 with 10M HCl);
  EDTA; 5mM
  1% NP-40 = 2.5mL of 100% stock (same as Triton X-100);
  0.5% Sodium deoxycholate = 12.5mL of a 10% stock;
  0.1% SDS (2.5mLs of a 10% stock)
  10% glycerol = 25mLs of 100% stock

- Lysis buffer — NH_4Cl; 155 mM, $KHCO_3$; 10 mM, EDTA; 100 µM

- TBS-T — Tris; (100mM), NaCl; (150mM), Tween 20; (1ml)
  2 L water; pH to 7.5

- ×5 SDS-PAGE sample buffer — Tris; (0.25M), SDS;10%, Glycerol; (50%v/v), H_2O; (5 ml)

Zymography gels and buffers	
• 8%Polyacrylamide gel (ml)	Acrylamide/Bis1M; 40%, Tris ;pH 8.8, MDPF-gelatine; 10mg/ml APS; 10%, SDS; 10% , H2O; TEMED
• 3% stacking gel (ml):	Acrylamide/Bis1M, 40%, Tris pH 6.8; MDPF-gelatine; 10mg/ml APS; 10%, SDS; 10%, H2O; TEMED
• Buffer I	(2.5% Triton X-100, 50 mM Tris-HCl, 1 µM ZnCl2, 0.02% NaN3)
• Buffer II	(2.5%Triton X-100, 50 mM Tris-HCl, 1 µM ZnCl2, 0.02% NaN3, and 10mMCaCl2)
• Buffer III	(50 mM Tris-HCl, 1 µM ZnCl2, 0.02% NaN3, 10mMCaCl2)