ROLE OF NITRIC OXIDE IN WOUND HEALING: FACILITATORY EFFECTS OF NITROSOGLUTATHIONE – A NITRIC OXIDE DONOR ON THE EXTRACELLULAR MATRIX DEPOSITION CHARACTERISTICS OF WOUND HEALING

ACHUTH HN, M.B.,B.S

A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
NATIONAL UNIVERSITY OF SINGAPORE

2002

To my wife Chetana

Avyay

Dad and Mom

Acknowledgements

I would like to thank A/Prof Shabbir M Moochhala who has been an excellent guide and a friend in my research. He has always inspired me to learn more about science. His knowledge and enthusiasm has been highly motivating. I have learnt science, interpersonal relationship and managerial skills from him.

Prof Walter Tan has played a key role in guiding me through my research work at all stages and has been kind enough to spare time from his busy schedule for scientific discussions. His timely advice and suggestions were highly effective in conducting my research.

Dr Ratha Mahendran has been generous to help me in learning laboratory techniques and scientific writing. She has been a good friend and made working in the lab enjoyable.

Ashvin and Dominic have helped me in doing all the biomechanics work. It has been a pleasurable experience working with them.

Shirhan, Siva and Viren have been brotherly in providing all the logistic and experimental help. I thank them for all the help that they have given me. I wish them well.

The project "Cellular Mechanisms of wound healing in battlefield injuries" was funded by Defence Medical Research Institute, Singapore. I would like to thank this organization for providing me an opportunity to serve.

I am thankful to National University of Singapore, for giving me all the facilities to do research and granting me a scholarship.

TABLE OF CONTENTS

Contents	Page
Table of Contents	i
List of Figures	xii
List of Tables	xv
List of Publications	xviii
Abbreviations used in text	xix
Summary	xxi
Introduction	1
1.0 The problem statement	2
1.1 Current concepts in wound management	3
1.1.1 Therapeutic agents in wound healing	3
1.1.1.1 Dressings	4
1.1.1.2 Pharmacological agents	5
1.1.1.3 Biological agents	5
1.1.1.3.1 Growth factors	5
1.1.1.3.2 Enzymes	6
1.1.1.3.3 Gene therapy	6
1.1.1.3.4 Miscellaneous	7
1.1.2 Pitfalls in Current Wound Management	8

Contents **Page**

1.2 Quantitative indicators of wound healing 8

1.2.1 Rate of wound contraction 8

1.2.2 Collagen content 9

1.2.3 Biomechanical strength 9

1.3 Physiology of wound healing 10

1.3.1 Phases of wound healing 11

1.3.1.1 Coagulation and Inflammation 12

1.3.1.2 Cell proliferation and matrix deposition 13

1.3.1.2.1 Re-epithelialisation 13

1.3.1.2.2 Fibroplasia 14

1.3.1.2.3 Neovascularisation 15

1.3.1.2.4 Matrix deposition 16

1.3.1.3 Matrix Remodeling 16

1.4 Factors regulating wound healing 20

1.4.1 Growth factors 20

1.4.1.1 Coagulation and Inflammation 20

1.4.1.2 Cell proliferation and matrix deposition 22

1.4.1.3 Matrix Remodeling 23

Contents **Page**

1.4.2 Collagen 27

1.4.2.1 Coagulation and Inflammation 27

1.4.2.2 Cell proliferation and matrix deposition 28

1.4.2.3 Matrix Remodeling 30

1.4.2.4 Regulation of collagen production 30

1.4.3 Enzymes 32

1.4.3.1 Matrix-metalloproteinases 32

1.4.3.1.1 72-kDa gelatinase (MMP2) 33

1.4.3.1.1.1 Regulation of Gelatinase A activity 34

1.4.3.1.1.2 Functions of Gelatinase A in cellular processes 36

1.4.3.1.2 92-kDa gelatinase (MMP9) 36

1.4.3.1.2.1 Regulation of Gelatinase B activity 37

1.4.3.1.2.2 Functions of Gelatinase B in cellular processes 38

1.4.3.1.3 Gelatinases as applied to wound healing 39

1.4.3.2 Enzymes in free radical metabolism 41

1.4.4 Free radicals 41

1.4.4.1 Free radicals in skin 44

1.4.4.1.1 Anti-oxidant systems 46

1.4.4.2 Free radical scavengers in wound healing 47

Contents **Page**

1.4.5 Metal Ions **48**

1.5 Biology of Nitric oxide **48**

1.5.2 Mechanism of action of NO in wound healing **51**

1.5.2.1 Coagulation and Inflammation **51**

1.5.2.2 Cell proliferation and Matrix deposition **52**

1.5.2.3 Matrix remodeling **54**

1.5.3 Pharmacological studies of NO in wound healing **57**

1.5.3.1 Nitric Oxide donors previously studied in wound healing **58**

1.5.3.2 Nitric Oxide inhibitors previously studied in wound healing **59**

1.5.3.3 Nitrosothiols **60**

1.5.3.4 Interaction of Nitric Oxide with antioxidants. **60**

2.0 Hypothesis **62**

Contents **Page**

Materials and Methods 65

3.0 Materials and Methods 66

3.1 Materials 66

3.1.1 Chemicals and reagents 66

3.1.1.1 Anaesthetic agents 66

3.1.1.2 General Chemicals 66

3.1.1.3 Other Chemicals 67

3.1.1.4 Biological products 67

3.1.1.5 Commercial kits 68

3.1.1.6 Instruments 68

3.1.2 Experimental animals 68

3.2 Methods 69

3.2.1 Animal Study 69

3.2.1.1 Animal care 69

3.2.1.2 Grouping of animals based on wound models 70

3.2.1.2.1 Excisional Square wound group 70

3.2.1.2.2 Incisional wound group 70

3.2.1.3 Surgical Procedure 71

3.2.1.3.1 Animal Anaesthesia 71

3.2.1.3.2 Square wound model 71

3.2.1.3.3 Incisional model 73

3.2.1.3.4 Intra-abdominal catheterization 74

3.2.1.3.5 Sephanous vein catheterization 75

3.2.1.4 Treatment of animals with pharmacological agents 75

3.2.1.5 Sampling of the scar tissue 79

3.2.1.6 Storage of the scar tissue 79

3.2.2 Determination of Collagen content 79

3.2.3 Biomechanical testing 80

3.2.3.1 Sample preparation 81

3.2.3.2 Tensile strength measurement 82

3.2.4 Tissue protein measurement 83

3.2.5 Matrix metalloproteinase activity assay 83

3.2.5.1 Extraction of MMP's from the scar tissue 84

3.2.5.2 Gelatinase (MMP) activity assay 85

3.2.6 Determination of Total Nitrite 85

3.2.6.1 Determination of Nitrite in wound lysates 86

3.2.6.2 Determination of Nitrite in plasma 86

3.2.7 Glutathione assay 87

3.2.7.1 Sample preparation 88

Contents **Page**

3.2.7.2 De-proteination 88

3.2.8 Flow cytometry of peritoneal cells 88

3.2.8.1 Sample collection 89

3.2.8.2 Fluorescent staining 89

3.2.9 Histology 90

3.2.9.1 Preparation of histological sections 90

3.2.9.2 Hematoxylin and eosin staining 90

3.2.9.3 Immunocytochemistry 91

3.2.9.3.1 MMP immunostaining 91

3.2.9.3.2 Immunostaining of iNOS and eNOS 92

3.2.9.3.3 Evaluation of slides 93

3.2.10 Statistical analysis 94

Contents **Page**

Results 95

4.0 General health of the animals 96

4.1 Square wounds 96

4.1.1 Gross observation 96

4.1.2 Effects of GSNO on rate of wound contraction 97

4.1.3 Effects of AG on wound contraction 97

4.2 Biomechanical strength of scars treated with NO donors and inhibitors 100

4.2.1 Load to failure of the scars 102

4.2.1.1 Effects of GSNO, SNAP and GSH on load to failure 102

4.2.1.2 Effects of AG on load to failure 102

4.2.2 Stiffness of the scars 104

4.2.2.1 Effects of GSNO, SNAP and GSH on scar stiffness 104

4.2.2.2 Effects of AG on scar stiffness 104

4.3 Collagen content 108

4.3.1 Effects of GSNO, SNAP and GSH on collagen content 108

4.3.2 Effects of AG on collagen content 109

4.4 Tissue protein content 112

Contents **Page**

4.5 Gelatinase Activity 113

4.5.1 Effects of GSNO, SNAP and GSH on wound gelatinase activity 113

4.5.2 Effects of AG on wound gelatinase activity 114

4.6 Nitrite and nitrate (total nitrite) content 116

4.6.1 Scar nitrite and nitrate content 116

4.6.1.1 Effects of GSNO, SNAP and GSH on scar nitrite content 116

4.6.1.2 Effects of AG on scar nitrite content 117

4.6.2 Plasma nitrite content 118

4.6.2.1 Effects of GSNO and SNAP on the plasma nitrite 118

4.6.2.2 Effects of AG on plasma nitrite 119

4.7 Glutathione content 123

4.7.1 Effects of GSNO, SNAP and GSH on scar glutathione content 123

4.7.2 Effects of AG on scar glutathione content 123

4.8 Peritoneal macrophage flow cytometry 127

4.8.1 Effects of GSNO, SNAP and AG on MHC Class I surface marker 129

4.8.1.1 Effects of GSNO and SNAP on MHC Class I marker 129

4.8.1.2 Effects of AG on MHC Class I marker 129

4.8.2 Effects of GSNO, SNAP and AG on MHC Class II surface marker 131

4.8.2.1 Effects of GSNO and SNAP on MHC Class II marker 131

4.8.2.2 Effects of AG on Class II marker 131

4.8.3 Effects of GSNO, SNAP and AG on ICAM-1 surface marker 133

4.8.3.1 Effects of GSNO and SNAP on ICAM-1 surface marker 133

4.8.3.2 Effects of AG on ICAM-1 expression 133

4.9 Histology 135

4.9.1 Immunohistochemistry 137

4.9.1.1 Immunohistochemistry of MMP2 & MMP9 138

4.9.1.1.1 Effects of GSNO on MMP2 expression in scars 138

4.9.1.1.2 Effects of AG on MMP2 expression in scars 139

4.9.1.1.3 Effects of GSNO on MMP9 expression in scars 141

4.9.1.1.4 Effects of AG on MMP9 expression of scars 141

4.9.1.2 Immunohistochemistry of NOS isoenzymes 143

4.9.1.2.1 Effects of GSNO on eNOS expression in scars 143

4.9.1.2.2 Effects of AG on eNOS expression in scars 143

4.9.1.2.3 Effects of GSNO on iNOS expression in scars 145

4.9.1.2.4 Effects of AG on iNOS expression in scars 145

Contents **Page**

Discussion **147**

5.1 Experimental design **148**

5.1.1 Rate of wound contraction **148**

5.1.2 Tensile strength **148**

5.1.3 Collagen content **149**

5.1.4 Gelatinase activity **149**

5.1.5 Glutathione content **150**

5.2 Effects of NO donors and inhibitor on wound healing **151**

5.2.1 Effects of GSNO on wound healing **151**

5.2.2 Effects of SNAP on wound healing **155**

5.2.3 Effects of GSH on wound healing **157**

5.2.4 Effects of AG on wound healing **158**

5.3 Summary of discussion **159**

5.4 General discussion **163**

Conclusions & Future Directions **165**

6.1 Conclusions **166**

6.2 Future Directions **168**

References **170**

Appendix A-D

LIST OF FIGURES

Figures		Page
Fig. 1.1	Schematic representation of phases of wound healing.	11
Fig. 1.2	Phases of wound healing.	19
Fig. 1.3	Growth factors regulating wound healing.	25
Fig. 1.4	Enzymes acting during re-epithelialization.	40
Fig. 1.5	Formation and metabolism of active oxygen species.	43
Fig. 1.6	Metabolism of L-Arginine by NOS isoenzymes.	50
Fig. 3.1	Square excisional wound model.	72
Fig. 3.2	Incisional wound model.	73
Fig. 3.3	Intra-abdominal catheterization.	74
Fig. 3.4	Chemical structure of glutathione and S-nitrosoglutathione.	76
Fig. 3.5	Chemical structure of N-Acetyl-DL-penicillamine (NAP) and S-Nitroso N-acetyl-DL-penicillamine (SNAP).	77
Fig. 3.6	Chemical structure of aminoguanidine and L-arginine.	78
Fig. 3.7	Experimental set-up for biomechanical testing.	81
Fig. 3.8	Instron materials testing machine.	82
Fig. 3.9	Schematic representation of DTNB recycling in glutathione assay.	87
Fig. 4.1	Log curve of wound contraction.	98
Fig. 4.2	Rate of wound contraction in Control, AG and GSNO treated animals.	99
Fig. 4.3	Load deformation curve.	101
Fig. 4.4	Load to failure of scars treated with GSNO, SNAP, GSH and AG.	103
Fig. 4.5	Stiffness of scars treated with GSNO, SNAP, GSH and AG.	106

LIST OF FIGURES

Figures		Page
Fig. 4.6	Sample tracing of load displacement of a 5 day Control scar.	107
Fig. 4.7	Standard straight line graph of hydroxyproline assay.	110
Fig. 4.8	Hydroxyproline concentration of scars treated with GSNO, SNAP, GSH and AG.	111
Fig. 4.9	Standard straight line graph for protein estimation.	112
Fig. 4.10	Gelatinase activity in the scars of animals treated with GSNO, SNAP, AG and GSH.	115
Fig. 4.11	Standard straight line graph of nitrite concentrations.	120
Fig. 4.12	Nitrite content of scar samples obtained at 3, 5, 7 and 10 days post-wounding.	121
Fig. 4.13	Plasma nitrite concentration following the administration of GSNO, SNAP and AG (n=6).	122
Fig. 4.14	Standard straight line graph of glutathione determined by Cayman Glutathione assay kit.	125
Fig. 4.15	Total glutathione concentration of scars at 3, 5, 7 and 10 days post-wounding.	126
Fig. 4.16	Dot-Plot representation obtained by plotting the A) unstained peritoneal cells (negative control) and B) IgG isotype control.	128
Fig. 4.17	Expression of MHC Class I surface markers on the peritoneal cells.	130
Fig. 4.18	Expression of MHC Class II surface markers on the peritoneal cells.	132
Fig. 4.19	Expression of ICAM-1 surface markers on the peritoneal cells.	134
Fig. 4.20	Haemotoxylin and Eosin staining of normal skin and scars.	136
Fig. 4.21	Negative control slides counterstained with methyl green.	137

LIST OF FIGURES

Figures		Page
Fig. 4.22	Expression of MMP2 in scars of animals treated with A) Control B) GSNO and C) AG.	140
Fig. 4.23	Immunohistochemistry of MMP9 enzymes in scars treated with A) Saline (control) B) GSNO and C) AG.	142
Fig. 4.24	Immunohistochemistry of eNOS enzymes in scars treated with A) Saline (control) B) GSNO and C) AG.	144
Fig. 4.25	Immunohistochemistry of iNOS enzymes in scars treated with A) Saline (control) B) GSNO and C) AG.	146

LIST OF TABLES

Tables		Page
Table 1.1	Therapeutic agents in wound healing	5
Table 1.2	Growth factors regulating wound healing	26
Table 1.3	Decomposition of hydro peroxides and hydrogen peroxides by enzymes	41
Table 1.4	Summary of previous studies on the administration of free radical scavengers/anti-oxidants in wound healing.	47
Table 1.5	Summary of previous studies on the effects of NO donors in wound healing.	58
Table 1.6	Summary of previous studies on the effects of NO inhibitors in wound healing.	59
Table 4.1	Load to failure of scars following treatment of GSNO, SNAP, AG and GSH.	102
Table 4.2	Stiffness of scars following treatment of GSNO, SNAP, AG and GSH.	105
Table 4.3	Collagen content of scars.	109
Table 4.4	Gelatinase activity of the scars.	114
Table 4.5	Nitrite and Nitrate content of the scars.	117
Table 4.6	Nitrite and Nitrate content of the plasma.	119
Table 4.7	Glutathione (GSSG) content of the scars.	124

LIST OF PUBLICATIONS

1) **Achuth HN, Moochhala SM, Mahendran R, Tan WTL, Lu JH, Shirhan Md. Nitrosoglutathione triggers enhanced collagen deposition in cutaneous wound repair. Manuscript revised and submitted to Experimental dermatology.**

2) **Achuth HN, Tambyah A, Moochhala SM, Dominic TKK. Nitric oxide and glutathione in wound healing: A biomechanical study (manuscript in preparation)**

3) **Moochhala SM, Achuth HN. Nitric oxide and anti-oxidant equilibrium in wound repair: A review (manuscript in preparation).**

4) **Achuth HN, Mahendran R, Moochhala SM. Effect of aminoguanidine on the biomechanical strength and expression of matrix metalloproteinases in scar formation. (Manuscript in preparation).**

LIST OF PRESENTATIONS /ABSTRACTS

Oral presentations

1) **Achuth HN, SM Moochhala, Walter Tan TL. Expression of MMP in scar tissue of nitric oxide synthase inhibited animals. In: 2nd SAF Military Medicine Conference, New Changi Hospital, Singapore, 16-17 Jan 1999.**

2) **Achuth HN, WTL Tan, SM Moochhala, Andrea Rajnakova, TC Lim. Aberrant expression of Nitric oxide synthase in normal human skin and Keloid: Effect of Steroids on the expression of NO in keloids. In: Wound Healing Society Conference, Institute of health, Singapore, Oct 1999.**

3) **Achuth HN, SM Moochhala, WTL Tan**
Wound healing- Role Of nitric oxide in the reparative process. In: Wound Healing Society Conference, Institute of health, Singapore, Oct 1999.

4) **Achuth HN, SM Moochhala, WTL Tan**
Effect of nitric oxide on the expression of Matrix metalloproteinases 1 and 3 in wound healing. In: Wound Healing Society Conference, Institute of health, Singapore, Oct 1999.

5) **Achuth HN, WTL Tan, R Mahendran, SM Moochhala**
The effect of aminoguanidine (nitric oxide synthase inhibitor) on the biomechanical strength and the expression of matrix metalloproteinases in scar formation. In: First World Wound Healing Congress, Melbourne, Australia, Sep 2000.

6) **Achuth HN, Moochhala SM, Mahendran R, Tan WTL. Effects of nitric oxide donors on wound collagen and matrix metalloproteinases - A rodent model. In: Fourth Joint Meeting of the European Tissue Repair Society and the Wound Healing Society, Baltimore, U.S.A, May 2002 (abstract published in Wound Repair and Regeneration, March-April 2002, Volume 10, Number 2:A1)**

Poster Presentations

1) **Achuth HN, SM Moochhala, Walter TL Tan. Role of Nitric Oxide In Wound Healing. Asia Pacific Miliitary Medicine Conference, Singapore, May 7-12 / 2000.**

2) **Achuth HN, SM Moochhala, Walter Tan TL. Biomechanics of wound healing. Musculoskeletal Bioengineering Symposium, Nov 1998.**

ABBREVIATIONS USED IN TEXT

NO	**Nitric Oxide**
GSH	**Reduced Glutathione**
GSNO	**S-nitrosoglutathione**
AG	**Aminoguanidine**
iNOS	**Inducible Nitric Oxide Synthase**
SNAP	**S-nitroso-N-acetylpenicillamine**
eNOS	**Endothelial nitric oxide synthase**
PDGF	**Platelet Derived Growth Factor**
VEGF	**Vascular Endothelial Growth Factor**
TGF	**Transforming Growth Factor**
FGF	**Fibroblast Growth Factor**
TNF	**Tumor Necrosis Factor**
IL	**Interlieukin**
KGF	**Keratinocyte Growth Factor**
EC	**Endothelial Cells**
SMC	**Smooth Muscle Cells**
VSMC	**Vascular Smooth Muscle Cells**
bFGF	**Basic Fibroblast Growth Factor**
IGF	**Insulin like Growth Factor**
HB-EGF	**Heparin-binding Epidermal Growth Factor**
MMP	**Matrix metalloproteinases**

MT	**Membrane Type**
TIMP	**Tissue Inhibitor of metalloproteinases**
AP	**Activator Protein**
mRNA	**Messenger RiboNucleic Acid**
APMA	**Amino Phenyl Mercurial Acetate**
kDa	**Kilo Dalton**
ECM	**Extra Cellular Matrix**
IFN	**Interferon**
GSSG	**Glutathione di sulphide**
ROS	**Reactive Oxygen Species**
PMN	**Polymorphonuclear cells (neutrophils)**
FMN	**Flavim mononucleotide**
FAD	**Flavin adenine dinucleotide**
cNOS	**Constitutive nitric oxide synthase**
LPS	**Lipopolysaccharide**
NFκB	**Nuclear factor-kappa B**
MCP	**Macrophage chemotactic protein**
MIP	**Macrophage inhibitory protein**
cGMP	**Cyclic guanosine monophosphate**
PVA	**Poly vinyl alcohol**
NSAID	**Non-steroidal anti inflammatory drug**
NP	**Nitroprusside**

Summary

Wound healing is a dynamic process, which is governed by many signaling molecules. Nitric oxide (NO) is one such molecule, which regulates the inflammatory response, cell proliferation, differentiation and matrix deposition in wound healing. Previous in vitro and in vivo studies on the administration of NO donors and inhibitors have pointed towards the facilitatory effects of NO in wound healing. Similarly the importance of anti-oxidants (GSH) in wound healing has also been described. Interaction between NO and GSH is one of the important mechanisms in inflammatory processes. In this study we have examined the beneficial effects of administering a NO donor S-nitrosoglutathione (GSNO) in wound healing. The effects of this agent are compared to S-nitroso-N-acetyl-penicillamine (SNAP), which belongs to the same group of compounds and a well-known NO donor. As GSNO contains a thiol component i.e glutathione, the effects were compared to reduced glutathione.

Sprague dawley male rats were all subjected to wounding. The two methods of wounding in this study were excisional square wounds and incisional-sutured wounds. The square wound model was the initial part of the study to examine the overall effects of GSNO on wound healing. This was compared to AG, an iNOS specific inhibitor.

In the incisional wound study, the animals were injected with GSNO, SNAP, GSH and AG. The drugs were administered daily to respective groups. Six animals (n=6) from each group were sacrificed at 3, 5, 7 and 10 days after wounding. GSNO

improved the rate of wound contraction by 55%. Aminoguanidine did not have any noticeable effect on rate of wound healing. Quantitative improvement in wound healing was monitored by 1) measuring the material property of the scar in the form of load to failure and maximum stiffness 2) collagen content in the scars 3) gelatinase activities 4) scar nitrite and nitrate content 5) glutathione concentration of the scars.

	GSNO	SNAP	GSH	AG
Biomechanical Strength	↑	—	—	↓
Collagen content	↑	—	—	↓
MMP activity	—	↓	—	—
Glutathione	—	↓	↑	—
Nitrite and Nitrate	↑	↑	—	↓

Results obtained from our study have been summarized in the table given above. ↑ indicates increase in the values of the parameters and ↓ indicates significant reduction compared to control and — shows no significant difference.

Nitrosothiols are thought to represent a circulating reservoir of NO and have potential as NO donors, distinct from currently used agents. Because of its wide range of effects on wound healing, GSNO has great potential as a therapeutic agent. The future applications of GSNO lie in the possibility of increasing GSH levels in pathological conditions such as ulcers and sores.

Chapter 1

Introduction

1.0 The problem statement

The primary function of the skin is to serve as a protective barrier against the environment. Loss of integrity of this barrier results in wounds, which are one of the most common pathological conditions. Improper wound healing can cause serious concerns in the form of major disability or even death. With increasing age of life expectancy, incidence of wounds with various etiologies, have also increased. Chronic wounds are a major challenge in health care. Significant part of health care expenditure is on wound treatment. Disturbed wound healing may manifest in various forms such as ulcers, scars and sores. Excoriations around discharging ulcers, repeated infections, malnourishment, severe contractures and physical disabilities are the main long-term complications due to delayed or non-healing wounds (Prem P. Gogia, 1995). In a study conducted by Ferrell BA (2000) the incidence of wounds in the elderly was as follows: 9.12% had pressure injuries, 37.4% had more than one ulcer and 14.0% had three or more ulcers. About 30% of subjects were at risk for new pressure ulcers. On an average the costs of management of pressure ulcer is 1000 US$, full thickness venous ulcer is 2000 US$, diabetic foot ulcer 1000 US$ and ischaemic ulcer 2000 US$ (http://www.medicaledu.com/outcomes.htm). The total wound care expenses globally runs into billions of dollars. The complications associated with chronic wounds are wide such as the cost involved in wound care, psychological and physical debilitation.

1.1 Current concepts in wound management

Continuous advances made in the study of the wound microenvironment, an ever-broadening understanding of the pathophysiology of wounds, and improved techniques in monitoring the response of healing have led to continuing developments in the treatment of chronic wounds.

The practice of wound management varies from the use of simple gauze dressings to complicated skin substitutes. Most commonly adopted strategies in wound management are antiseptics and antibiotics in the form of topical ointments to prevent contamination. There are various other therapeutic agents, pharmacological and biological, available for wound management. However, the current challenge is to identify the basic underlying mechanism and appropriate therapeutic agent, which enhances wound healing. An ideal wound enhancer must be able to prevent contamination, act as a chemotactic to resident host cells, enhance reparative tissue deposition and finally prevent the development of a scar. In order to enhance wound healing, the agent must also have a facilitatory effect on one or all the phases of this reparative process.

1.1.1 Therapeutic agents in wound healing

Wound management involves hemostasis, antisepsis, analgesia and antimicrobiostasis. The pharmacological and biological agents presently available to assist wound management are described below.

1.1.1.1 Dressings

Dressings are now available specifically for individual variety of wounds because of the multi-etiologic nature of wounds. They vary from simple cotton gauzes to bioactive dressings such as hydrocolloids, moist dressings and hygroscopic agents (polymeric agents). The main purpose served by dressings is mainly antisepsis. The moist dressings, pressure dressings and cavity dressings are tailor made for venous ulcers, cavitating wounds and sores.

Advanced dressings attempt to specifically maintain a moist wound environment. Although they supersede conventional dressings, such as paraffin-impregnated non-adherent gauze, temporally, they are not always more appropriate and can be more expensive. Hydrocolloids, alginates and foams maintain the moist wound environment by absorbing exudates, and hydrogels and films donate or maintain moisture. Infection-controlling properties of some wound dressings have been evaluated recently. Cadexomer iodine dressing composed of starch lattice into which 0.9% w/v iodine is trapped, is highly absorptive facilitating autolytic debridement while slow release of iodine maintains levels in the wound bed, where it has a broad spectrum of antibacterial activity. Hydrofibre dressing made of carboxymethylcellulose, effectively sequesters and retains micro-organisms upon exposure to simulated wound fluid, thus providing a passive mechanism for reducing the microbial load in wounds and in the surrounding environment.

1.1.1.2 Pharmacological agents

Treatment	Clinical application	Advantages	Disadvantages
Anti-microbials	Prophylactic agents against extensive contamination	Prevention of microbial contamination	Resistance and cross resistance, toxic to some parenchymal cells
Anaesthetic agents	Topical application in surgical sutures, pediatric wounds and tooth extraction	Relieves pain in severe cases	Not extensively used as it does not help the healing process per se
Non-steroidal anti-inflammatory agents	Controls extensive inflammatory response	Decreased scarring, improves mobility and decreases pain	Inhibit certain important processes in healing
Fibrin glue	Extensive hemorrhage	Immediate hemostasis	Not easily available and difficult to store
Cyanoacrylate glues	Clean incisional bleeding	Substitutes suture	Not applicable to laceration and irregular edges of wounds

Table 1.1: Therapeutic agents in wound healing. This table summarizes currently available pharmacological agents which support wound healing (Prem P. Gogia, 1995).

1.1.1.3 Biological agents

1.1.1.3.1 Growth factors

The ability study and manipulate the wound milieu has led to the identification and separation of a variety of growth factors such as the platelet-derived growth factor, epidermal growth factor, transforming growth factor, fibroblast growth factor-β,

tumor necrosis factor and interleukin-1. The characterization of the effects of these factors and the ability to prepare them in large supply has led to trials of various growth factors. The reported effects of growth factor therapy include stimulation of cell movement and cell division and increases in matrix synthesis and cell mass, thus leading to rapid wound closure. Preparation of the wound bed, choice of growth factor appropriate to the stage of healing, and quantity and duration of administration are all important considerations in growth factor usage, with the presence of protein degrading enzymes in chronic wound fluid constantly challenging the survival of these growth factors.

Currently, the Platelet Derived Growth Factor (PDGF) is available commercially for clinical use. It has been used successfully in the treatment of chronic wounds. The major drawbacks in the use of growth factors are: 1) they are expensive 2) require stringent storage conditions and 3) require expert handling.

1.1.1.3.2 Enzymes

Collagenase has been effectively used in the enzymatic debridement of burn wounds, pressure ulcers, necrotic ulcers and infected wounds. Superoxide dismutase encapsulated in liposomes has been shown to improve wound healing. The drawbacks of this treatment are it is an expensive mode of treatment and is not applicable in most of the cases.

1.1.1.3.3 Gene therapy

With the technology to introduce and express genes in human somatic cells, sustained delivery of wound healing-promoting products is now a real possibility.

Vascular endothelial growth factor (VEGF) has increased angiogenic effect in wound healing. PDGF is an efficient treatment for chronic diabetic ulcers. Genes encoding various growth factors, such as platelet-derived growth factor and epidermal growth factor, have been transferred into and induced in wounds, thus providing a constant supply of a product that can induce optimal repair. The disadvantages are: 1) it is expensive and not extensively used and 2) unlimited expression of the gene is undesirable.

1.1.1.3.4 Miscellaneous

Hyperbaric oxygen is studied in prevention of necrosis of skin, chronic non-healing open wounds and diabetic wound therapy. The advantage is that it limits necrosis in ischaemic wound and increases re-epithelialisation. The drawback is that it is difficult to administer and accidents such as lung damage are expected.

Maggots, honey and certain plant extracts such as aloe vera are all reported to enhance healing. The potential for maggots to rapidly debride wounds in a nontoxic manner has been recognized for centuries. Recent studies directed at larval secretions suggest that constituent of the secretions may also act directly as growth factors, or alternatively stimulate appropriate cytokine production to facilitate wound healing. Honey has also been used in the treatment of wounds for centuries as a result of its efficacy against antibacterial-resistant pathogens as well as its ability to debride and promote granulation and epithelialization within wounds.

1.1.2 Pitfalls in Current Wound Management

Current wound management involves providing supportive measures in preventing infections, pain and disfigurement. Drugs altering the actual mechanism of inflammation, matrix deposition or tissue remodeling are not yet clinically used.

Recent advances in cellular and molecular biology have greatly expanded our understanding of the biologic processes involved in wound repair and tissue regeneration and have led to improvements in wound care. As these biologic processes are tightly regulated by redox mechanisms we have examined the effects of nitric oxide, a highly reactive radical and a key secondary signaling molecule in wound healing.

1.2 Quantitative indicators of wound healing

In-order to monitor the prognosis of wound healing, it is important to measure certain important parameters. They are a) rate of wound healing, b) collagen content of the scar and c) Biomechanical strength.

1.2.1 Rate of wound contraction

Clinically, evaluation of wound healing is done by tracking the time taken for the complete closure of the wound and the formation

of mature scar. This is classically known as the rate of wound healing. The decrease in the wound area is technically termed as wound contraction and is the main component in wound healing. This also represents the rate of epithelialisation and

growth of the granulation tissue which are the key mechanisms regulating wound healing.

1.2.2 Collagen content

It is the chief indicator of the reparative tissue deposited in the wound environment. Collagen deposition begins during the phase of connective tissue deposition and granulation formation. The time course of various subtypes of collagen deposition has been studied. Briefly, in the early phase of matrix deposition the collagen type III is secreted by the fibroblasts, but it slowly matures into type I, which is a thinner and mature form of collagen. In an experimental set-up it is important to determine the collagen content of the scar as a measure of quality of wound healing.

1.2.3 Biomechanical strength

Biomechanical strength is a key factor in determining the final outcome of healing. The progressive increase in biomechanical strength of the tissue results from the formation and turnover of granulation tissue. Hence the physical quality of the scar is measured as the tensile strength. The material properties of the scar are measured and the changes in the strength indicate the effects of various treatments on the collagen deposition in the scar.

1.3 Physiology of wound healing

Wound healing is a dynamic process requiring the collaborative efforts of many different tissues and cell lineages. The behavior of each of the contributing cell types during the phases of proliferation, migration, matrix synthesis, and contraction, as well as the growth factors and matrix signals present at a wound site, are now roughly understood. Details of how these signals control wound cell activities are beginning to emerge and are discussed below.

A temporary repair is achieved in the form of a clot that plugs the defect, and over subsequent days, steps to regenerate the missing parts are initiated. Inflammatory cells and then, the fibroblasts and capillaries invade the clot to form a contractile granulation tissue that draws the wound margins together. Meanwhile, the cut epidermal edges migrate forward to cover the denuded wound surface. Fundamental to our understanding of wound-healing biology is, the knowledge of the signals that trigger relatively sedentary cell lineages at the wound margin to proliferate, to become invasive, and then to lay down new matrix in the wound gap. Studies in the last decade have provided a list of the growth factors and matrix components that are available to provide these "start" signals, and one of the tasks now begun is to relate these factors specifically to the starting and stopping of each of the many cell activities by which the wound is healed. Most skin lesions are healed rapidly and efficiently within a week or two. However, the end product is neither aesthetically nor functionally perfect. Epidermal appendages that have been lost at the site of damage do not regenerate, and when the wound has healed there remains

a connective tissue scar where the collagen matrix has been poorly reconstituted, in dense parallel bundles, unlike the mechanically efficient basket-weave meshwork of collagen in unwounded dermis. A major goal of wound-healing biology is to understand the mechanisms by which skin is induced to reconstruct the damaged parts more appropriately. Wound healing has been clearly divided into three overlapping phases (Fig. 1.1), each of which is predominated by a specific physiological response. These phases are described in detail below.

1.3.1 Phases of wound healing

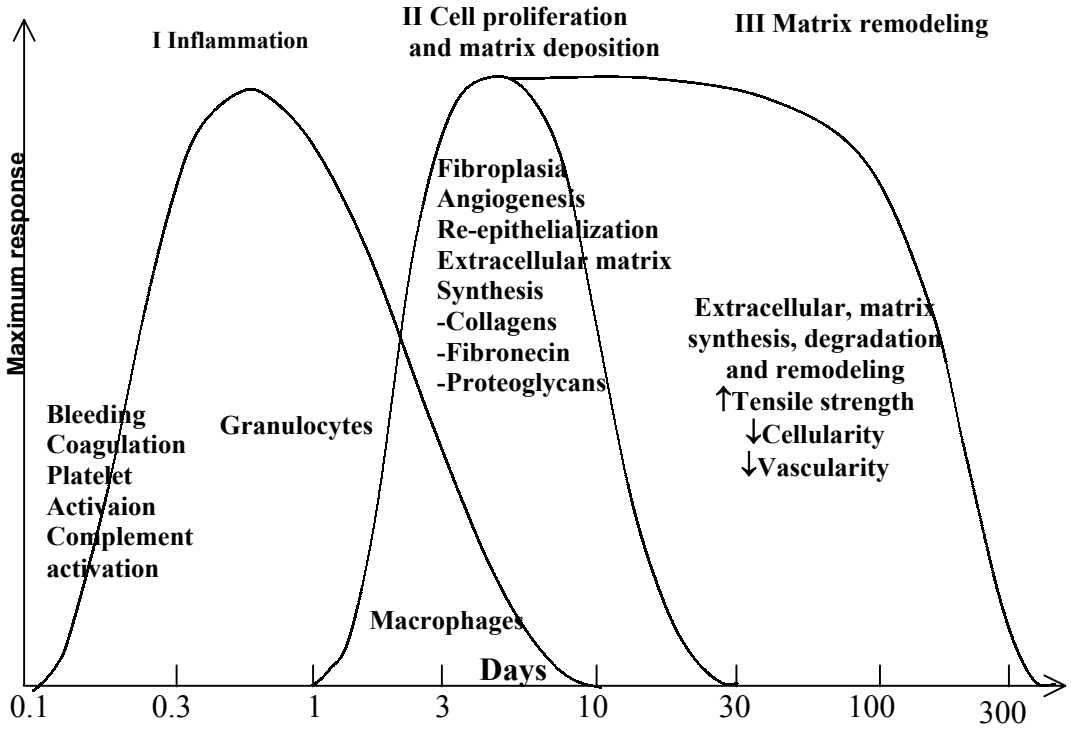


Fig. 1.1: Schematic representation of phases of wound healing. X-axis represents time (days) in log scale and Y-axis represents maximum response. The physiological events and the predominant cell type at each phase are depicted in this diagram.

1.3.1.1 Coagulation and Inflammation

Dermal wounds cause leakage of blood from damaged blood vessels. The formation of a clot then serves as a temporary shield protecting the denuded wound tissues and provides a provisional matrix over and through which cells can migrate during the repair process (Fig 1.2A). Importantly, the clot also serves as a reservoir of cytokines and growth factors that are released as activated platelets degranulate. The activated platelets release a cadre of biologically active substances that promote cell migration and growth into the site of injury. Additionally the platelets also release their alpha (∝) granules, which contain fibrinogen, fibronectin, thrombospondin and von Willebrand factor VIII (Detwiler & Fienman, 1973; Plow E.F, 1986). Fibrin and fibronectin act as provisional matrix for the influx of monocytes and fibroblasts (Turk, 1976). Neutrophils are the first leukocytes to enter the wound area (Fig. 1.2B). This early cocktail of growth factors "kick starts" the wound closure process. It provides chemotactic cues to recruit circulating inflammatory cells to the wound site, initiates the tissue movements of re-epithelialization and connective tissue contraction, and stimulates the characteristic wound angiogenic response. They ingest the microbial flora, acting as the first line of defense. The neutrophils are predominant in the early inflammatory phase and later replaced by the monocytes. This marks the end of the early inflammatory phase. They transform into tissue macrophages, which in turn ingest the foreign organisms, digest out the effete neutrophils and release mediators for the recruitment of the other cells (Newman, 1982). The macrophages release a plethora of growth factors, vasoactive mediators, chemotactic factors and enzymes. The chemotactic factors and the

growth factors are responsible for the initiation of the granulation tissue (Leibovich, 1975). Thus the macrophages play an important role in the transition between wound inflammation and wound repair.

1.3.1.2 Cell proliferation and matrix deposition

1.3.1.2.1 Re-epithelialisation

Re-epithelialization of a wound begins within hours after injury. In the skin, keratinocytes of the stratified epidermal sheet or hair follicle appear to move one over the other in a leapfrog fashion (Winter, 1962) (Fig. 1.2C). Alongwith migration, epithelial cells undergo marked phenotypic alteration. This metamorphosis includes retraction of intracellular tonofilaments, dissolution of most intercellular desmosomes and formation of peripheral cytoplasmic actin filaments (Gabbiani, 1978). One to two days after injury, epithelial cells at the wound margin begin to proliferate (Krawczyk, 1971). However, a few days after injury, fibronectin is deposited by wound fibroblasts, macrophages, or the migrating epidermal cells themselves (Clark, 1982). Wound keratinocytes express functionally active integrin receptors for fibronectin in contrast to normal epidermal cells. Thus, wound keratinocytes can pave the wound surface with a provisional matrix and express cell surface receptors that facilitate their migration across this matrix (Clark, 1982). The epidermis dissects through the wound, separating desiccated or otherwise non-viable tissue from viable tissue (Clark, 1982). Epidermal movement through tissue depends on collagenase production by epidermal cells (Woodley, 1982) and plasminogen activator. The latter enzyme activates collagenase as well as plasminogen (Fig.1.4). The driving forces for epithelial cell movement are

chemotactic factors, active contact guidance, loss of nearest neighbor cells, or a combination of these processes. As re-epithelialization ensues, basement membrane proteins reappear in a very ordered sequence from the margin of the wound inward in a zipperlike fashion (Clark, 1982). Epidermal cells differentiate into their normal phenotype, once again firmly attaching to the reestablished basement membrane through hemidesmosomes and to the underlying neodermis through type VII collagen fibrils (Gipson, 1983).

1.3.1.2.2 Fibroplasia

Matrix formation begins simultaneously with the formation of granulation tissue (Fig. 1.2C). During the dissolution of granulation tissue, the matrix is constantly altered, with relatively rapid elimination of fibronectin from the matrix and slow accumulation of large fibrinous bundles of type I collagen that provide the residual scar with increasing tensile strength. The composition of the granulation tissue varies from center to periphery (Bailey, 1975). Extracellular matrix components serve several critical functions for effective wound repair. This process includes accumulation of macrophages and migration of fibroblasts, deposition of connective tissue and angiogenesis. The granular appearance of the tissue is due to the numerous newly formed blood vessels. Macrophages, fibroblasts and blood vessels move into the wound space as a unit. Fibroplasia and angiogenesis are stimulated by the numerous growth factors that are released by platelets and macrophages (Gauss-Muller, 1980). Fibroblasts respond to these stimuli by proliferation, migration, matrix deposition and wound contraction (Grillo, 1964). The connective tissue matrix formed by the fibroblasts provides a substrate on which the

macrophages, new blood vessels and fibroblasts themselves migrate into the wound area. Thus macrophages, wound fibroblasts and blood vessels are absolutely dependent on each other during granulation tissue formation.

1.3.1.2.3 Neovascularisation

Angiogenesis is a complex process that depends on an appropriate extracellular matrix in the wound bed as well as phenotype alteration, stimulated migration, and mitogenic stimulation of endothelial cells. Endothelial cells are phenotypically modified during angiogenesis (Ausprunk and Folkman, 1977). Factors such as FGF, TGF-α, TGF-β, TNF-α, angiogenin, angiotropin, vascular endothelial growth factor (VEGF), interlieukin-8 (IL-8) and PDGF all promote angiogenesis. The above mentioned factors may also induce angiogenesis in vivo by stimulating chemotaxis of endothelial cells or by recruiting monocytes or other cells to produce angiogenic factors (Ryan, 1977). Proteolytic enzymes released into the connective tissue degrade extracellular matrix proteins, including fibronectin. Activated macrophages and injured tissue cells release FGF, which stimulate endothelial cells to release plasminogen activator and procollagenase. Plasminogen activator converts plasminogen to plasmin and procollagenase to active collagenase, and in concert, these two proteases digest basement membrane constituents. The fragmentation of the basement membrane allows endothelial cells to migrate into the injured site. As endothelial cells migrate into the fibrin-fibronectin-rich wound, they form tubes that express integrin to facilitate adhesion and migration. The neovasculature first deposits its own provisional matrix containing fibronectin and proteoglycans, and ultimately forms a true basement membrane (Shelly, 1984; Sten, 1979). In

summary, angiogenesis is a complex process depending on at least four interrelated phenomena: cell phenotype alteration, chemoattractant-driven migration, mitogenic stimulation, and an appropriate extracellular matrix deposition.

1.3.1.2.4 Matrix deposition

After a 5-day lag, increased rate of type I collagen synthesis begins, which coincides with increased wound-breaking strength (Diegelmann, 1975; Gabbiani 1972). Types I and III fibrillar collagen deposition peaks between 7 and 14 days (Clore, 1979). Rigid helical collagen macromolecules oriented into fibrillar bundles gradually provide the healing tissue with increasing stiffness and tensile strength (Levenson, 1965). As with types I and III collagen mRNAs, type VI collagen mRNA peaks between 1 and 2 weeks after injury (Bruns, 1986). Besides providing structural support and strength to the new tissue, collagen affects the activity of the cells within the matrix. These effects may be mediated, through activation of the integrin collagen receptors (Kurkinen, 1980). The newly deposited matrix rich in collagen alters the fibroblasts into myofibroblasts. Perhaps the collagen-rich extracellular matrix, which accumulates in mature granulation tissue, reduces the ability of wound fibroblasts to produce further collagenous matrix, but promotes the ability of these cells to remodel collagen-rich matrix already present.

1.3.1.3 Matrix Remodeling

After the initial synthesis and deposition of collagen, myofibroblasts remodel the matrix by wound contraction (Fig. 1.2D). Fibroblasts undergo a series of phenotypic changes during granulation tissue formation that continually modify their

interactions with the extracellular matrix (Gabbiani, 1971). Fibroblasts assume a migratory phenotype initially and a synthetic phenotype at a later phase to produce collagen (Gabbiani, 1971; Welch, 1990). Subsequently, during the second and third week of healing, fibroblasts begin to assume a myofibroblast phenotype characterized by large bundles of actin-containing microfilaments disposed along the cytoplasmic face of the plasma membrane and the establishment of cell-cell and cell-matrix linkages (Welch, 1990). The appearance of the myofibroblasts corresponds to the commencement of connective tissue compaction and the contraction of the wound. New collagen bundles in turn have the capacity to join end-to-end with collagen bundles at the wound edge and to ultimately form covalent cross-links among themselves and with the collagen bundles of the adjacent dermis (Yamauchi, 2002). These cell-cell, cell-matrix, and matrix-matrix links provide a network across the wound whereby the traction of fibroblasts on their pericellular matrix can be transmitted across the wound (Singer, 1984). Wound contraction is now ascribed to the actin-rich myofibroblasts that, in fact, are the most numerous cells in mature granulation tissue and are aligned within the wound along the lines of contraction. Collagen condensation is a result from a "collection of collagen bundles" executed by fibroblasts as they extend and retract pseudopodia attached to collagen fibers (Bell, 1979). The transmission of these traction forces across the in vitro collagen matrix depends on two linkage events: fibroblast attachment to the collagen matrix through the integrin receptors (Schiro, 1991) and cross-links between the individual collagen bundles (Woodley, 1985). Thus, wound contraction represents a complex and masterfully orchestrated interaction of cells, extracellular

matrix and cytokines. Collagen remodeling during the transition of granulation tissue to mature scar is dependent on both continued collagen synthesis and collagen catabolism. The degradation of wound collagen is controlled by a variety of collagenase enzymes from granulocytes, macrophages, epidermal cells, and fibroblasts. These collagenases are specific for particular types of collagens, but most cells probably contain two or more different types of these enzymes (Veli-Matti Kahari, 1997). Wounds gain only about 20% of their final strength by the third week, during which time fibrillar collagen has accumulated rapidly and has been remodeled (Levenson, 1965). Thereafter, the rate at which wounds gain tensile strength is slow, reflecting a much slower rate of collagen accumulation. Infact, the gradual gain in tensile strength depends on collagen remodeling with formation of larger collagen bundles and an alteration of intermolecular cross-links (Bailey, 1975). Even so, wounded tissue fails to attain the same breaking strength as uninjured skin. At maximum strength a scar is only 70% as strong as intact skin (Levenson, 1965). The early formation of types I, III and V collagen fibrils provides nascent tensile strength for the wound. As the matrix matures, the fibronectin and hyaluronic acid disappear. The collagen bundles grow in size lending increased tensile strength to the wound site and the deposited proteoglycans add resilience to the tissue.

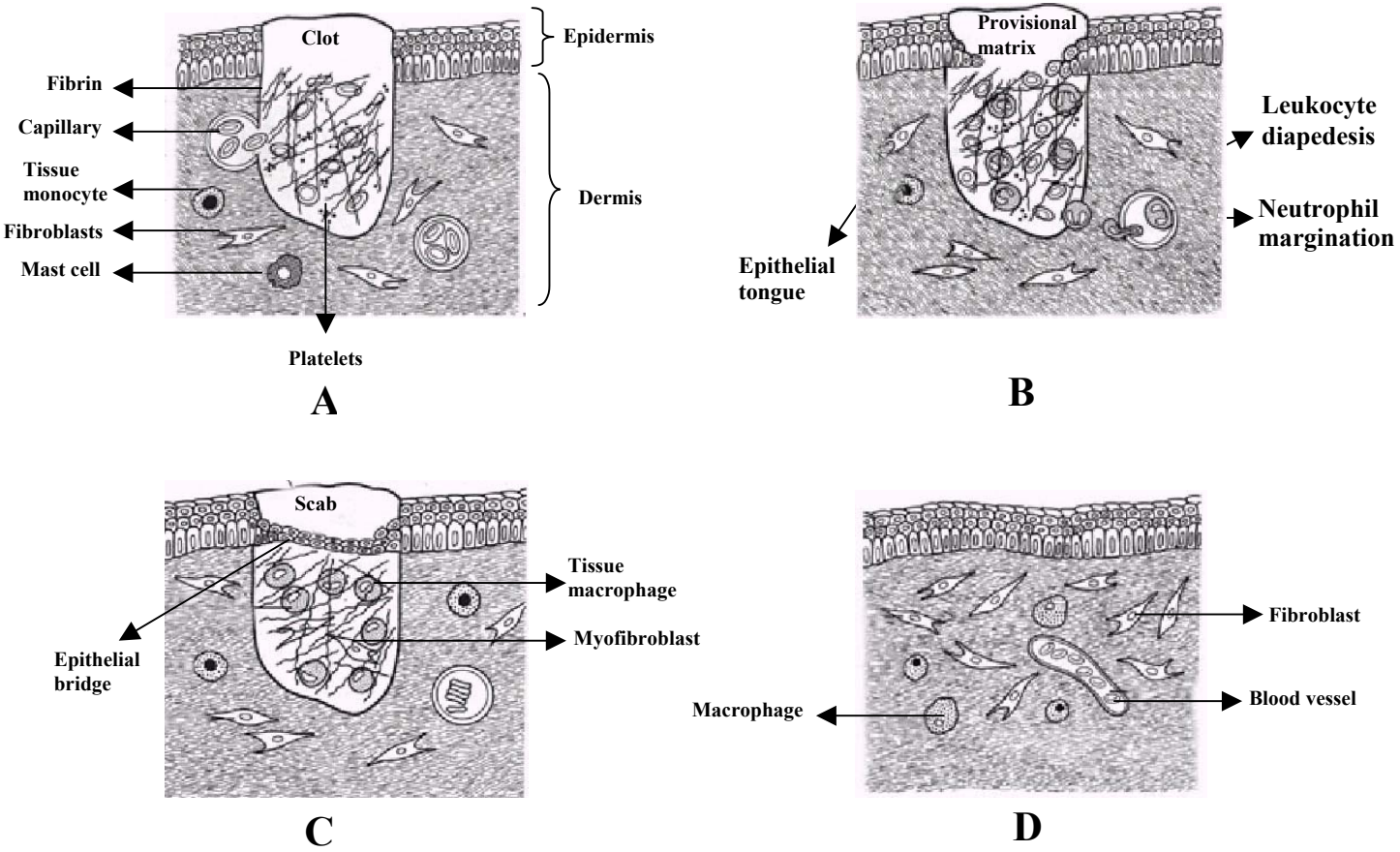


Fig. 1.2: Phases of wound healing. A) Clot formation – Platelets, fibrin and dead cells form the clot. B) Inflammation – Neutrophils migrate into the clot from the surrounding areas initiating inflammatory response C) Re-epithelialisation – Keratinocytes migrate into the provisional matrix differentiating the outer scab and the inner neomatrix. D) Matrix remodeling – The collagen deposited undergoes remodeling to give the tissue its nascent strength

1.4 Factors regulating wound healing

Factors that regulate wound healing are many and are inter-related. Cells either secrete growth factors, enzymes or extracellular matrix components which coordinate wound healing. The cells and their secreted products in turn affect the functioning of other cells through several signaling pathways, thus mutually regulating each other's functions. The factors that regulate wound healing are described as follows.

1.4.1 Growth factors

Growth factors are polypeptides that are secreted by various cells in response to particular stimuli. These factors present in serum and platelet extracts are considered to play important roles in wound healing. The effects of growth factors are clearly studied in each phase of wound healing as described below. The growth factors released from the cells during wound healing and their primary targets are listed in Table 1.2.

1.4.1.1 Coagulation and Inflammation

Platelet derived growth factor (PDGF)

In the early phase of wound healing platelets release platelet derived growth factor. PDGF is a dimeric glycoprotein with a molecular weight of 30,000, composed of A and B chains. It exists as both a heterodimer and a homodimer. The major platelet and macrophage isoforms (AB and BB) of PDGF were found to stimulate fibroblasts to contract collagen matrix, while the major fibroblast isoform (AA) had no activity

(Clark, 1983). It is undetectable in normal human plasma and has a very short half-life in vivo (Ross R, 1986; Pierce, 1994). Platelets, macrophages, ECs and vascular SMCs have been shown to secrete PDGF. It is a chemoattractant for neutrophils, monocytes, endothelial cells, fibroblasts, smooth muscle cells, and macrophages (Bowen-Pope, 1984). PDGF-BB is now available as an adjunct to wound healing. The activity of PDGF is thus thought to result from local (i.e., paracrine) effects rather than circulating effects. PDGF has mitogenic and chemotactic activity, on the target cells. Many cells express receptors for PDGF, including some microvascular ECs, dermal fibroblasts, and vascular SMCs (Fig. 1.3). Perhaps tissue macrophages release PDGF-BB or -AB approximately 1 week after cutaneous injury, a time when myofibroblasts have filled the wound and are linked to each other and to the extracellular matrix. This then may be the signal for wound contraction to commence (Lepisto, 1995).

Basic fibroblast growth factor (bFGF)

Macrophages, smooth muscle cells, vascular ECs, fibroblasts, and some malignant tumor cells have all been shown to contain bFGF (Nguyen, 1993). It is a cell-associated protein that has been localized to the cytoplasm and extracellular matrix. It does not have a secretion signal peptide, and its mechanism of release has not been clearly elucidated. bFGF can be released from heparan sulfate binding sites on the cell surface and in the matrix, or by proteinase degradation of the extracellular matrix (Baird, 1986). Basic fibroblast growth factor has been shown to be angiogenic in vitro in a number of studies. Although the mechanism of bFGF release remains unknown, a unifying hypothesis in all of these cases is that bFGF may be

either an effect of, or a reaction to, injury and thus aid in the healing process. bFGF has therefore earned the nickname of a "wound hormone."

1.4.1.2 Cell proliferation and matrix deposition

Almost all the growth factors so far studied have shown to influence this phase of wound healing. Epidermal growth factor (EGF), PDGF and FGF all together influence re-epithelialisation. Keratinocyte growth factor (KGF), insulin like growth factor (IGF) and transforming growth factor (TGF) all regulate epidermal growth. Majority of the growth factors include the EGF family, especially TGF-α (Barrandon and Green, 1987), heparin-binding epidermal growth factor (HB-EGF) (Higashiyama, 1992) and the FGF family (Werner, 1998). Growth factors may be derived from macrophages or dermal parenchymal cells and act on epidermal cells through a paracrine pathway (Baird, 1986), TGF-α, and perhaps other growth factors, originate from keratinocytes themselves and act directly on the producer cell or adjacent epidermal cells in an autocrine or juxtacrine fashion (Dlugosz A. A, 1994).

Vascular endothelial growth factor (VEGF)

VEGF is the key factor that has been studied in angiogenesis of the neomatrix. VEGF is a 34 - 42kd homodimeric glycoprotein. The primary target of VEGF is a vascular endothelium that possesses two high-affinity tyrosine kinase receptors called Fit-1 and Flk-1 (Millauer, 1994). Early studies of the expression patterns of VEGF in tumors revealed elevated expression in cells bordering necrotic areas of tumors. These observations, in turn, lead to the suggestion that VEGF might be

regulated by low local oxygen tensions, that is, hypoxia (Shima, 1995). A number of subsequent studies have shown this to be true and have begun to investigate the molecular basis of the hypoxic regulation of VEGF. The ability of VEGF to be regulated by hypoxia and the knowledge that the wound is a particularly low oxygen environment suggest a role for VEGF in wound healing. In addition to its angiogenic capacity, VEGF has been shown to alter local protease production including plasminogen activator and interstitial collagenase (Pepper, 1991;Unemori 1992). In addition, VEGF has been shown to induce monocyte migration and activation events critical to the successful wound healing response (Clauss, 1990).

1.4.1.3 Matrix Remodeling

Transforming growth factor (TGF)

The effects of TGF-β on extracellular matrix are more complex and more profound than those of any other growth factor and are central to its effects on increasing the maturation and strength of wounds, (Roberts, 1992; Sporn, 1992; Border, 1994). In a dermal site, the target cell is the wound fibroblast, which is first stimulated to migrate chemotactically in response to very low concentrations of TGF-β at the periphery of the wounded area. This is activated transcriptionally by higher concentrations of TGF-β within the wound site. It also regulates the transcription of a wide spectrum of matrix proteins including collagen, fibronectin, and glycosaminoglycans. Evidence suggests that effects of TGF-β on matrix may be mediated through a receptor complex or signaling pathway. They also suggest that pathological accumulation of matrix may result not only from excessive production of TGF β1, but also from defects in the signaling pathways emanating from matrix

that would ordinarily function to terminate or suppress expression of TGF β. One way to assess healing is to measure the strength of an incisional wound. TGF-β increases the breaking strength in incisional wounds in rats (Thomas A. Mustoe, 1987). The effect of TGF β on collagen synthesis is transient and parallels the increase in tensile strength. Subsequent collagen cross-linking, on the other hand, is not a major determinant of TGF β dependent improvement in repair (Pierce G. F, 1991). Individual growth factors may regulate different aspects of repair. Specifically, with respect to their effects on collagen synthesis, it was shown that ulcer wounds treated with TGF β, appeared to bypass the inflammatory phase of wound repair (Pierce G. F, 1994). In contrast, PDGF-BB stimulated wound closure by augmenting deposition of provisional matrix composed of glycosaminoglycans and fibronectin at the edge of new granulation tissue and did not stimulate new collagen synthesis until rather late in the repair process. Wounds treated with bFGF contained little to no collagen even after complete closure (Pierce G. F, 1991). These results are consistent with the action of these growth factors on fibroblasts in vitro.

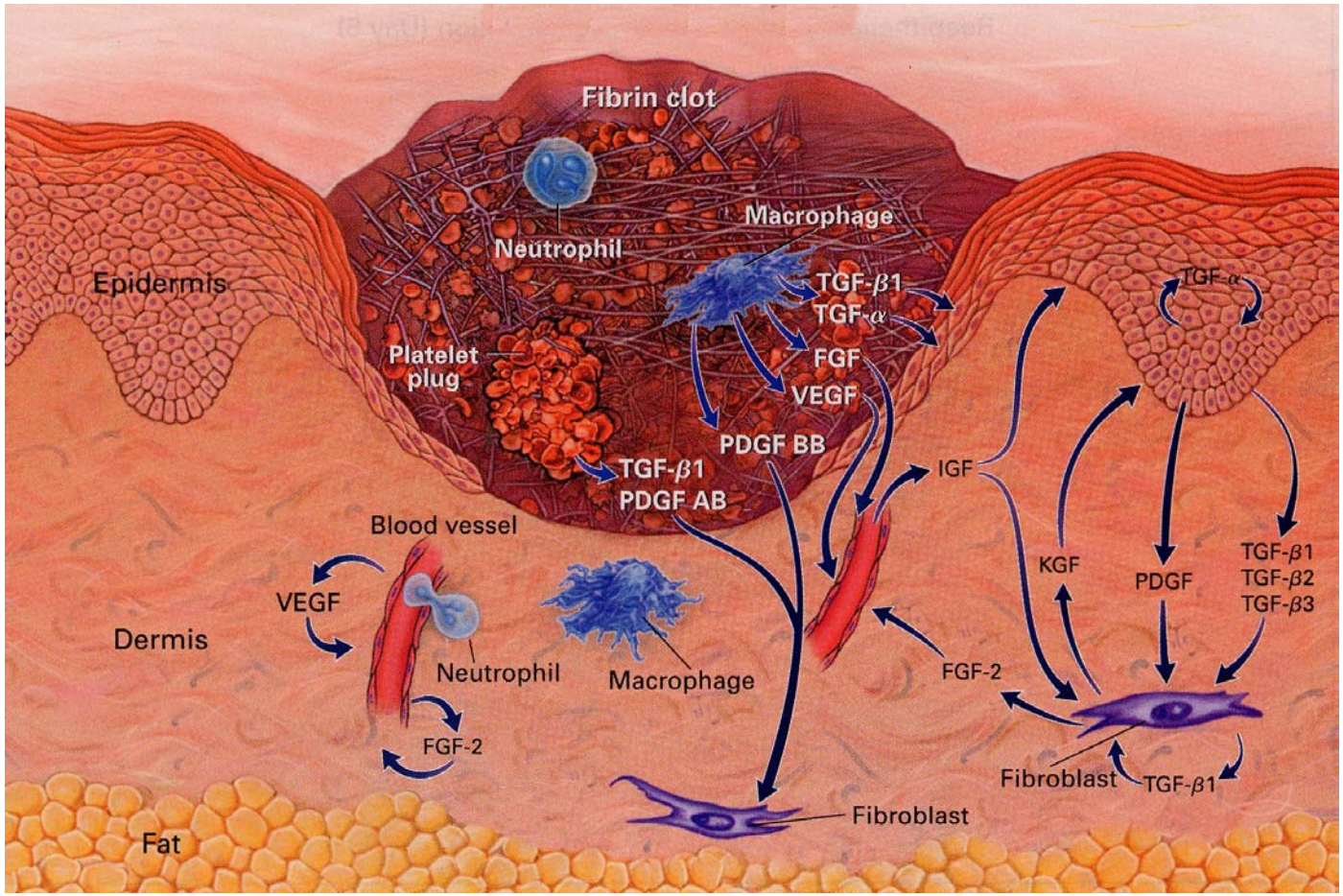


Fig. 1.3: Growth factors regulating wound healing. TGF-β1, β2 and β3 are secreted from keratinocytes and macrophages. KGF and FGF are secreted by fibroblasts, VEGF by endothelial cells and PDGF by macrophages and fibroblasts. The sources of growth factors and their sites of action are clearly depicted in this diagram (Adam J. Singer, 1999).

Growth factor	Source	Primary target cells and effect
EGF	Platelets	Keratinocyte motogen and mitogen
TGF-α	Macrophages; keratinocytes	Keratinocyte motogen and mitogen
HB-EGF	Macrophages	Keratinocyte and fibroblast mitogen
FGFs 1, 2, and 4	Macrophages and damaged endothelial cells	Angiogenic and fibroblast mitogen
FGF7 (KGF)	Dermal fibroblasts	Keratinocyte motogen and mitogen
PDGF	Platelets; macrophages; keratinocytes	Chemotactic for macrophages, fibroblasts; macrophage activation, fibroblast mitogen, and matrix production
IGF-1	Plasma; platelets	Endothelial cell and fibroblast mitogen
VEGF	Keratinocytes; macrophages	Angiogenesis
TGF-β1 and -β2	Platelets; macrophages	Keratinocyte migration; chemotactic for macrophages and fibroblasts; fibroblast matrix synthesis and remodeling
TGF-β3	Macrophages	Antiscarring
IL-1α and -β	Neutrophils	Early activators of growth factor expression in macrophages, keratinocytes, and fibroblasts
TNF-α	Neutrophils	Similar to the IL-1s

Table 1.2: Growth factors regulating wound healing. The cellular origin and the target cell of action of the growth factors in wound healing are shown in this table.

1.4.2 Collagen

Collagens are a family of glycoproteins containing triple helices, and are main components of extracellular matrix. At present, there are 18 collagen types designated type I-XVIII according to their chronological order of discovery (Fukai, 1994). Type I collagen is the major structural component of skin, tendon, bone, and many minor structures. Type III collagen is present in skin in association with type I. Type VI collagen forms distinctive 100-nm periodic microfibrils intercalated between the types I and III collagen of the dermis (Bruns, 1986). Type VII collagen forms the anchoring fibrils of epidermal basement membranes (Sakai, 1986). Most studies on the collagen content of healing wounds have examined types I and III collagens. The function of collagen in the different phases of wound healing and regulation of collagen synthesis are described below.

1.4.2.1 Coagulation and Inflammation

The inflammatory phase of dermal wound repair is initiated almost immediately following tissue injury by either activation of tissue complement or by activation of the coagulation cascade. Activation of both of these pathways leads to the recruitment of inflammatory cells into the area via the generation of C5a or the release of platelet factor, fibrin-degradation fragments. These molecules are chemotactic agents in vitro for specific cell types and may play a critical role in the early events of wound healing. Fibrillar collagens (types I and III) play a pivotal role in the initial stages of wound healing since they are believed to be a key element involved in the promotion of platelet aggregation following vascular injury (Shoshan, 1981). The binding of platelets to fibrillar collagen in the surrounding

connective tissue results in the release of several large glycoproteins such as fibronectin and thrombospondin. This collagen-induced aggregation of platelets, along with other events in the coagulation cascade, results in the formation of a physical plug that provides hemostasis following vascular injury. Platelet aggregation also results in the release of the factors, such as PDGF, fibronectin which are chemotactic to inflammatory cells, fibroblasts and smooth muscle cells (Gauss-Muller, 1980;Grotendorst, 1985;Seppa 1982). Thus, fibrillar collagen plays a crucial role in the early stages of wound healing by such as hemostasis and recruitment of connective tissue cells.

1.4.2.2 Cell proliferation and matrix deposition

Matrix deposition occurs in an ordered sequence of fibronectin, type III collagen, and type I collagen (Kurkinen, 1980). Type IV collagen, together with other components, including a unique heparan sulfate proteoglycan and the glycoprotein laminin, makes up both the epidermal and endothelial basement membranes. Type VII collagen forms anchoring fibrils that attach the basement membrane to underlying connective tissue (Bentz, 1983). Type V collagen in fibrillar form, may play a similar role. The migration of fibroblasts into the wounded area and rapid vascularization signal the initiation of granulation phase in the wound-healing. This phase is generally considered to begin 3-5 days after wounding and persist for 10-12 days, during which time there is rapid synthesis of type I and III collagen and an associated increase in the tensile strength of the wound (Heughan, 1975). In rat dermal wounds, significant collagen synthesis begins within 24 hrs of wounding and is localized in the area of the panniculus carnosus, the muscle layer underlying the

dermis (Diegelmann, 1975). The collagenous matrix of early granulation tissue synthesized by fibroblasts in the wound, in conjunction with non-collagenous proteins such as fibronectin and fibrin, provides support for epidermal cell migration and proliferation (Clark 1982;Woodley, 1982). Exposure of a granulating wound to air results in the formation of a scab (eschar), which is composed primarily of dead cells, and dehydrated serum, which attaches itself to the underlying granulation tissue. Epithelial cells burrow between the eschar and granulation tissue by expressing collagenase and other hydrolases dissolving the collagenous matrix in front of them as they move (Harris, 1974). Once wound closure is affected, the scab is sloughed off. During wound closure, the provisional matrix provided by the early granulation tissue and serum-derived components is quite different from the basement membrane. This results in quite different cellular behavior characterized by lateral cell movement onto the wound bed and cell proliferation rather than the vertical movement and terminal differentiation characteristic of epithelial cells, when they rest on an intact basement membrane. Once cell closure of the epidermis is complete, a basement membrane is rapidly synthesized. This signals the end of rapid fibroplasia and the beginning of remodeling and maturation of the wound. During the first 3-4 days of wound healing, increased amount of type III collagen deposition is seen. Subsequently, a rapid increase in the amount of type I collagen was noted (Gay, 1978). The relative amounts of other collagen types that participate in the wound healing process are low compared with type I and III collagen. Particularly important in the process of dermal wound healing is type IV collagen synthesis by epidermal cells and the

regeneration of the basement membrane. The synthesis of type V collagen by epidermal cells during this migratory phase has also been reported (Stenn, 1979). The production of type IV collagen and regeneration of the basement membrane, including associated glycoproteins such as laminin, is delayed until the wound is covered and the epidermal cells are no longer in a migratory phase.

1.4.2.3 Matrix Remodeling

Remodeling and cross-linking of the collagen follows fibroplasia, resulting in generation of fibrillar collagen bundles or fibers. These fibers become oriented according to lines of stress and provide a slow increase in the tensile strength of the healing wound (Heughan, 1975). This remodeling phase in the human ultimately results in the formation of a fibrous connective tissue commonly called a scar. The role of type I and III collagen in the process of contraction remains to be completely elucidated. These matrices provide anchoring points and connecting cables to which myofibroblasts bind and attempt to reduce the wound volume through an active contraction process (Gabbiani, 1972). Wound contraction is a relatively early event in the healing process, which as stated above, involves an interaction between myofibroblasts and the extracellular matrix.

1.4.2.4 Regulation of collagen production

The factors regulating collagen production during wound healing can be divided into those that participate in the early, intermediate, and later stages of the process. In the early inflammatory stage of wound repair, platelet factors released during clotting may play a key role in regulating collagen synthesis. One such factor is

PDGF (Knighton, 1982). This factor could promote collagen synthesis by recruiting connective tissue cells into the wound via its chemotactic activity (Seppa, 1982) and causing them to proliferate via its mitogenic effect (Rutherford and Ross, 1976). Another platelet factor, TGF-β, has been reported to enhance the rate of connective tissue deposition (Sporn 1982). Large-scale proliferative response of fibroblasts and associated deposition of connective tissue in the wound bed does not begin until the acute inflammatory phase begins to subside at 3-5 days (Clore J. N, 1979). The released growth factors promote collagen synthesis by increasing the number of connective tissue cells in the wound site. Several other lines of evidence implicate macrophage in the control of collagen deposition in wounds. Thus, it now appears that macrophages play an important role in fibroplasia and associated collagen deposition during the intermediate stages of wound healing by secreting a host of factors that enhance fibroblast proliferation. They promote these cells to synthesize and secrete extracellular matrix proteins. In addition to the requirements for chemoattractants and mitogens to promote new collagen synthesis, some investigators have hypothesized that reduced oxygen tension in the wound, due to the high metabolic activity of cells in the wound bed, also plays an important role in the wound healing process. These investigators suggest that reduced oxygen tension results in local hypoxia and lactate production, which in turn promotes new collagen synthesis (Levene and Bates, 1976). On the contrary, re-epithelialization of the wound is negatively affected by reduced oxygen and positively influenced by increased oxygen levels (Winter, 1978). Thus, while there is circumstantial evidence that oxygen tension may play a role in the regulation of new collagen synthesis in

wounds, the mechanisms by which this effect may be regulated remains to be determined. Another regulatory event of collagen production in the wound healing, particularly at the later stages of the process, is the catabolism of newly synthesized collagen. The rate of collagen degradation changes as wound healing proceeds, starting out at a relatively slow rate during the early stages of the process and increasing as wound maturation occurs (Zeitz, 1978).

1.4.3 Enzymes

Wound healing is a dynamic reparative process involving debridement, inflammation and matrix deposition. All these metabolic processes require efficient expression and activity of enzymes. These include matrix metalloproteinases (MMPs) and enzymes involved in the free radical metabolism. The following section provides a detailed description of these enzymes and their role in wound healing.

1.4.3.1 Matrix-metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases collectively capable of degrading essentially all components of extracellular matrix. The members of this family are divided into four groups namely collagenases, gelatinases, stromelysins and membrane type MMPs (MT MMP) according to their substrate type specificity and primary structure. Controlled breakdown of ECM by MMPs plays an important role in detachment and migration of cells as well as in tissue remodeling. MMPs play an important pathogenic role in excessive breakdown of connective tissue components e.g., arthritis, ulcers, dermal photoageing, periodontitis, tumor cell invasion etc. In skin, several different types of cells are

capable of producing MMPs: keratinocytes, fibroblasts, macrophages, endothelial cells, mast cells, eosinophils and neutrophils. These enzymes are primary mediators of collagen turnover and are secreted as zymogens that can be activated by certain other proteases such as plasmin (Werb, 1989). This activation of the collagenase, as well as inhibition of its enzymatic activity by protease inhibitors such as α-- macroglobulin, have been proposed to play critical roles in the regulation of expression of collagenolytic activity in vivo (Werb, 1989; Diegelmann, 1990). The activities of these enzymes are controlled by various inhibitor counterparts called tissue inhibitor of metalloproteinases (TIMP), which play an important role during development (Overall, 1991) and wound repair. Cytokines such as TGF β, PDGF, and IL-1 and the extracellular matrix itself may play an important role in the modulation of collagenase and TIMP expression in vivo (Werb, 1989; Circolo, 1991; Sporn and Roberts, 1992). With certain exceptions the MMPs are not constitutively expressed in the skin but are induced temporarily in response to exogenous signals such as the cytokines or growth factors, cell- matrix interactions etc. The primary MMP's involved in wound healing are described in the following section.

1.4.3.1.1 72-kDa gelatinase (MMP2)

Gelatinase A is unique in that it is constitutively expressed by many cells, has a ubiquitous tissue distribution and has a cell surface mode of activation that differs from the other MMPs. Structurally this enzyme is similar to the rest of the MMPs. In addition to the shared domains, both gelatinases (A and B) have an additional fibronectin-like gelatin binding domain that provides substrate specificity of these enzymes (Cao, 1995).

1.4.3.1.1.1 Regulation of Gelatinase A activity

The activity of Gelatinase A is controlled by many regulatory mechanisms. They are as described as follows

a) Transcriptional regulation of Gelatinase A expression

Gelatinase A is constitutively expressed in many cells. The gelatinase promoter is characterized by the absence of AP-1, PEA-3 which is present in the other protease promoter regions. This promoter site has a unique TATA box as well as an enhancer element at –223 to –422 nucleotides. The distinctive features of this enzyme promoter include SP-1 sequence at 120bp from the start site and adenovirus E1A repressor element. As Gelatinase A is constitutively expressed and not well regulated it has led to the understanding that it acts as a housekeeping enzyme.

b) Post-transcriptional regulation of Gelatinase A expression

TGF β-1 supresses the overall MMP activity but enhances the levels of Gelatinase A secreted by human fibroblasts and rat osteoblasts (Overall, 1991). It is shown that TGF β increases the stability of the gelatinase mRNA.

c) Regulation of the activity of Gelatinase A by TIMP's

Progelatinase A is usually found complexed with TIMP-2. It binds to the C-terminal domain of progelatinase-A and regulates its activity. Recent studies show that TIMP-2 may mediate the cell surface activation of progelatinase A by binding to a MT1-MMP containing complex on the cell surface. Thus TIMP-2 facilitates the cell-

surface mediated activation of the progelatinase-A and also the inhibition of the active enzyme.

d) Regulation of activation of Gelatinase A proenzyme

In-vitro activation by APMA and chaotropic agents: The MMPs are latent because their unpaired cysteine residue in the pro-domain and the Zn atom at the active site are well co-ordinated. Upon the dissociation of the cys-Zn interaction, progelatinase-A undergoes an autolytic cleavage. This results in a removal a 8-kDa peptide by hydrolysis and activation of the proenzyme.

e) Regulation of Gelatinase A activity by MT1-MMP and $\alpha 5\beta 3$

MT-MMP acts as a receptor for TIMP-2. The MT-MMP-TIMP complex can bind to progelatinase-A and form a trimeric complex. Formation of this complex leads to the activation of the progelatinase A. Binding, localization and activation of the progelatinase A on the cell surface depends on the cooperative interaction of all the proteins. Interestingly in some tumor cell lines it has been shown that $\alpha 5\beta 3$ enhances the surface binding of the progelatinase A. It has also been shown that thrombin and urokinase facilitate the cell surface activation of progelatinase A. This suggests that multiple cellular control mechanisms may be evoked to control the activation of progelatinase A.

1.4.3.1.1.2 Functions of Gelatinase A in cellular processes

a) Regulation of Cell proliferation and differentiation

In a mesangial cell model Turck et al (1996) have demonstrated that the expression of Gelatinase A coincides with the phenotypic transformation of the cells into those of the inflammatory stage. The inhibition of transcription of the Progelatinase A protein results in the failure of the cells to change into those of the inflammatory phase. Gelatinase A also cleaves fibronectin which is one of the key extracellular matrix components along with collagen (Stetler-stevenson, 1989).

b) Modulation of cell adhesion and migration

Recent studies in melanoma and breast cancer cells have shown that gelatinase A decreases the adhesion of the cells to their substrates and increases their migration. Studies' involving the cleavage of the Ln-5γ2 subunit of Laminin-1 by gelatinase A exposes the putative cryptic pro-migratory site that triggers cell motility but not cell adhesion (Calof, 1994). During cell migration as in tumor cell invasion, the degraded protein products serve as stimulus for cell movement. While it is established that proteolysis is required for tumor invasion, certain studies suggest that excessive proteolysis may inhibit cell-matrix interactions and matrix signals that are required for migration and invasion.

1.4.3.1.2 92-kDa gelatinase (MMP9)

This enzyme is homologous to the gelatinase A and degrades the type IV collagen and gelatins. The protein consists of several structural domains. Both the gelatinases have an additional fibronectin like domain. The protein is maintained in the inactive

state by a cysteine switch mechanism. When the interaction between the zinc and cysteine is disrupted the enzyme is activated. The activation depends on the state of the enzyme as it is mostly linked with TIMP-1. Gelatinase activation is also brought about by cathepsin G, trypsin, α chymotrypsin and stromelysin. The activation of the progelatinase-B TIMP-1 complex by APMA or trypsin results in poorly active Gelatinase B whereas activation by other MMPs results in a higher activity (Montogomery, 1993). Gelatinase B is widely thought of as a type IV collagenase as it cleaves native type IV collagen molecules. It also cleaves aggrecan, a cartilage proteoglycan. Other non-ECM substrates of Gelatinase B are Myelin basic protein, galactoside-binding proteins etc.

1.4.3.1.2.1 Regulation of Gelatinase B activity

a) Regulation by growth factors and cytokines

Most of the growth factors have been found to increase the activity of Gelatinase B, for e.g., TGF-β, EGF, bFGF, interleukins, TNF-α and IFN-γ. A number of proteins such as the granulocyte chemotactic protein and leukocyte inhibitory protein also induce the expression of this enzyme (Mertz, 1994).

b) Regulation by Cell-Cell and Cell-matrix interaction

Gelatinase expression is also controlled by cell adhesion molecules, ECM and agents that change the shape of cells. Laminin peptide SIKVIV induces gelatinase expression in human monocytes. Cell-cell contact also up-regulates gelatinase B activity (Huhtala, 1995). Certain co-culture experiments have proven that the cell-cell contact is important for the expression of gelatinase B.

1.4.3.1.2.2 Functions of Gelatinase B in cellular processes

a) Tissue injury, inflammation and wound healing

Most of the inflammatory cells such as the neutrophils, lymphocytes, eosinophils, mast cells and macrophages express gelatinase B (Leppert, 1995). The degradation of the subendothelial basement membrane during the process of inflammation is brought about by gelatinase B. Increased MMP-9 is found in infarcted heart, acute respiratory distress syndrome and burns. MMP-9 appears during the early phases of burns, whereas MMP-2 is expressed at a later stage along with stromelysin (Young, 1994). Gelatinase B is expressed in the early inflammatory phase of tissue repair whereas Gelatinase A appears at a later stage.

b) Implantation

The embryonic trophoblast invades the uterine epithelium by the secretion of gelatinase B. It is also expressed in the perital endoderm that may play an important role in the expansion of Reichert's membrane (Behrendtsen, 1992).

c) Bone development

The degradation of cartilage and the resorption of bone are carried out by osteoclasts (Okada, 1995). These cells express high levels of gelatinase B during the bone remodeling and initial migration from perichondrium into the cartilaginous bone model before the initiation of ossification process.

1.4.3.1.3 Gelatinases as applied to wound healing

MMP 2 and 9 are thought to play a key role in the final degradation of fibrillar collagens after initial cleavage by collagenases. MMP 2 cleaves native type I collagen to N-terminal ¾ and C-terminal ¼ fragments identical to those generated by collagenases. In addition, MMP 9 has been shown to cleave type I, II and V collagens in the N-terminal non-helical telopeptide. Therefore it is possible that MMP 2 and MMP 9 play a more important role in the remodeling of collagenous ECM than has been previously thought. In a murine model the MMPs have been localized to the basal cells of the hyperproliferative epithelium (Liotta, 1979). The Gelatinase B is absent in the non-wounded epidermis. Gelatinase A is released later during the repair i.e. in the granulation tissue formation. Maximum activity of 72 kDa Gelatinase is seen between day 5-7, post- wounding (Akiko, 1997). Thereafter the activity decreases and by day 13 it reaches that of the normal skin. MT1-MMP is co-localized with 72kDa Gelatinase. It is a physiological activator of the 72kDa Gelatinase. This suggests that there is a high level of gelatinolytic activity in the provisional matrix. TIMP-1 which binds to most activated MMPs is expressed with similar kinetics as collagenase, 92kDa Gelatinase and stromelysins. TIMP 2 that is secreted as a complex with 72kDa progelatinase shows a constant level during entire time period. The various MMP's released and their actions are briefly summarized in Fig. 1.4.

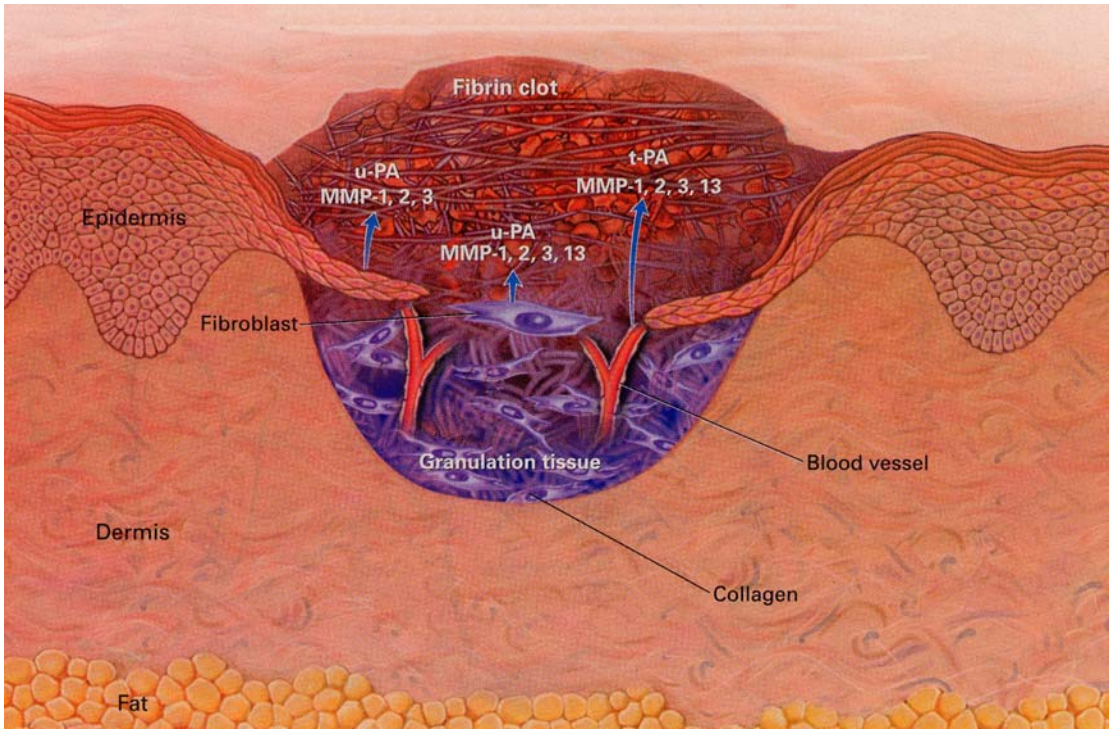


Fig. 1.4: Enzymes acting during re-epithelialization. Matrix Proteases such as matrix metalloproteinase (MMP), urokinase type plasminogen activator (u-PA) and PA are secreted into the fibrin clot. This promotes re-epithelialization and fibroblast migration into the matrix (Adam J. Singer, 1999) (Permitted by Massachusetts Medical Society – Appendix B).

1.4.3.2 Enzymes in free radical metabolism

Cells possess various mechanisms to protect themselves from the free radical-mediated damage defined as scavengers. And among these, some enzymes have the role of anti-oxidants. A brief summary of the role of these enzymes in the free radical metabolism is given in Table 1.3.

Enzymes	Function
Glutathione peroxidase	Reduction of hydrogen peroxide $H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$
Glutathione-S-Transferase	Reduction of fatty acid hydroperoxide
Peroxidase	Reduction of fatty acid hydroperoxide and hydrogen peroxide $H_2O_2 + AH2 \rightarrow 2H_2O + A$
Catalase	Reduction of hydrogen peroxide $2H_2O_2 \rightarrow 2H_2O + O_2$

Table 1.3: Decomposition of hydro peroxides and hydrogen peroxides by enzymes. The enzymes that catalyze the peroxidation reactions are shown in this table.

1.4.4 Free radicals

Free radicals are chemical species, which contain one or more unpaired electrons and are capable of independent existence. In biological systems, these reactive radicals tend to combine with cellular and extracellular components. Lipids, proteins, nucleic acids, sugars and metabolic intermediates are the targets of these radicals. The consequences are either in the form of chromosomal aberrations, cytotoxicity, carcinogenesis, cellular degeneration and apoptosis.

Glutathione, a free radical plays a unique role in the cellular defense against active oxygen species and reactive intermediates. GSH functions both as a reductant in the metabolism of hydrogen peroxide and organic hydroperoxides and as a nucleophile, which can conjugate electrophilic molecules. During glutathione peroxidase-catalysed metabolism of hydroperoxides, GSH serves as an electron donor, and the glutathione disulfide (GSSG) formed in the reaction is subsequently reduced back to GSH by glutathione reductase, at the expense of NADPH. Under conditions of oxidative stress, when the cell must cope with large amounts of H_2O_2 or organic hydroperoxides, the rate of glutathione oxidation exceeds the slower rate of GSSG reduction by glutathione reductase, and GSSG accumulates. To avoid the detrimental effects of increased intracellular levels of GSSG (e.g., formation of mixed disulfides with protein thiols), the cell excretes GSSG, which can lead to depletion of the intracellular glutathione pool (Fig. 1.5).

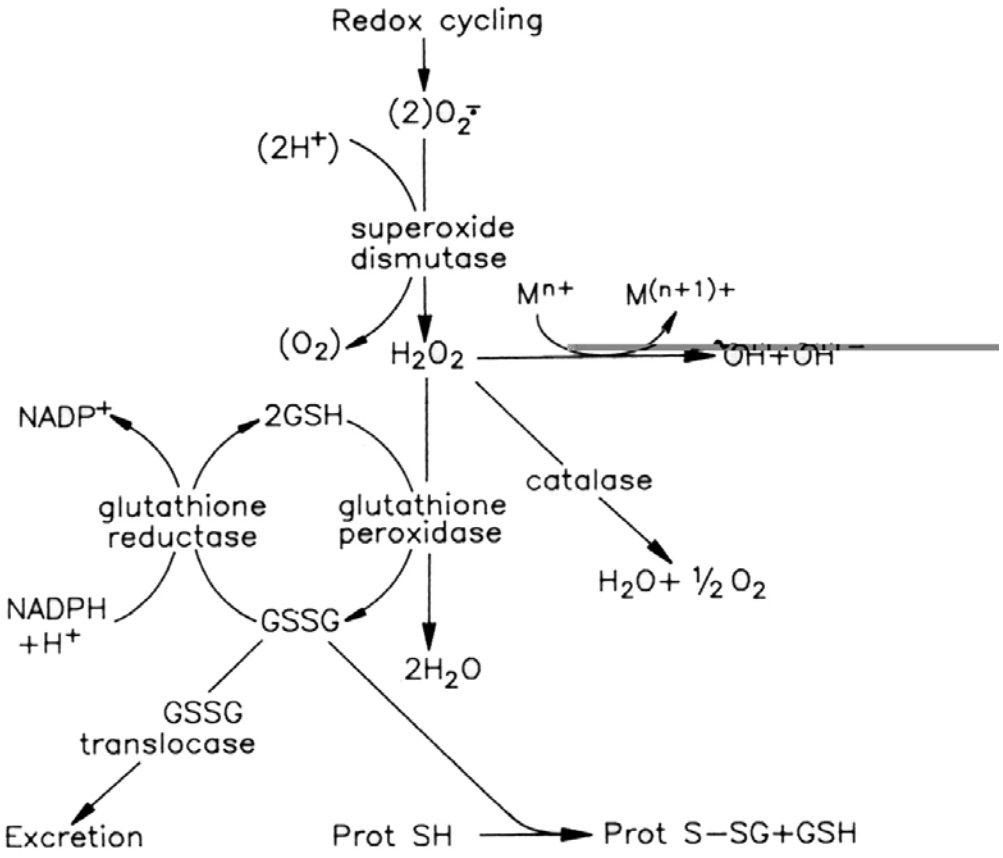


Fig. 1.5: Formation and metabolism of active oxygen species. Metal ions (M^n) and the enzymes catalyzing the metabolism of oxygen species are shown in this figure. Protein thiol formation is also represented (prot-SH).

1.4.4.1 Free radicals in skin

Wound healing involves the initiation of events, which involve the release of reactive oxygen species (ROS) as part of their defense mechanism. In the inflammatory phase of wound healing numerous PMN and macrophages invade the injured area (Trenam, 1992). Once activated, they produce large quantities of reactive oxygen species (ROS) as part of the defense mechanism (Darr D, 1994). Although this process is beneficial, increased levels of ROS can inhibit cell migration and proliferation and can even cause severe tissue damage. Therefore cells adopt strategies to detoxify these molecules. The PMN and macrophages destroy the contaminating bacteria and debride the dead tissue by phagocytosis and the release of ROS (Kanegasaki S, 1992). If the wound contamination is limited, the neutrophils cease to infiltrate and the reparative phase begins. The production of ROS in the inflammatory response is termed respiratory burst (Clark R. A. F, 1990). Neutrophils and tissue macrophages produce ROS by cytochrome-b dependent oxidase to reduce molecular oxygen to superoxide anions. Simultaneously the other cells, such as the fibroblasts are stimulated to release ROS by the interleukin-1 and tumor necrosis factor α (Epstein J. H, 1977). Excess of free radicals are adverse for the normal functioning of cells. Therefore it is important for the cells to detoxify the excess ROS. The main mechanisms through which the cell can detoxify the ROS are 1) small antioxidant molecules like ascorbate, poly-unsaturated fatty acids or sugars and 2) enzymes such as superoxide dismutase, catalases and various peroxidases (Schroder, 1995). They have the tendency to react more or less rapidly with other free radicals or non-radicals by donating or

accepting an electron and therefore the process proceeds as a chain reaction. When generated these free radicals tend to combine with several cellular and extracellular components. Unsaturated lipids, proteins, nucleic acids, sugars, metabolic intermediates etc., react with free radicals. The biological consequences of these reactions include mutations, chromosomal aberrations, cytotoxicity, carcinogenesis, cellular degeneration and death. Oxygen free radicals are known to be the mediators of tissue damage during catabolic states (Griffits C. E, 1991). The body utilizes antioxidant substances made endogenously or provided in the diet as free radical scavengers, to attenuate oxidant-mediated peroxidation of lipid membranes and disruption of cell proteins in extracellular fluid and in tissues (Picardo, 1992). Several dietary nutrients, including zinc, selenium, vitamins A, C, and E, and glutamine, appear to function as antioxidants or are involved directly or indirectly in the generation of critical antioxidants such as glutathione (GSH) (Picardo, 1990). Maintenance of GSH concentrations in tissues and in the circulation, serves to inhibit lipid peroxidation of cell membranes. Thus, GSH (a glutamate-cysteine-glycine tripeptide) is a key antioxidant in the body (Picardo, 1990). The metabolism of nutrient antioxidants is interrelated. For example, tissue protection by ascorbate and glutathione against microsomal lipid peroxidation is dependent on vitamin E. Enzymes involved in GSH generation, such as glutathione peroxidase, are dependent on adequate supplies of the trace metal cofactors such as zinc and selenium. Several studies indicate beneficial effects on tissue GSH production with GSH inhibitor administration in animal models of tissue injury and inflammation. Emerging clinical trials suggest that administration of single antioxidants, such as

vitamins C and E, or antioxidant nutrient "cocktails" reduces indices of oxidant damage and tissue injury in selected groups of catabolic patients, including those with pancreatitis or burn injury and after operation (Wang Y, 1995). In addition, several studies suggest that beneficial clinical effects occur with administration of enteral diets enriched in combinations of antioxidant and "immunomodulating" nutrients, including arginine, glutamine, fish oil, selenium, zinc, and vitamin C (Bissett, 1990). Although there is as yet little evidence for interactions between growth factor hormones and antioxidant nutrient action pathways, it is likely that diminution of oxidative tissue damage by certain nutrients serves to facilitate growth factor-induced tissue growth and repair.

1.4.4.1.1 Anti-oxidant systems

a) Primary defense systems

Superoxide dismutase is an enzyme that catalyses the dismutation of O_2^- into oxygen and water.

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \rightarrow (1)$$

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \rightarrow (2)$$

Reaction (1) is catalysed by superoxide dismutase and (2) by catalase and glutathione peroxidase.

b) Secondary defense systems

One of the most secondary defense systems is α tocopherol. It quenches and reacts with almost all the radicals known. It is reduced by ascorbic acid and glutathione

and this in-turn maintains the antioxidant status. Ubiquinone is another reducing agent found in the mitochondria and cellular membranes of mammalian cells.

1.4.4.2 Free radical scavengers in wound healing

Agent	Experimental model	Wound parameter	Reference
S.O.D encapsulated Liposomes (injection & gel)	Full thickness burns (rabbit)	Scald size	Karola Vorauer (2002)
Vit C	Keratinocytes	↑ collagen	Steven T Boyce (2002)
Vit C	I.W (guinea pig)	↑ strength	Silverstein R.J (1999)
Malotilate	fibroblasts	↑migration ↑collagenase	Osamu Ohgoda (1999)
Ascorbic acid	Human wounds	↑strength	Vaxman.F (1996)
EGF + Nafomostat	Full thickness burns (rat)	↑ S.O.D	Kiyohara.Y (1993)
Taurine-Chitosan gel	I.W (mice)	↑ collagen, ↑ strength	Degim.Z (2002)
Raxofelast	I.W (diabetic mice)	↑M.P.O, ↑strength, ↑ collagen	Galeno. M (2001)
α tocopherol + FC-43 perflurocarbon	Rats-burns	↓ free radicals	Bekyavora (1996)

Table 1.4: Summary of previous studies on the administration of free radical scavengers/anti-oxidants in wound healing. SOD - superoxide dismutase, VitC – Vitamin C, VitE – Vitamin E, EGF – Epidermal Growth Factor, IW – Incisional wound.

1.4.5 Metal Ions

Metal ions are the regulating factors of the activity of the enzymes. The main ions that are of importance are Zinc ions, which regulate the activity of metalloproteinases and copper, selenium and ferric ions that regulate enzymes in the Fenton pathway.

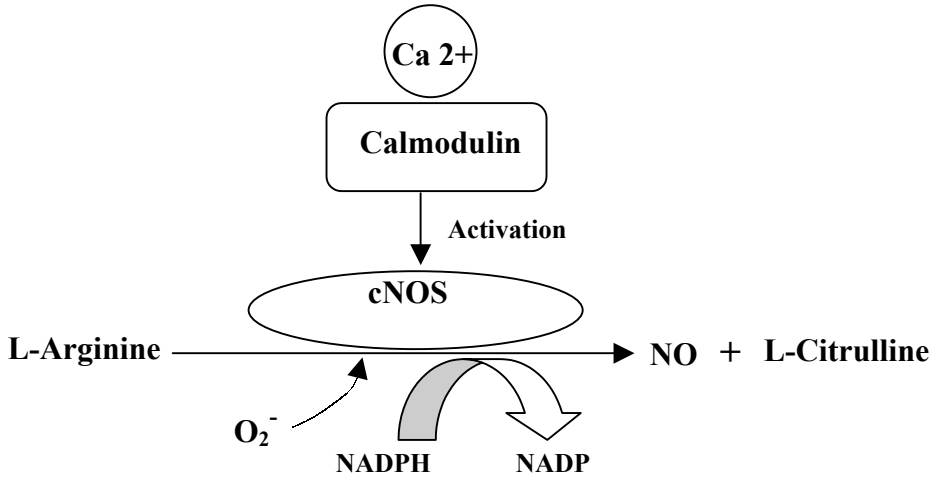
1.5 Biology of Nitric oxide

Nitric oxide was initially identified as a blood vessel relaxing factor derived from endothelia (Palmer, 1988) and has been shown to possess a variety of physiological and pathophysiological activities in living systems. In the central and peripheral nervous systems, it appears to be an inter and intra cellular messenger molecule, which functions as a neurotransmitter (Nathan C, 1992). In the vascular system, this molecule plays an important role in maintaining the tone of the vessel (Palmer, 1988). In the immune system, all mononuclear leukocytes and neutrophils have the capacity to synthesize NO upon stimulation. These stimulated cells in turn perform the cytotoxic and inflammatory functions (Nathan C, 1992). NO also serves as a signaling molecule between the immune system and other organ systems by transmitting cytokine signals to the regulatory machinery of a variety of cells (Jeanette R Hill, 1996; Schneemann M, 1993).

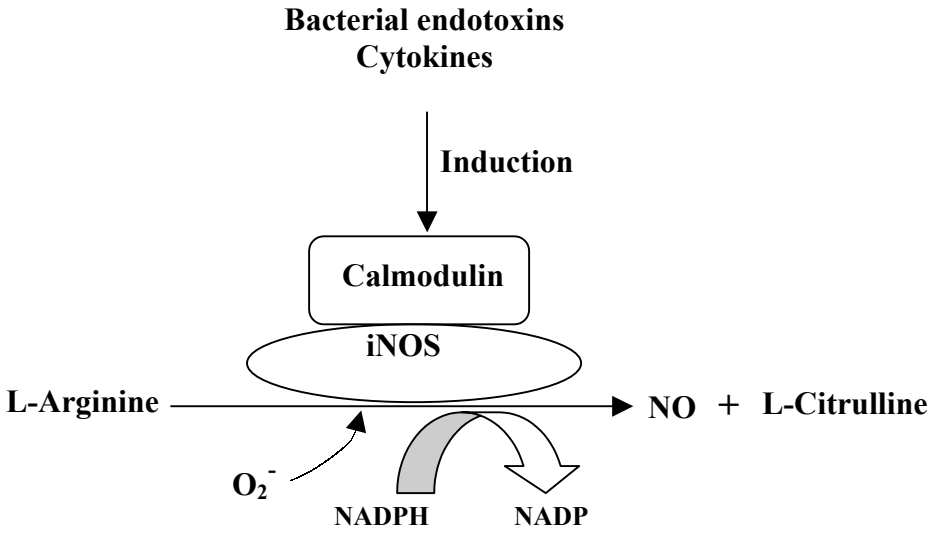
1.5.1 Nitric oxide biosynthesis: The Arginine NOS pathway

The biosynthesis of NO is mediated by Nitric Oxide Synthase (NOS), an NADPH dependent enzyme that catalyses the oxidation of L- arginine with molecular oxygen

to form NO and L-citrulline (Kwon, 1990). NOS is a complex enzyme involving several tightly bound redox co-factors (NADPH, FAD, FMN and pterin) that are clearly organized into distinct domains (Marletta, 1998). There is a structural similarity between the NOS and NADPH cytochrome P- 450 reductase since it contains a heme moiety and the C- terminal part of the protein shows sequence homology to cytochrome P-450 reductase (Bredt, 1991). Three isoforms of NOS are now distinguished by cloning of their genomic and complementary DNA (Bredt, 1991). Activities of two of these are dependent on elevated intracellular Ca^{2+} and extrinsic calmodulin. These are constitutively expressed (cNOS) (Fig 1.6). One is restricted to endothelial cells (endothelial NOS, eNOS) while another is expressed in specific neurons (neural NOS, nNOS) of the central and peripheral nervous system and in skeletal muscles (Nakane M, 1991). These two isoforms of NOS oxidize L-Arginine to form small amounts of NO (n moles) and citrulline in response to signals that elevate intracellular Ca^{2+}. The third isoform, the Inducible NOS (iNOS) is independent of the elevated intracellular Ca^{2+} (Fostermann U, 1994) (Fig 1.6). This isoform is not expressed under normal conditions, but can be induced in many types of cells by inflammatory cytokines and bacterial lipopolysaccharide (LPS). In the process of inflammation and immune responses, cytokines and bacterial products stimulate the expression of iNOS in a wide variety of cells that converts L-arginine to form high levels of NO, resulting in a diversity of beneficial and detrimental consequences (Petros, 1994). All of the NOS isoforms require NADPH and molecular oxygen as co-substrates.



Constitutive enzyme



Inducible enzyme

Fig. 1.6: Metabolism of L-Arginine by NOS isoenzymes. A) Constitutive isoenzyme (cNOS) metabolizes L-Arginine to NO and L-citrulline. Calmodulin activation by calcium is important. B) Inducible isoenzyme (iNOS) metabolizes L-Arginine to NO and L-citrulline. This enzyme is induced by bacterial endotoxins and lipopolysaccharide. NADPH is nicotinamide adenine di nucleotide phosphate in the reduced form. O_2^- is the superoxide anion.

1.5.2 Mechanism of action of NO in wound healing

The initial finding that, in cases of protein calorie malnutrition arginine could enhance wound healing sparked interest in identification of NO as a key molecule in the process of wound healing. Intravenous alimentation enabled/enhanced appropriate wound healing (Adrian Barbul, 1985). The cellular mechanisms affected by NO are the oxidation of Sulfhydryl groups, formation of complexes with amines in organic solvents and in the activity of metal containing proteins. The vital role either as a physiological messenger or as a cytotoxic agent played by NO depends on other free radicals present and it's concentration. NO reacts with molecular oxygen or superoxide to produce reactive Nitrogen Oxide species (RNOS), which mediate certain important reactions (Nathan, 1992). By the virtue of its high reactivity and simple chemical structure NO is implicated in promotion of wound healing. Enough evidences have shown biphasic effect of NO on wound healing. Increase in NO concentration increased collagen syntheses in some studies while others have shown inhibition of protein synthesis and in-turn collagen. Different cells such as macrophages, neutrophils, lymphocytes, fibroblasts and keratinocytes involved in wound healing liberate NO. This radical regulates certain key pathways in their performance as important metabolic components in wound healing.

1.5.2.1 Coagulation and Inflammation

The initial platelet rich coagulation phase is paralleled by increase in NO concentration. Platelet aggregation is inhibited following administration of NO donors. Certain other studies have indicated that NO is a primary mediator in

platelet neutrophil interactions (Aki Hirayama, 1999). Thus various opinions and observations exist on the role of NO in platelet function. PDGF mediated vascular smooth cell migration, an important event in wound healing is regulated by NO (Asha Jacob, 2002). Neutrophils when stimulated produce increasing amounts of NO (Nathan, 1992). These activated neutrophils release many cytokines such as IL-8 and TGF- β which are important mediators of wound healing. The release of these cytokines is known to be regulated by NO (Vodovotz, 1994). Certain other cytokines such as, monocyte attracting chemotactic agents released by marginal keratinocytes are known to be downregulated by NO (Christian Wetzler, 2000). NO inhibits the NFκB activation, a key initiating event in inflammatory process (Ann F. Haas, 1998). This in turn results in lowered inflammatory response. As NO plays distinct roles in various cells and in different pathways, the exact role of NO in coagulation and inflammation which is the initial event in wound healing has yet to be clearly understood.

1.5.2.2 Cell proliferation and Matrix deposition

In the second phase of wound healing the ongoing physiological responses are fibroplasia, angiogenesis, re-epithelisation and the initiation of extracellular matrix synthesis (Clark R.A.F, 1982). The predominant process during this phase is re-epithelisation and angiogenesis. The highly proliferating epithelial tongue covers the wounded area. NO represents a potent mediator for epithelial regeneration and is independent of the presence of other protein type mitogens (Heck D. E., 1992). Wounds fail to epithelialize in the event of NO inhibition inspite of the presence of optimum concentration of keratinocyte growth factor. The inhibition of

keratinocyte proliferation of UV damaged skin following administration of NOS inhibitor supports the fact that NO initiates the keratinocyte proliferation. In low concentration, NO promotes keratinocyte proliferation and in higher concentration mediates differentiation. Thus NO accelerates wound closure and offers an effective signaling pathway in re-epithelization. There has been evidence of co-expression of iNOS and GTP-CH1, which are important in melanogenesis and keratinocyte differentiation. The endothelium derived NO maintains the surface expression of certain adhesion molecules in endothelial cells that are necessary for cell migration (Wei. X. Q., 1995). Angiogenesis occurs due to a switch of the endothelial cells from their stationary phase to a replicative phase (Justus Benrath, 1995). NO regulates angiogenesis which plays a central role in the revascularisation of the scar tissue. Macrophages play an important role in angiogenesis which is regulated by NO (Leibovich, 1975). VEGF secreted by keratinocytes is a potent inducer of angiogenesis. This in-turn is under the influence of NO produced endogenously. The action of NO induction of VEGF mRNA is on the transcriptional level in the keratinocytes. The response of NO on VEGF expression is primarily on gene activation. Inhibition of inducible nitric oxide-synthase (iNOS) enzymatic activity during cutaneous wound repair leads to severely impaired tissue regeneration. In vitro studies confirmed a regulatory role of NO for keratinocyte-derived chemokine expression, as NO attenuated IL-1β and TNF-α induced MCP-1 mRNA expression, whereas NO augmented IL-1β induced IL-8 (functional human homologue to murine MIP-2) mRNA expression in the human keratinocyte cell line HaCaT. In the wound environment certain cytokines such as IL and IFN act synergistically

with NO in induction of VEGF mRNA (Stefan Frank, 1999). Recently keratinocyte growth factor is found to be a powerful mitogen for keratinocytes. It has a synergistic action in inducing VEGF mRNA in keratinocytes. The cumulative effect of exogenous KGF and NO is increased induction of VEGF mRNA (Frank S, 1999). The effect of NO on VEGF expression is dependent on the type of the cell it acts. For e.g. in the smooth muscle cells VEGF expression is decreased by NO (Pipili-Synetos E, 1994). Thus one of the key processes regulated by NO is the regulation of growth factor and cytokine-triggered processes in wound healing. Thus the presence of constitutive concentration of NO is important in the regulation of angiogenesis. As NO is a very well known vasodilator the vascular permeability in the vicinity of wound is under the direct influence of its concentration. This process suggests that the secretion of the growth factors and migration of cells from the leaky vessels which allow formation of matrix across which keratinocytes proliferate and capillaries form are invariably under the control of NO. The study by Pipili of angiogenesis in-vitro and effect of NO donors has shown that it caused a dose-dependent inhibition of angiogenesis. It is suggested that NO exerts its anti-angiogenic effect as it is anti proliferative on endothelial cells through cGMP.

1.5.2.3 Matrix remodeling

Fibroplasia and extracellular matrix deposition are under the direct influence of NO concentration. NO phenotypically induces wound fibroblasts to myofibroblasts which possess increased capacity to secrete collagen and increased contractile characteristics. This helps the wound to heal and the scar to contract. NO secretion by fibroblasts starts as early as 3 days and is seen upto 21 days (Michael Schaffer,

1997). The wound fibroblasts are stimulated to synthesize NO by a number of cytokines (Beer H. D, 2000). Thus the major effect of NO on fibroblasts seems to be on the synthetic function. The increased concentration of NO in wound might negatively affect collagen secretion. The synthesis of collagen involves an important rate limiting step i.e hydroxylation of proline. The enzyme proline hydroxylase is the key enzyme in this step (Cao M, 1997). Certain evidences prove that NO may inactivate the enzyme by reacting with the Fe radical, as NO is found to inhibit the Oxygenase group of enzymes. This is brought about by the formation of Fe- nitrosyl complexes. The other possibility is that NOS and prolyl hydroxylase might compete for the free available oxygen. Thus NOS being highly reactive might decrease the available oxygen to prolyl hydroxylase, resulting in low collagen synthesis. One of the strong evidences suggesting that NO decreases wound healing is the observation of the fact that endotoxins inhibit wound healing and increases NO production. In another different observation it is found that nitric oxide promotes fibroblast proliferation (Michael Schaffer, 1997). The net effect on collagen metabolism could be direct or indirect by the involvement of the matrix metalloproteinases. In a separate observation a strong relation has been seen between the release of IL-1 and enhanced expression of iNOS mRNA and matrix-metalloproteinases(George Murrel, 1995). The ubiquitous transcription factor NF κB increases expression of genes for many enzymes, cytokines and adhesion molecules. NO exerts a negative feed back effect on NF KB. Thus the production of inflammatory cytokines and other enzymes are decreased with the inhibition of NF κB (Takahashi S, 2001). This stands in the favour of the fact that NO enhances wound collagen synthesis. There

are various and opposite observations of NO expression and diabetic wound (Jude E. B, 1991). In one set of experiments observers have seen a decreased expression of NO in diabetes whereas the other group has observed an increase. The variation could be related to the models as the former was on an animal model and the later on human diabetic foot ulcers (DFU). NOS and in turn NO expression is higher in DFU than that in the normal skin. The local cytokine concentration also determines the activity of NOS. There has been substantial evidence as regards to the inverse relation between transforming growth factor β (TGFβ). TGFβ increases arginase activity and decreases that of NOS (Boutard, 1995). In normal wound healing the arginase and NOS pathways are properly controlled and co-ordinated but in diabetes both the pathways seem to acting independently. Thus there is no down regulation of both the pathways. As a result there is an impaired synthesis of proline and NO. The interaction of NO and TGFβ have been demonstrated by Joseph. The effect of NO on collagen production is also related to the modulation of cGMP activity. cGMP activity is decreased by NO and and this furthur decreases collagen synthesis. On the contrary TGFβ, which is inhibitory to cGMP enhances wound collagen accumulation. Thus it is suggested that TGF-β inhibits NO which in-turn inhibits cGMP, which results in increased collagen synthesis (Chu A. J, 1999). One evidence in support of the decreased NOS in diabetes is that urinary nitrate is increased in normal wound healing but is decreased in diabetes-induced animals. The only explanation for this contrasting observation is that NOS expression might vary depending on the experimental model. Thus there is a possibility of variation in expression of NOS in diabetic patients and drug induced diabetes in animals.

1.5.3 Pharmacological studies of NO in wound healing

Previous studies suggest that nitric oxide has a direct influence on all the mediators and components of wound healing. It is important for mediation of epithelialization, angiogenesis, collagen deposition. Antimicrobial effects of the NO released also help in complete wound healing with minimal complications. So far most of the studies done on nitric oxide and wound healing has been either by supplementing Arginine, Ornithine and SNAP. In our study we have studied the effects of Nitrosoglutahione (GSNO), a nitrosothiol NO donor on wound healing.

1.5.3.1 Nitric Oxide donors previously studied in wound healing

Agent	Experimental model	Wound Parameter	Reference
Molsidomine	I.W. Diabetes (rat)	↑collagen, ↑ strength, ↑ MMP	Witte M.B (2002)
NO releasing PVA sponge (dressing)	I.W. Diabetes (mouse)	↑ wound contraction	Kristyn.S Bohl (2002)
(Sodium nitrite + Ascorbic acid) polymer	Bacterial culture Normal skin	Bacterial killing Vasodilation	Hardwick (2001)
HCT-3012 (NO-NSAID)	I.W, (rat)	↑ Collagen	Muscara M N (2000)
Sodium nitroprusside *	I.W (rat)	↓ collagen	Shukla A (1999)
Sodium nitroprusside	Diabetic leg ulcer (rat)	↑ accumulation of antimicrobial in ulcer	Shree.E.Cross (1996)
Nitrosoglutathione	Keratinocyte culture	↑ proliferation, ↑VEGF	Frank .S (1999)
Glyceryl trinitrite	Anal fissures	↑ healing	Lund J N (1997)
Polyethyleneimine/NO * adduct	I. W (rat)	↓ epithelialisation ↓ collagen ↑ inflammation	Joseph.A.Baeur (1998)
L-Arginine	I.W (human)	↑ lymphocyte immune response	Adrian Barbul (1990)
L-Arginine	I.W, Diabetic (rats)	↑collagen ↑ strength	Witte. M.B (2002)
L-Arginine	Full-thickness Burns (Rat)	↓ wound edema ↓ extravasation	Lindblom L (2000)
L-Arginine	I.W, iNOS knockout (mouse)	No improvement	Shi H P (2000)
L-Arginine	I.W, (rat)	↓ collagen	Shukla A (1999)
Ornithine	I.W, iNOS knockout (mouse)	↑collagen ↑ strength	Shi H P (2002)
SNAP	I.W, iNOS knockout (mouse)	↑collagen	Shi H P (2001)
INOS transfection (cationic)	I.W. (rat)	↑collagen	Thornton F J (1998)
Adenoviral iNOS transfection	I.W, INOS deficient mice	↑ rate of healing	Yamasaki K (1998)
DETA-Nonoate, NP, SIN-1 and SNAP	Keratinocytes	↑proliferation ↑ differentiation	Verena Krischel (1998)
GSNO & DETA-Nonoate	HaCaT keratinocyte	↑ proliferation	Stefan Frank (2000)

Table 1.5: Summary of previous studies on the effects of NO donors in wound healing. I. W. indicates incisional model. * indicates the reports which describe inhibition of collagen deposition by NO donors. @ is the only report which describes the effect of NO donor on wound concentration.

1.5.3.2 Nitric Oxide inhibitors previously studied in wound healing

Agent (NO inhibition)	Experimental model	Wound parameter	Reference
AG	Full thickness burns (rat)	↓ Granulation ↓ angiogenesis	Akcay (2000)
NG-L-nitro L-Arginine	Full thickness burns (rat)	No effect	Lindblom.L. (2000)
L-NAME *	I.W (rat)	↑ collagen	Shukla A (1999)
L-NIL	Keratinocytes	↓ VEGF	Frank S (1999)
L-NMMA	Keratinocytes	↓ VEGF	Frank S (1999)
AG	I.W (rat)	↓ collagen	Michael Schaffer (1999)
SMITU	I.W (rat)	↓ collagen	Michael Schaffer (1999
L-NAME	I.W (rat)	No effect	Michael Schaffer (1999)
L-NMMA	I.W (rat)	No effect	Michael Schaffer (1999)
L-NMMA	Sponges from wounds (in-vitro)	↓ nitrite and nitrate	Michael Schaffer (1997)
Tacrolimus	I.W (rat)	↓ collagen	Michael schaffer (1998)
EGF @	Keratinocytes	Increased proliferation	Heck D.E (1992)
iNOS knockout	I.W (mouse)	No change	Most D (2002)
iNOS knockout	I.W (mouse)	↓ collagen ↓strength	Shi H P (2002)
iNOS knockout	Fibroblast populated Collagen gel	↓ contraction	Shi H P (2001)
L-NIL	I.W (mouse)	↓ epithelialisation ↓keratinisation	Brigit Stallmeyer (1999)

Table 1.6: Summary of previous studies on the effects of NO inhibitors in wound healing. I. W. indicates incisional model. * is the only report where an iNOS inhibitor has improved wound collagen content. @ is the study in which EGF has positive effects on wound healing by inhibition of NO production.

1.5.3.3 Nitrosothiols

These are the sulfur analogues of the alkyl nitrites. Due to the widespread presence of cystiene in most of the tissues also the bioactive peptides these compounds are readily distributed in the tissues. Nitrosothiols may be formed by nitrosation reactions thiols (RSH + HONO $\rightarrow$RS-N=O +H_2O) but alternatively thiols react with NO directly and form nitrosothiols. The release of NO from these compounds is by spontaneous dissociation or one electron reduction. The storage of NO in-vivo is maintained by the interconversion of nitrosothiols and NO by various biochemical reactions. The main forms of nitrosothiols in the blood are S-nitrosocystiene, S-nitrosoglutathione and S-nitrosoalbumin. These compounds are involved as the active intermediates in several NO dissociation of other NO donor compounds.

1.5.3.4 Interaction of Nitric Oxide with antioxidants.

NO is a highly toxic compound present in every cell. Antioxidant systems counteract all the destructive pathways. Ascorbic acid rapidly detoxifies the NO produced. Glutathione effectively reacts with NO and acts as an efficient intracellular NO antagonist. Majority of the NO scavenging by GSH is undertaken in the mitochondrial matrix. GSH inhibits the peroxynitite –mediated nitration of tyrosine. N2O3 also reacts with glutathione yielding S-nitrosoglutathione (Goldstein and Czapski 1996). This is found to have protective roles in preventing nitrosation of amines (David A. Wink, 1996). The other secondary antioxidant mechanisms that enhance protection against NO mediated cellular damages are Vitamin E, β-carotene and carotenoids. All these antioxidants are found to have excellent synergistic effects in cell protection against nitrogen dioxide (Bohm, 1998). GSH is

abundantly present in the cytoplasm and the NO released intracellularly reacts with GSH forming GSNO.

$$2^{\cdot}NO + O_2 \rightarrow 2^{\cdot}NO_2$$

$$^{\cdot}NO + ^{\cdot}NO_2 \rightarrow N_2O_3$$

$$GSH + N_2O_3 \rightarrow GSNO + HNO_2$$

The reaction of NO with GSH is described by the reaction above (Neil Hogg, 1996). GSH in the cytoplasm and mitochondria reacts with NO and avoid damaging side reactions (peroxynitrite formation) and facilitates inactivation of this highly reactive molecule.

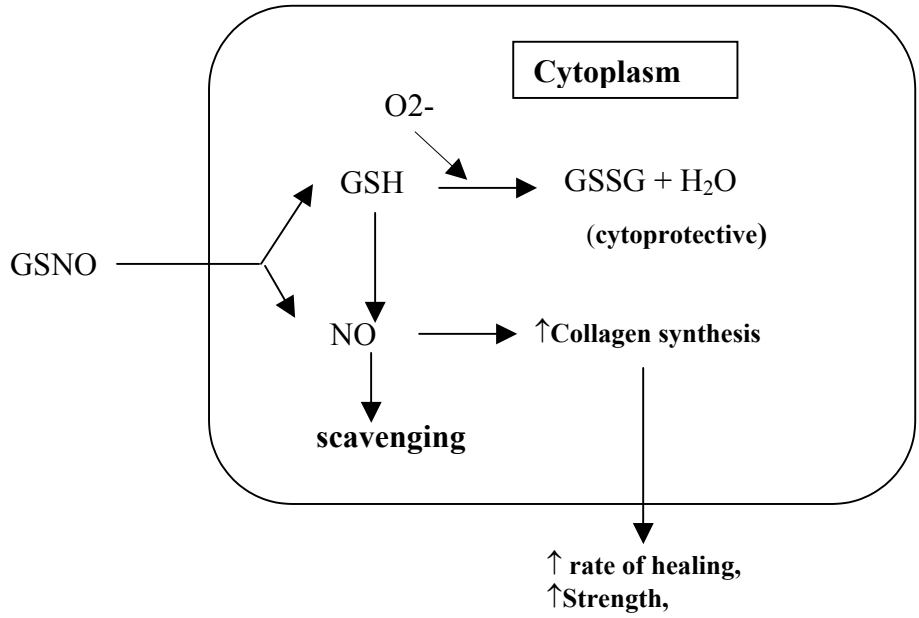
Chapter 2

Hypothesis

2.0 Hypothesis

Nitric oxide is a biologically active messenger molecule important in the regulation of critical cellular functions. The cellular effects of Nitric Oxide are influenced by the presence of glutathione, an anti-oxidant. We hypothesize that administration of NO donors containing both NO and glutathione positively regulate wound healing.

1) **A combination of NO and GSH could regulate the cellular responses due to injury by influencing the activity of enzymes involved in extracellular metabolism (MMPs)**
2) **The maintenance of appropriate levels of NO and GSH could enhance wound collagen content by regulating the activity of MMP's**
3) **Administration of nitrosoglutathione a compound which contains both NO and GSH components facilitates wound healing by improving collagen deposition and regulating the redox status of the wound.**



The hypothesized role of S-nitrosoglutathione in a cell. The proposed beneficial effects are in the form of increased collagen content, improvement in the rate of wound contraction and biomechanical strength. GSSG is the oxidized state of glutathione which is formed by the interaction of GSH and O_2^-. GSH interacts with NO which prevents the formation of peroxynitrite.

Chapter 3

Materials and Methods

3.0 Materials and Methods

3.1 Materials

3.1.1 Chemicals and reagents

3.1.1.1 Anaesthetic agents

Hypnorm was purchased from Jansen Pharmaceutica, Beerse, Belgium and Dormicum was purchased from, Roche, Basel, Switzerland.

3.1.1.2 General Chemicals

Merck & Co., Inc. (U.S.A)

Ammonium Chloride

Calcium Chloride

Potassium Chloride

Sodium Chloride

Sodium Acetate

Sodium Hydroxide

Sodium Carbonate

Tris base

Sigma Aldrich (St Louis, USA)

Amino-phenyl-mercurial-acetate (APMA)

Aminoguanidine

Chloramine T

Dithiothreitol

Glutathione

Hydroxyproline

Iodoacetamide

Methylcellosolve

Triton X-100

p-dimethylaminobenzaldehyde

Glycerol

3.1.1.3 Other Chemicals

Calbiochem - Novabiochem Corp., (U.S.A)

S-Nitrosoglutahione (GSNO)

S-Nitroso-N-acetyl-D-penicillamine (SNAP)

Leo Pharmaceuticals (Ballerup, Denmark)

Heparin sodium

3.1.1.4 Biological products

iNOS and eNOS monoclonal antimouse I_gG antibodies (*Transduction Laboratories KY, USA*).

MMP2 (Ab-1) and MMP9 (Ab-7) antibodies (*Neomarkers CA, USA*).

Vectastain, mouse and Rabbit IgG kits (*Vecta Laboratories, CA, USA*).

Bovine anti-mouse IgG_1 FITC as a isotype control cat. no MCA 1421 (*Serotec Inc., NC, USA*).

Mouse anti-rat MHC class I FITC cat no. MCA 51T (*Serotec Inc., NC, USA*).

Mouse anti-rat IA FITC cat. no MCA 46 FT (*Serotec Inc., NC, USA*).

Mouse anti rat CD 54 RPE cat. no 773 PE (*Serotec Inc., NC, USA*).

Protein assay kit (*Bio-rad, Hercules, CA, USA*).

Fetal Bovine Serum (*Gibco-BRL*).

Trypsin-EDTA, DMEM and Penicillin-Streptomycin (*Sigma, USA*).

3.1.1.5 Commercial kits

Type IV Collagenase activity assay kit cat. no ECM 478 (*Chemicon Intl Inc. Temecula, USA*).

Glutathione assay kit cat. No. 703002 (*Cayman chemical, USA*).

1 part of dormicum (*Roche, Basel, Switzerland*).

3.1.1.6 Instruments

Instron material testing machine (*Massachusetts, USA*)

Schimadzu Spectrophotometer (*Kyoto, Japan*)

Fluorescence Spectrophotometer LS 50B (*Perkin Elmer Inc., USA*)

Microfilter tubes (*Whatman Intl Ltd., England*)

3.1.2 Experimental animals

Sprague Dawley male rats were chosen for this study. They were between the age group of 8-10 weeks and weighed around 200-250gms. All the animals were obtained from Laboratory Animal Center (Sembawang, Singapore). The animals were housed in Animal Holding Unit, National University of Singapore.

3.2 Methods

3.2.1 Animal Study

The effect of drugs on wound healing was studied using intact animal models. All animal experiments were conducted according to the institutional guiding principles for animal research, National University of Singapore. The weight of the animals was measured once every 3 days as a general health indicator. Constant weight gain and activity were the indices of general health. The official approval of animal experiments is given in the Appendix A.

3.2.1.1 Animal care

Sprague Dawley male rats, weighing 200-250 gm, obtained from the Laboratory Animals Centre (LAC, Sembawang, Singapore) were used throughout the study. To avoid overcrowding each rat was individually caged. All animals were kept at the Animal Holding Unit (National University of Singapore) and were allowed to acclimatize for a duration of 1 week before operation. All rats were weighed prior to the study, and thereafter monthly till the date of sacrifice.

All animals were kept in a photo-periodic room with 12 hour day/night cycle. Animals were kept in artificial light. Animals were fed on standard laboratory chow and water given ad libitum. All procedures were performed between hour 8 and 12 of the day cycle. The room temperature was maintained at 21°C with a relative humidity of about 50%. Soft white wood shavings were used as bedding material.

3.2.1.2 Grouping of animals based on wound models

Experimental animals were grouped based on two separate models of wound for the purpose of this study. They were 1) excisional square wound model and 2) Incisional wound model. These two groups were further divided into various treatment groups as described below.

3.2.1.2.1 Excisional Square wound group

The excisional wound model was adopted to compare the overall effects of Nitric oxide donor and inhibitor on wound healing. Totally, Eighteen rats underwent square wound explants on their dorsal surface. They were divided into control, GSNO and AG group, with 6 animals 'n' in each group.

3.2.1.2.2 Incisional wound group

In-order to examine the effects of drug treatments at different time points, the animals underwent incisional wound. This allows to sample out the reparative tissue deposited between the margins at earlier time points. The incisional wound groups consisted of 4 different time points at which the scar tissue was harvested. The time points were 3, 5, 7 and 10 days after wounding. At each time point there were 6 animals (n=6) that were sacrificed from each group. Therefore there were twenty-four animals in each group. The study design involved administration of Saline (control), GSNO, GSH, AG and SNAP. Therefore a total of one hundred and twenty animals were recruited for our experiments

3.2.1.3 Surgical Procedure

3.2.1.3.1 Animal Anaesthesia

All rats included in the study requiring tranquillisation were anaesthetised using a Clinical Research Center (CRC) cocktail obtained with the consent of the in-house veterinary surgeon. This cocktail containing 1 part of Hypnorm, 1 part of Dormicum and 2 parts of saline was used for injection. The aneasthetic was administered at a dose of 0.1ml/100mg kg body weight.

3.2.1.3.2 Square wound model

To evaluate the effect of the agents on wound healing, the changes in a square wound model were examined. This model is representative of all the phases of wound healing. Following acclimatization for a week, the animals were subjected for the operative procedure. The hair on the dorsum of the anaesthetized rats was shaved with an electrical shaver. Full thickness square wounds measuring 15mm X 15mm were made by excising the skin within the confines of square down to the level of the panniculous carnosus (Fig. 3.1). All dissections were performed using a size 15 blade and care was taken not to contuse the borders. The wounds were left to heal for 12-14 days on their own without any dressing being applied. The animals were placed in individual cages. The outline of the wounds were measured every 24 hrs. The animals were held immobile with head covered and the length (L) and breadth (B) of the wound was measured and the area calculated (LXB). The rate of contraction was obtained by plotting the log of area Vs time.

The gradient of each regression line is the log co-efficient of wound contraction k, for each animal. The k value of each wound was taken as a measure of the rate of wound contraction (Cross S. E, 1995).

Fig. 3.1: Square excisional wound model. Four excisional wounds, each measuring 15mm X 15mm were created. Daily measurements of the area (length and breadth) of the wound were done in the same position.

3.2.1.3.3 Incisional model

Each animal underwent a seven-centimeter long dorsal incision along the head-tail axis, through the depth of the epidermis, dermis and panniculous carnosus under hypnorm anaesthesia (Fig. 3.2). The margins were properly approximated and sutured with intermittent 3-0 silk surgical sutures. The animals were observed till they recovered from anaesthesia.

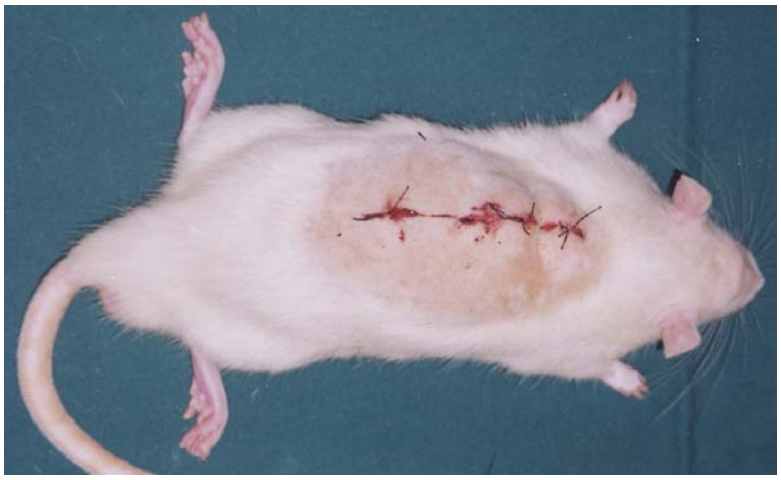


Fig. 3.2: Incisional wound model. 7-8 cms long incisions were made on the dorsum of the rats. Wounds were closed by interrupted sutures and the scars were harvested at 3, 5, 7 and 10 days, post-wounding.

3.2.1.3.4 Intra-abdominal catheterization

Animals were anaesthetized, their abdominal wall was shaved and a small incision was made in the anterior wall. The peritoneum was opened and a silastic tube with a wide perforated tip was inserted into the abdomen under sterile conditions (Fig. 3.3). The tube was then drawn through the dorsum under the skin and let out at the top of the head. The free end was corked with a metallic stopper. The tube was sealed with a dental cement on the skull. The catheters were flushed with saline and the animals were kept under observation. The catheter exiting from the skull were flushed again with saline on the following day and 500µl of peritoneal fluid was aspirated and preserved at 4 ^{0}C for flow cytometry of peritoneal cells.

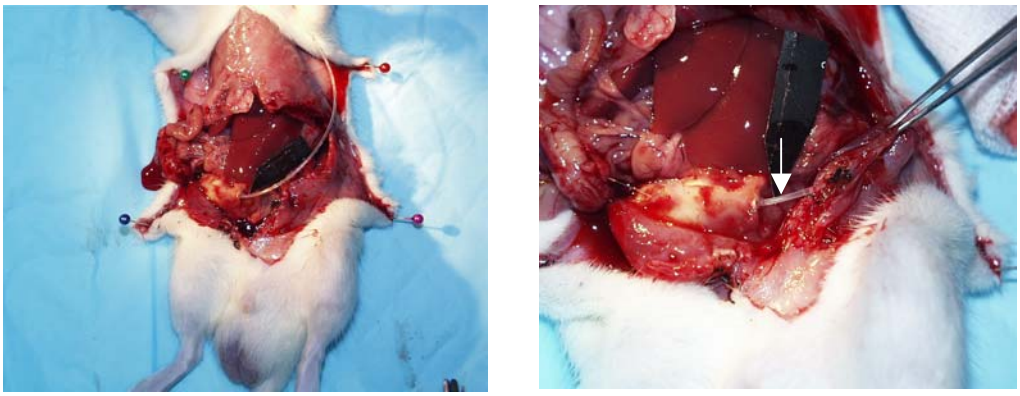


Fig. 3.3: Intra-abdominal catheterization. A silastic tube was placed in the abdominal cavity (arrow) to aspirate fluid and cells.

3.2.1.3.5 Sephanous vein catheterization

A different catheter was inserted into the sephanous veins of the animals. Briefly the inguinal region was dissected out and the sephanous vein was identified. The vein was cannulated by a thin elastin tube attached to P10 silastic tube. The catheter was drawn up to the base of the skull along with that from the abdomen. Both the catheters were secured safe at the skull base by dental cement.

3.2.1.4 Treatment of animals with pharmacological agents

Control animals

Animals were administered normal saline, which was the vehicle (solvent) for all the other drugs.

Nitric oxide donors

Nitrosoglutathione, (GSNO) and S-nitroso-N-Acetylpenicillamine (SNAP) was administered i.p to animals at 24 hour intervals at 0.3 mg kg^{-1} body weight.

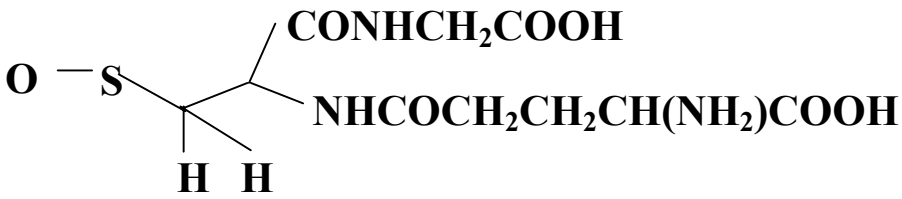
Nitric oxide inhibitor

Aminoguanidine was administered i.p to animals at 24 hour intervals at a dosage of 100 mg/kg body weight.

Reduced glutathione

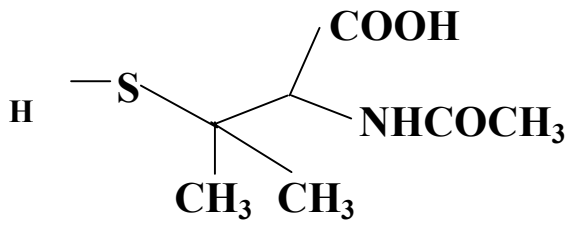
Reduced glutathione was administered i.p to animals at 24 hour intervals at 0.3 mg/kg body weight.

Glutathione (GSH)

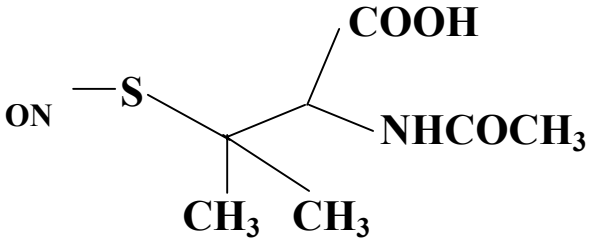


S-NitrosoGlutathione (GSNO)

Fig 3.4 Chemical structure of glutathione and S-nitrosoglutathione

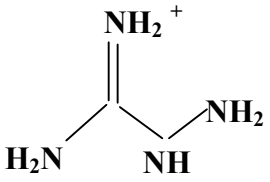


N-Acetyl-DL-penicillamine (NAP)

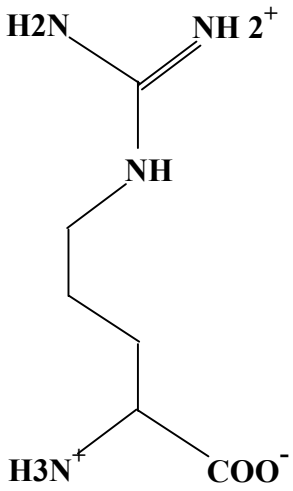


S-Nitroso N-acetyl-DL-penicillamine (SNAP)

Fig 3.5: Chemical structure of N-Acetyl-DL-penicillamine (NAP) and S-Nitroso N-acetyl-DL-penicillamine (SNAP).



Aminoguanidine



L-arginine

Fig 3.6: Chemical structure of aminoguanidine and L-arginine

3.2.1.5 Sampling of the scar tissue

The scar tissue from the animals was excised completely. The surrounding normal tissue in the scar sample was clearly dissected out. The scar was divided into four equal parts for A) measurement of collagen content B) MMP activity assay C) Nitrate and Nitrite measurement and D) Glutathione content. The following diagram represents schematic plan of scar excision.

3.2.1.6 Storage of the scar tissue

Samples were snap frozen in liquid nitrogen and stored at –80°C in labeled cryovials for MMP activity assay, hydroxyproline estimation, nitrite and nitrate assay and glutathione estimation. Few samples of the same tissue were placed in plastic cassettes and preserved in 4% formalin for preparation of paraffin blocks.

3.2.2 Determination of Collagen content

Hydroxyproline (Hyp) is a post-translational product of proline hydroxylation catalyzed by an enzyme prolylhydroxylase. The occurrence of this imino acid is thought to be confined almost exclusively to the connective tissue collagen (Nemethy, 1986). Because of its restricted and unique distribution in connective tissue collagen, the metabolism of collagen and its regulation is conveniently studied by measuring the Hyp content in a number of normal and clinical situation. In this study, the Hyp content has been measured which indicates the total collagen content of the scar

tissue. This imino acid was quantified by a spectrophotometric method (Woessner 1961) as described below.

Experiment: Pre-weighed frozen scar tissue was treated with 6 N HCl for 30 minutes at 130^0C in a microwave oven. The solution thus obtained was neutralized with 1M Sodium hydroxide. The neutral solution was reduced with chloramine T in acetate buffer. Following 20 minutes of incubation at room temperature, perchloric acid was added to furthur neutralize the solution. Para-aminobezaldehyde dissolved in methyl-cellosolve was added to derivatize hydroxyproline. The absorbance was read at 557 nm in a Shimadzu spetrophotometer. The hydroxyproline content was read off a standard straight line obtained using known concentrations of the compound. Therefore, collagen content was quantified for each sample of the scar tissue from each group of animals.

3.2.3 Biomechanical testing

The biomechanical testing of the tissue clearly provides information regarding it's material properties. The study of the effects of the treatment on the material property indicates the change in the quality of the scar. The instron materials testing machine tests the material property of the scar. The parameters that are being measured are a) Load to failure and b) stiffness of the scar.

3.2.3.1 Sample preparation

The specimen to be tested is prepared in a manner that is appropriate to conduct a load displacement measurement of the scar. Fresh rectangular strips of skin (1cm) with the scar at the mid-point were harvested from anaesthetised animals, following wounding (both 5 days & 10 days). The biomechanical testing is done at 5 and 10 day intervals as these are the two time points at which the collagen deposition is significant and the remodeling of the new matrix takes place. For biomechanical testing the scars are harvested from the caudal end of the linear scar on the back of the rats. The strip of skin, orthogonal to the axis of the scar, was taken to ensure that the tensile load applied to the specimen is 90^0 in direction to the axis of the scar.

Fig. 3.7: Experimental set-up for biomechanical testing. Fresh skin for testing and sandpaper interfaces to enhance the gripping by the materials testing machine are seen in this picture

3.2.3.2 Tensile strength measurement

The specimen was held between clamps of an INSTRON 5543 materials testing system (Fig 3.7 & 3.8) with a 500 N load cell. The accuracy of this machine was upto 1% of maximum readings i.e. 2N. The clamps were modified to include a perspex and sandpaper interface to optimize specimen grip with minimum damage to the tissue at the grip site during testing. The gauge length (distance between the clamps) for each specimen was set at 15mm with the specimen positioned to ensure that the horizontal scar-line lay in the center, equidistant from either of the clamped ends. Uniaxial load at a deformation rate of 25mm/min was applied along the length of the skin *(*Paul R.G, 1997*).* From the load-deformation curves obtained after the mechanical testing, the load to failure and maximum stiffness were determined.

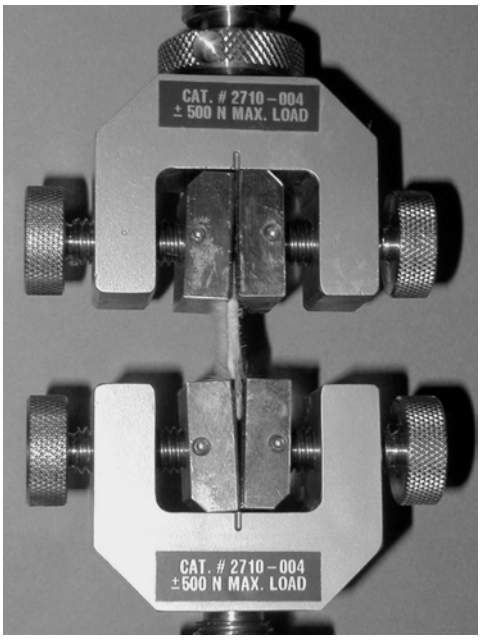


Fig. 3.8: Instron materials testing machine. Skin mounted between the clamps of the material testing machine is seen in this picture.

3.2.4 Tissue protein measurement

The Bio-Rad protein assay, based on the Bradford method was adopted to determine the protein content in the tissues. All the protein assays were done according to the standard procedure provided by Bio-Rad Protein assay instruction manual. The microtiter plate protocol was followed. The Bio-Rad reagent is diluted 1:5 with double distilled water. The dye is filtered and stored at room temperature. Protein standards from bovine serum albumin are prepared between a range of 0.05 mg/ml – 0.5mg/ml. 10 μl of standard or test protein solution is added into each well of a microtiter plate. 200 μl of diluted reagent is added to each well containing the samples or standards. The plate is incubated at room temperature for 5 minutes and the absorbance was read at 595 nm in a Shimadzu (Japan) spectrophotometer. The readings are tabulated in Graphpad Prism software (USA) and protein concentration of the samples are derived from standard curve obtained by plotting the absorbance of standards against concentration.

3.2.5 Matrix metalloproteinase activity assay

Gelatinases are the key enzymes in breakdown pathway of collagen. Extended activity of MMP's in healing environment inhibits re-epithelialisation and matrix deposition. As NO and GSH are effective mediators of inflammation, and a high MMP activity is seen in this phase, it is important to examine the effects of these molecules on Gelatinase activity.

Fluorogenic peptide substrate assay for activity of matrix metalloproteinase in the scar samples was carried out. The first mammalian MMP peptidase activities demonstrated were cleavage of Dnp-Pro-Leu-Gly~Ile-Ala_gly-D-Arg and Dnp-Pro-Gln-Gly~Ile-Ala-Gly-Gln-D-Arg by MMP 2 (Seltzer, 1981). Fluorogenic substrates provide a particularly convenient enzyme assay method. The basic principle that governs the method is as follows: When the substrate is cleaved by the enzyme, diffusion of the donor-containing substrate fragment cleaves away from the acceptor-containing substrate fragment results in loss of energy transfer and subsequent appearance of fluorescence. This method was adopted to measure the gelatinase activity in the scars of animals treated with various pharmacological agents.

3.2.5.1 Extraction of MMP's from the scar tissue

Preweighed fresh frozen scar tissue was utilized for the activity assay. The specimens were pulverized in liquid nitrogen and then homogenized in buffer containing 0.25% triton X-100 in 0.01M $CaCl_2$. The homogenized sample was centrifuged at 6000 g and the pellet was resuspended in 0.1 M $CaCl_2$, 0.15 M Nacl and 0.05 M Tris HCl (pH 7.5). The suspension was heated for 6 minutes at 60°C, chilled and centrifuged at 20,000 g. The extract was reduced with 2 mM di-thiothrietol for 30 minutes at 37°C and alkylated with 5 mM iodoacetamide for 30 minutes at 37°C. The samples were filtered through microfilter tubes (Whatman intl Ltd England) of 30 kDa cutoff size. The eluate was utilized for scar MMP activity assay.

3.2.5.2 Gelatinase (MMP) activity assay

The assay was carried out on the scar samples of all the animals according to the manufacturers instructions (Appendix C). The extracted MMP's were immediately activated by 0.5 mM amino phenyl mercurial acetate (APMA). The fluorescent collagen substrate provided in the Gelatinase assay kit is incubated with 25 µl of the sample. The fluorescence of the reaction mixture is read in a luminescence spectrometer LS 50 B (Perkin Elmer England). The readings are calculated according to the formula

FS-FB / FT-FB x 1 / Reaction time (mins) x Yµg of substrate / (X ml sample / tube)

Where FS implies Florescence of the sample, FB is the fluorescence of the blank and FT is the fluorescence of the total.

The result is expressed as units where in each unit is the activity of the enzyme per µg of collagen substrate added.

3.2.6 Determination of Total Nitrite

The main breakdown products of Nitrosothiols are nitric oxide which rapidly forms peroxynitrite and inturn nitrite and nitrate. Therefore the end-products of NO metabolism were measured through Griess reaction.

3.2.6.1 Determination of Nitrite in wound lysates

The stable NO oxidation product, nitrite was determined in wound homogenates by the Griess reaction. Skin samples were homogenized in lysis buffer containing 2% triton X-100, 40 mM tris HCl (Merk, Germany), pH 8.0, 274mM NaCl, 20% glycerol (Merk, Germany), 10mM phenylmethylsulfonyl fluoride and 30(g/ml leupeptin. The tissue extract was cleared by centrifugation and the supernatant was diluted with equal volume of water. The extract thus diluted was centrifuged at 100,000 g for 30 minutes. The supernatant was further filtered through ultra spin cups (Whatman intl Ltd England) with 30 kda cut off. The eluate obtained was analysed for the nitrite content. Sulphanilamide 50 µl and 50 µl N-naphthylendiamine dihydrochloride were added to 50 µl of sample. Each reaction mixture contained 0.1 U/ml nitrate reductase (Roche,USA), 5(M FAD, 0.03mM NADPH for the complete reduction of nitrate. The absorbance of the reaction mixture was read at 540 nm after 15 minutes of incubation.

3.2.6.2 Determination of Nitrite in plasma

Blood was obtained from the rats by sephanous vein catheterization. 500 µl of blood samples was collected in microtainer tubes (Becton Dickinson) containing EDTA. The samples were centrifuged at 10,000 g and the supernatant plasma was collected. The plasma was diluted with equal volume of distilled water and filtered through microfilters (Whatman Intl UK). The eluate was transferred into fresh eppendorf tubes and stored at –20°C until further procedure. 40 µl of the sample was used for the nitrite assay. The readings obtained were further standardized against the

protein content. The results are expressed as mM of nitrite / mg of protein content in the tissue.

3.2.7 Glutathione assay

Glutathione is one of the important molecules present in the cells and it readily interacts with NO^{-}. Therefore in any study involving the usage of NO modulating drugs, the effect on the GSH status has to be monitored. The scar glutathione was quantified by Cayman's GSH assay kit. This utilizes the recycling method of DSTNB by glutathione reductase for the quantification of GSH. The sulfhydryl group of GSH reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman's reagent) and produces a yellow colored 5-thio-2 nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and (produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which is in turn proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 or 412 nm provides an accurate estimation of GSH in the sample.

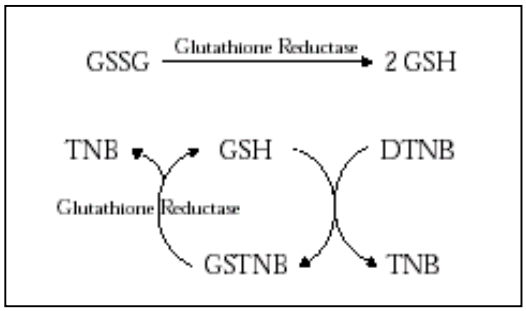


Fig. 3.9: Schematic representation of DTNB recycling in glutathione assay.

3.2.7.1 Sample preparation

The frozen scar tissues were pulverized and homogenized in 1 mM tris HCl, pH 6.5. The samples were centrifuged at 11,000 g and the supernatant was aliquoted into labeled microcentrifuge tubes. The samples thus prepared were subjected for de-proteination.

3.2.7.2 De-proteination

The samples were de-proteinated by metaphosphoric acid (MPA). Equal volumes of MPA is added to the sample and incubated for 5 minutes. The mixture is centrifuged at 2000 g for 5 minutes and the supernatant is aliquoted. 50 µl of Triethanolamine is added to the supernatant. The sample is ready for Glutahione quantification. The procedure is followed according to the manufacturers instruction (Appendix D). The absorbance is measured at 412 nm.

3.2.8 Flow cytometry of peritoneal cells

Nitric oxide is a secondary signaling molecule that influences the activity of immune cells. Following administration of NO donors or NOS inhibitors, changes in the immune response and macrophage activity are possible. So in order to characterize the effects of the drugs on the peritoneal immune cells we studied the expression of MHC Class I, Class II and ICAM-1, which indicate alteration in immune response.

3.2.8.1 Sample collection

The peritoneal washing obtained after 24 hours of abdominal catheterization is used in this method. Following the abdominal catheterization rats were allowed to recover overnight. 24 hrs after catheterization 1ml of PBS was flushed into the catheter and same amount of fluid was withdrawn carefully into labeled microfuge tubes.

3.2.8.2 Fluorescent staining

The effects of GSNO on the MHC Class I, Class II and ICAM-1 (CD54) expression on peritoneal cells was monitored by flow cytometry. Peritoneal lavage was centrifuged at 600 g for 10 minutes. The cell pellet was resuspended in RBC lysis buffer and incubated at 4°C for 10 minutes. The sample is again centrifuged at 600 g at 4 ^{0}C for 10 minutes and the cell pellet is resuspended in PBS containing 1% BSA. The cells were incubated in MHC Class I, Class II and ICAM-1 fluorescent antibodies at a concentration of 10 μl/ 10^{6} cells. The samples were incubated for 30 minutes. FITC conjugated Isotype control mouse IgG1, was used in this study. Cells were pelleted and washed twice inPBS-BSA and furthur fixed in 1% Paraformaldehyde. The stained cells were analysed by flow cytometry on a Becton Dickinson FACStation using the winMDI software, version 2.8, (The Scripps Research Institute, San Diego).

3.2.9 Histology

The effects of treatment were monitored histologically by H&E staining and immunohistochemistry. By H&E method, changes in the dermal or the epidermal morphology, inflammatory infiltration and changes in the capillary network can be appropriately studied. Immunohistochemical staning provides information on the distribution of the molecule at microanatomic level and variation in the pattern of expression can be noted.

3.2.9.1 Preparation of histological sections

The animals were sacrificed and the scars of all animals were excised, placed in cassettes and fixed in 10% formalin for histological examination. Two scars and one normal skin biopsy were taken from the dorsum of each animal. After processing, the samples were transferred to an Lieca embedding machine for preparing the paraffin blocks. The tissues were embedded and sectioned into 4 μm sections.

3.2.9.2 Hematoxylin and eosin staining

Slides were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol. The deparaffinized specimens were placed in eosin for 5 minutes. Immediately the slides were placed in hematoxylin solution for 3 minutes. The slides were then washed under running water till all the excess stains were washed away clearly. The slides were dehydrated in increasing concentrations of alcohol and xylene. The samples were mounted and coverslipped in Canada balsam. All the slides were viewed in Olympus microscope at 20 X and 40 X magnifications.

3.2.9.3 Immunocytochemistry

3.2.9.3.1 MMP immunostaining

The sections are dewaxed in xylene by immersing thrice. The sections are rehydrated through decreasing concentration of ethanol. [Absolute → Absolute → 90% → 90% → 70% → 70%] followed by immersing in water. Endogenous peroxidase activity is blocked by treating the sections with 0.3% hydrogen peroxide in methanolfor 30 minutes. The slides are then washed in PBS for 5 minutes. Digestion of the sections is done with 0.05% saponin solution for 30 minutes followed a thorough wash in PBS for 5 minutes. Non-specific binding is inhibited by blocking the sections with normal goat serum for 30 minutes. The excess serum is drained and, Avidin is applied for 15 minutes. The slides are drained and again treated with Biotin solution for 15 minutes. The slides are washed in PBS for 5 minutes. MMP primary antibody is applied at a concentration of 1:30 for MMP-9 and 1: 100 for MMP 2. The antibodies are incubated at room temperature for 2 hours. [Negative control slides will be treated with PBS]. The slides are further washed in PBS for 5 minutes. Biotinylated secondary antibody (goat anti-rabbit) is added for 45 minutes to each section. The sections are washed again in PBS for 5 minutes. Each specimen is treated with ABC reagent for 10 minutes and washed in PBS for 5 minutes. Freshly prepared chromogen DAB is applied for 10 minutes. The slides are washed well in running water as soon as the brownish discoloration is seen. Counterstaining was done with haematoxylin for 5 – 10 seconds.

The washed slides are dehydrated in increasing concentration of ethanol [70% → 70% → 90% → 90% → Absolute → Absolute]. The specimens are immersed in clear sections in xylene and mounted and coverslipped.

3.2.9.3.2 Immunostaining of iNOS and eNOS

The sections are dewaxed in xylene by immersing thrice. The sections are rehydrated through decreasing concentration of ethanol. [Absolute → Absolute → 90% → 90% → 70% → 70%] followed by immersing in water. Endogenous peroxidase activity is blocked by treating the sections with 0.3% hydrogen peroxide in methanol for 30 minutes. The slides are then washed in PBS for 5 minutes. Digestion of the sections is done with 0.05% saponin solution for 30 minutes followed a thorough wash in PBS for 5 minutes. Non-specific binding is inhibited by blocking the sections with normal horse serum for 30 minutes. The excess serum is drained and furthur, Avidin is applied for 15 minutes. The slides are drained and again treated with Biotin solution for 15 minutes. The slides are washed in PBS for 5 minutes. iNOS and eNOS primary antibodies are applied at a concentration of 1:30 and 1:100 respectively. All the slides were incubated overnight at 4°C. [Negative control slides will be treated with PBS]. The slides are further washed in PBS for 5 minutes. Biotinylated secondary antibody is added for 45 minutes to each section. The sections are washed again in PBS for 5 minutes. The sections are treated with ABC reagent for 10 minutes and washed in PBS for 5 minutes. Freshly prepared chromogen DAB is applied for 10 minutes. The slides are washed well in running water as soon as the brownish discoloration is seen. The slides are counterstained with haematoxylin for 5 – 10 seconds. The washed slides are dehydrated in

increasing concentration of ethanol [70% → 70% → 90% → 90% → Absolute → Absolute]. The specimens are immersed in clear sections in xylene and mounted and coverslipped. The slides are then viewed at 20 X and 40 X magnification.

3.2.9.3.3 Evaluation of slides

The present underlying technique was used to evaluate the immunohistochemical staining procedures. This method of staining takes into account both the intensity of staining in a given high power field and area stained (number of cells) in a high power field. All scoring was done based on staining patterns of a minimum 5 high power fields that were randomly chosen by the evaluator. The slides were evaluated blindly by two individuals and the scores obtained compared to verify the staining pattern in a given cell type.

Scoring: Samples were rated according to a visual score which was calculated by adding the scale of intensity of staining to the area of the staining i.e score = intensity +area. The intensity of staining had the following scale; 0, no staining of the dermal and the epidermal layer, 1+ mild staining; 2+ moderate staining; 3+ marked staining. The area of staining was evaluated differently using the following scale; 0, no staining of the scar tissue, 1+ <25% of the area stained positive, 2+ 25-50% positive staining, 3+ 50-75 % positive staining, 4+ >75% positive staining. The maximum score awarded was 7 (3+4) and the minimum was 0, indicating complete absence of the antigen under study.

3.2.10 Statistical analysis

The parametric data are reported as mean ±standard error of mean (SEM). Statistical significance was assessed by one-way ANOVA using Statistical Program for Social Sciences version 10 (SPSS Inc. USA). For the analysis of Non-parametric data, Kruskal-wallis test for multiple comparisons of Median of each group was adopted. Statistical significance value 'p' was chosen at 0.05.

Chapter 4

Results

4.0 General health of the animals

The animals were monitored daily for food and water intake. The wounds were examined daily and the activity of the animals was monitored. Most of the wounds healed without any infection. The animals were active and a constant weight gain was observed.

4.1 Square wounds

This model was adopted to investigate the effect of the drugs on the process of wound contraction. As an initial step to analyze quantitatively the progress of wound repair following administration of a NO donor (GSNO) and an iNOS inhibitor, we propose to study the effect of these drugs on wound contraction. Four square wounds were inflicted on each animal and the average area of the four wounds was taken as a single reading. This method of measurement was repeated three times on each animal. The rate of wound contraction is expressed in terms of log of wound area vs. time (Fig. 4.1). A regression line drawn along the points of log curve gives the constant co-efficient, which is interpreted as the rate of wound healing.

4.1.1 Gross observation

The wound area increased marginally over the area of the original template used for the first two days. This is a result of the extension forces exerted by the surrounding normal skin and its loose attachment to the deep fascia. Between the second and third day a sharp decrease in the area of the wounds was observed. After the third day, the wound area appeared to decrease at a constant rate, which progressively

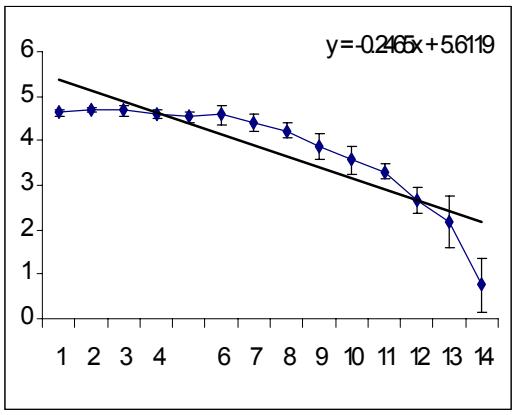
declined towards tenth to twelfth day. The measurements were all taken from the margins of the wounds, which were previously marked by permanent ink while creating the wounds. The healing progressed with the scabs drying from the periphery. Measurements were taken for a total period of 12 days by which time most of the wounds had completely healed (Fig. 4.2). Following complete healing a scar with a size smaller than the original wound was observed. The scar was square in shape and the edges were elongated. The measurements of the area were consistent and repeatable. Each wound was exposed to external environment. The ability of the wound to heal in the natural setting, wherein no dressing or any other supportive therapy is administered was a part of this study.

4.1.2 Effects of GSNO on rate of wound contraction

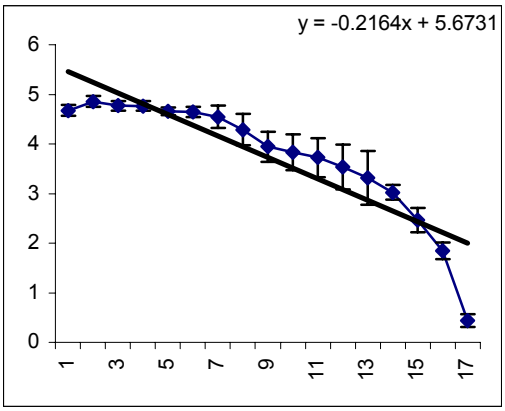
The log co-efficient (k) which indicates the rate of wound contraction was 0.125 ± 0.02 in the control group and 0.234 ± 0.06 in the GSNO group (Fig. 4.2). The rate of wound contraction was significantly higher ($p < 0.05$) in the GSNO treated group than the control group. The GSNO and control (saline) treated animals did not show any signs of wound infection and the wounds healed normally.

4.1.3 Effects of AG on wound contraction

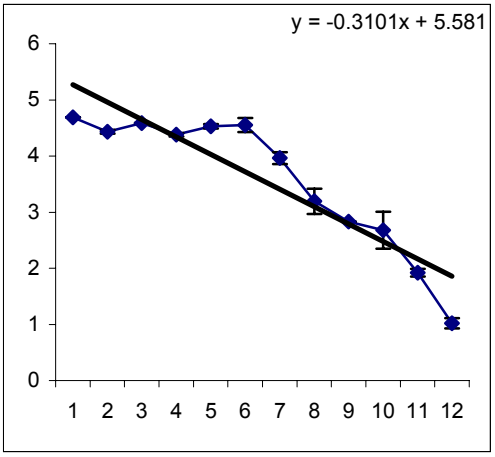
The log co-efficient (k) was 0.09±.0.009 in the AG treated group (Fig. 4.2). The administration of AG did not significantly alter the rate of healing. Though the rate of healing was decreased in the AG treated group there was no statistical significance observed in our study. A trend towards a decrease in the rate of healing was seen in the AG treated group.



A



B



C

Fig. 4.1: Log curve of wound contraction. The rate of contraction of wound in a) Control b) AG and c) GSNO treated animals are shown in this figure. Each point on the graph represents mean log area of the wound at that corresponding time (n=6) and the Error bars represent SEM. The correlation co-efficient is obtained by drawing a regression line through these points. X-axis represents number of days and Y-axis represents the log of the area of wound obtained by measuring the length and breadth.

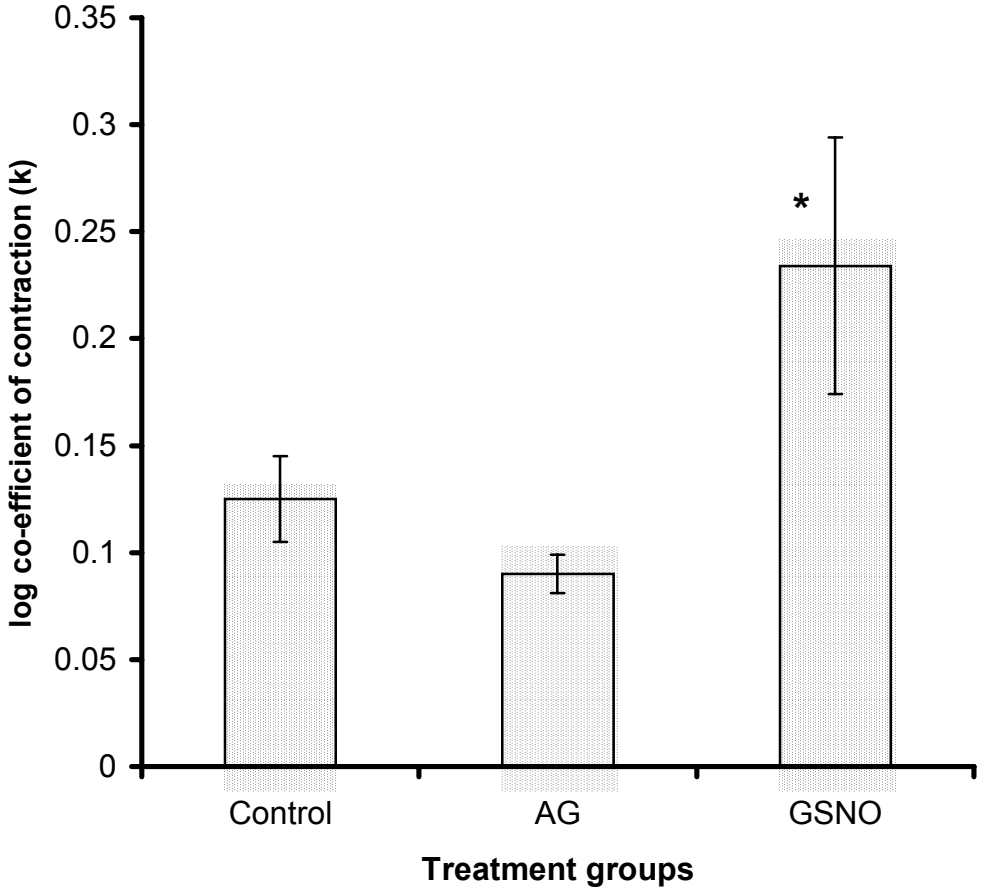


Fig. 4.2: Rate of wound contraction in Control, AG and GSNO treated animals. The mean rate of wound contraction is represented in this graph (n=6). Error bars correspond to SEM and * is statistical significance ($p<0.05$). Y-axis represents log co-efficient of contraction (k).

4.2 Biomechanical strength of scars treated with NO donors and inhibitors

The changes in the material properties (Load and stiffness) of the scar following treatment with GSNO, SNAP, GSH and AG were measured in an Instron 5543 materials testing machine. The load deformation curve for each sample was traced with the displacement (distance moved) on the X-axis and the force applied on Y-axis (Fig. 4.3). The load of each sample corresponds to the peak of the graph. The stiffness is obtained by measuring the slope of the straight line in the log phase of the curve.

Gross observation: The scars are elastic in the initial phase. Gradually a break appears at the site of the scars from the periphery to the center. The point at which the scar completely breaks is the end-point of tracing. Maximum strength (load) is required when the scar substance has to be broken initially following which, the rest of the sample gives way without much resistance.

The scar properties were characterized in terms of the force required to break the newly deposited tissue. This force is described as the load to failure and is expressed in Newton (N). Biomechanical testing of the scars was conducted at two time points which correspond to a) maximum deposition of extracellular matrix (day 5) and b) matrix remodeling (day 10). By 5 days the wounds were not completely healed and the granulation tissue did not offer great resistance to the force applied by the Instron machine. At the tenth day most of the wounds were totally healed and the biomechanical properties were clearly measured.

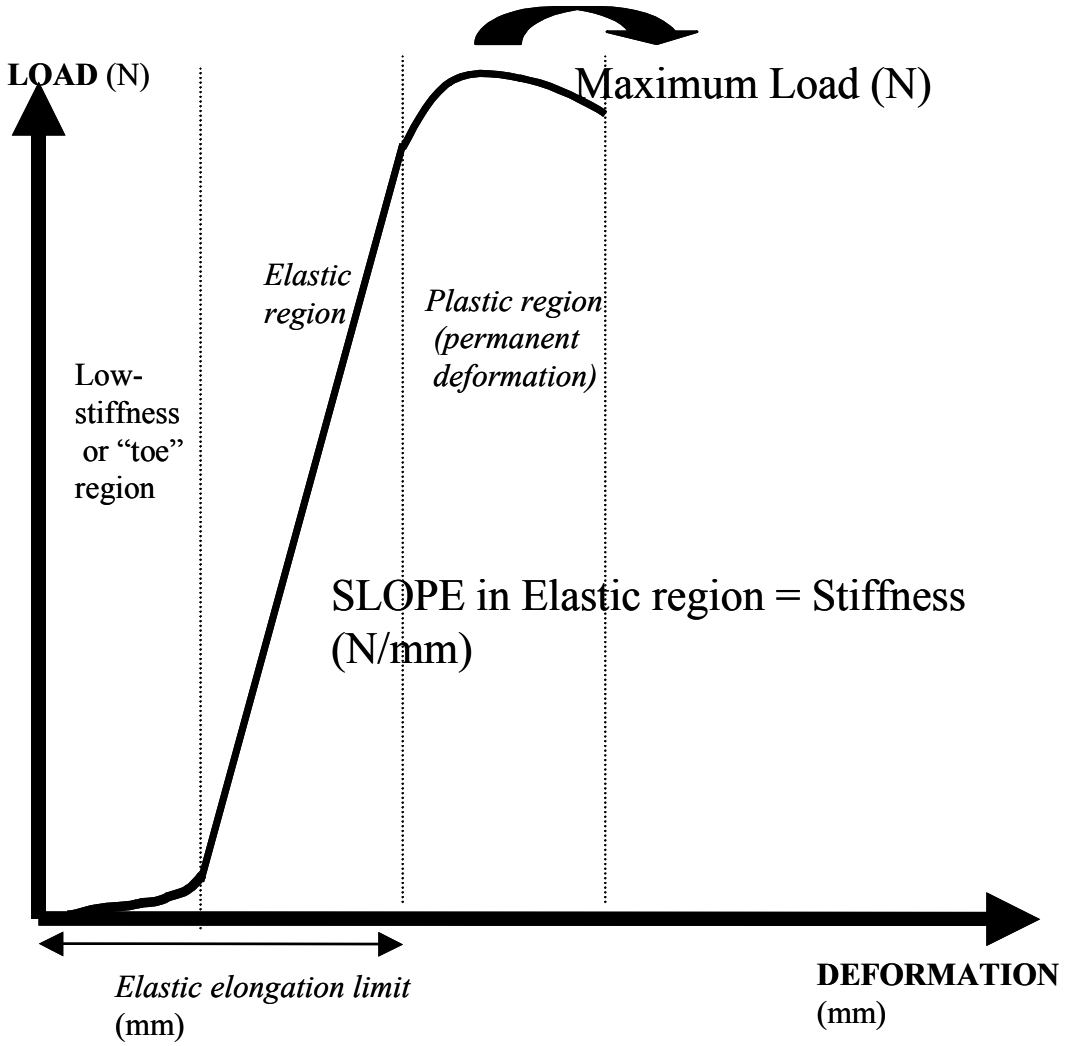


Fig 4.3: Load deformation curve of the scars. The X-axis is the displacement (mm) and Y-axis is the Load (N). The load displacement curve has various phases corresponding to the changes in the tissue, each representing a physical parameter. The tissue has the initial toe region with less resistance and a steep resistant region of increased load. This is the elastic region and gives the measure of the stiffness of the tissue. The tissue undergoes permanent deformation upon reaching maximum load.

4.2.1 Load to failure of the scars

4.2.1.1 Effects of GSNO, SNAP and GSH on load to failure

The maximum load to failure of the control group increased from 6.0 ± 0.47 N to 9.8 ±1.51 N from the 5th to the 10th post-wounding day (Fig. 4.4). All the groups showed an increase in the load from the 5th to 10th post-wounding day (Table 4.1). The load of the GSNO group was significantly higher than the control, SNAP and GSH treated scars on the tenth post-wounding day. SNAP and GSH treated groups showed no significant difference in the load to failure of the scars.

4.2.1.2 Effects of AG on load to failure

The maximum load to failure of AG treated scars was significantly lower ($p<0.05$) than the GSNO treated group at the 10th post-wounding day.

Treatment	5 Days	10 Days
Control	6.0 ± 0.47	9.8 ± 1.51
GSNO	7.84 ± 0.79 ^{e}	16.6 ± 2.65 ^{a,c,d,e}
SNAP	5.8 ± 0.15	10.1 ± 1.38
AG	5.1 ± 0.56	11.39 ± 1.91
GSH	4.34± 0.47	7.81 ± 1.51

Table 4.1: Load to failure of scars following treatment of GSNO, SNAP, AG and GSH. Measurements were taken at 5 and 10 days post-wounding. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) GSH and e) AG. Note that the comparisons were made between the groups of the same day.

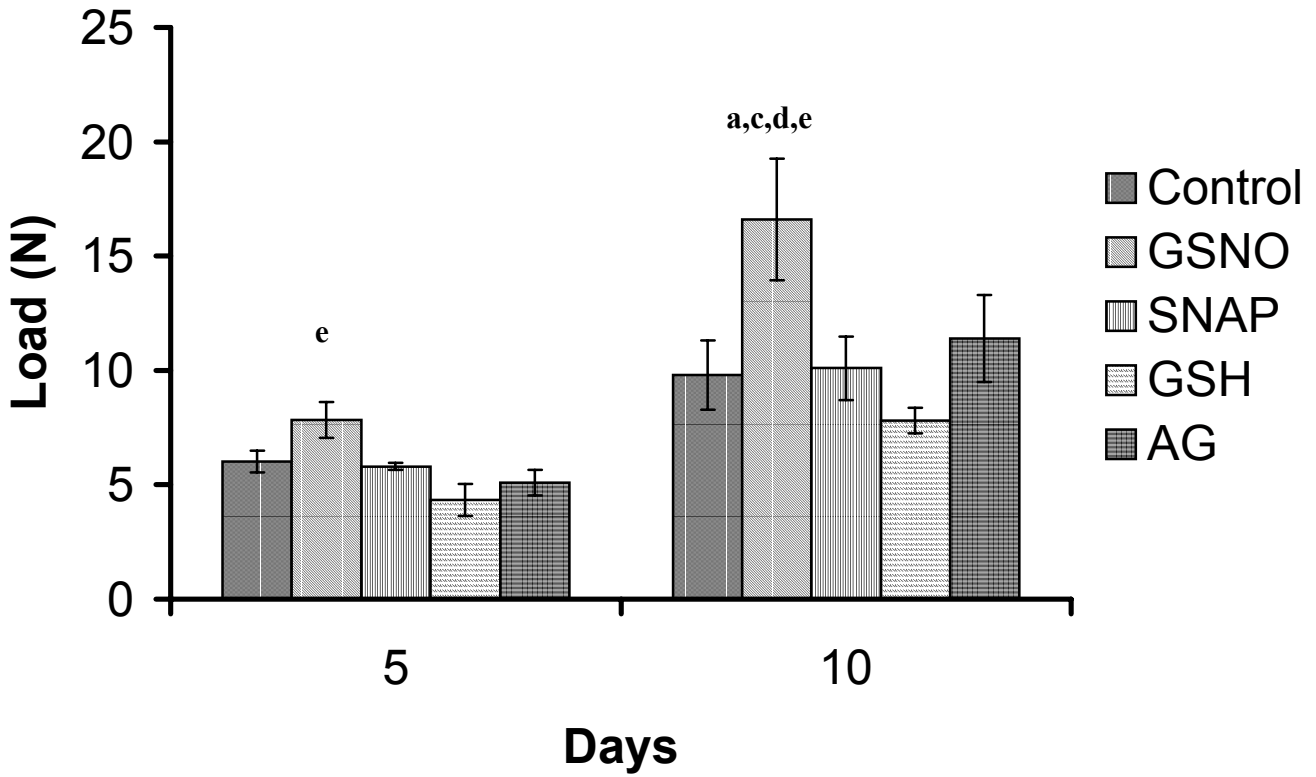


Fig. 4.4: Load to failure of scars treated with GSNO, SNAP, GSH and AG. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) GSH and e) AG. Note that the comparisons were made between the groups of the same day and error bars correspond to SEM. Y-axis represents load to failure (N).

4.2.2 Stiffness of the scars

Stiffness is defined as the maximum resistance offered by the scar to the force applied. This is interpreted as the slope of the log phase of the load deformation curve (Fig. 4.3).

4.2.2.1 Effects of GSNO, SNAP and GSH on scar stiffness

The stiffness of control group was 1.86 ± 0.26 N/mm at the 5th post-wounding day and 2.87 ± 0.45 N/mm at tenth post-wounding day (Fig.4.5). The stiffness of the GSNO group was significantly higher than the GSH group at 5 days. After 10 days of healing the stiffness of GSNO treated group was significantly higher than the control, SNAP and GSH groups (Table 4.2). SNAP treated group showed no significant change whereas GSH group had lower stiffness than the control, SNAP and GSNO groups at the tenth day ($p<0.05$).

4.2.2.2 Effects of AG on scar stiffness

AG treated scars had lower stiffness compared to GSNO group on the fifth day ($p<0.05$) (Fig. 4.5). After 10 days of treatment AG had significantly lower stiffness compared to control, SNAP and GSNO groups ($p<0.05$).

Treatment	5 Days	10 Days
Control	1.86 ± 0.26	2.87 ± 0.45
GSNO	2.82 ± 0.60 [d,e]	4.56 ± 0.64 [a,d,e]
SNAP	2.03 ± 0.16 [e]	2.91 ± 0. 52 [e]
AG	1.54 ± 0.16	1.7 ± 0.491 [a]
GSH	0.992 ±0.17	1.79 ± 0.25 [a]

Table 4.2: Stiffness of scars following treatment of GSNO, SNAP, AG and GSH. Measurements were taken at 5 and 10 days post-wounding. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) GSH and e) AG. Note that the comparisons were made between the groups of the same day.

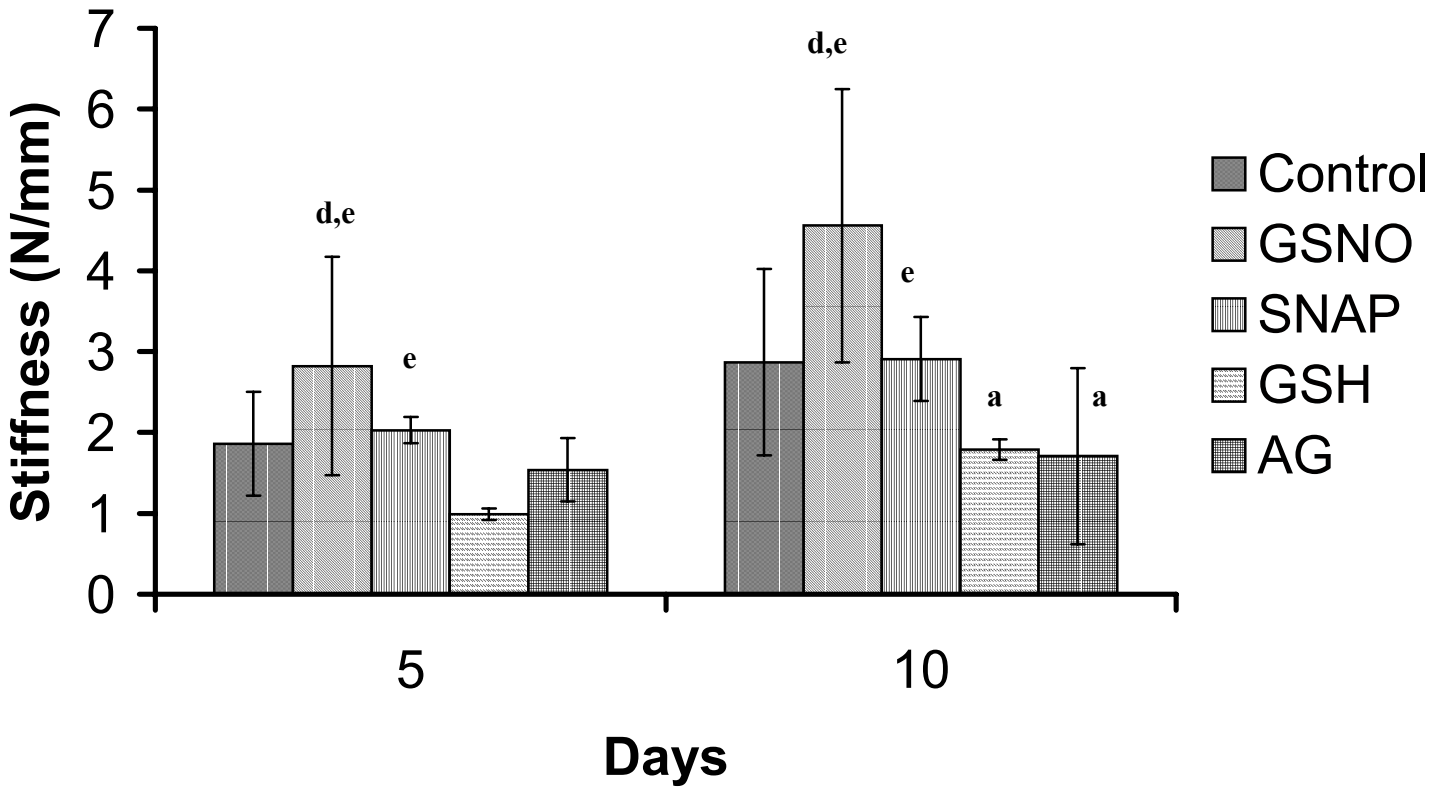


Fig. 4.5: Stiffness of scars treated with GSNO, SNAP, GSH and AG. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) GSH and e) AG. Note that the comparisons were made between the groups of the same day and error bars correspond to SEM. Y-axis represents stiffness (N/mm)

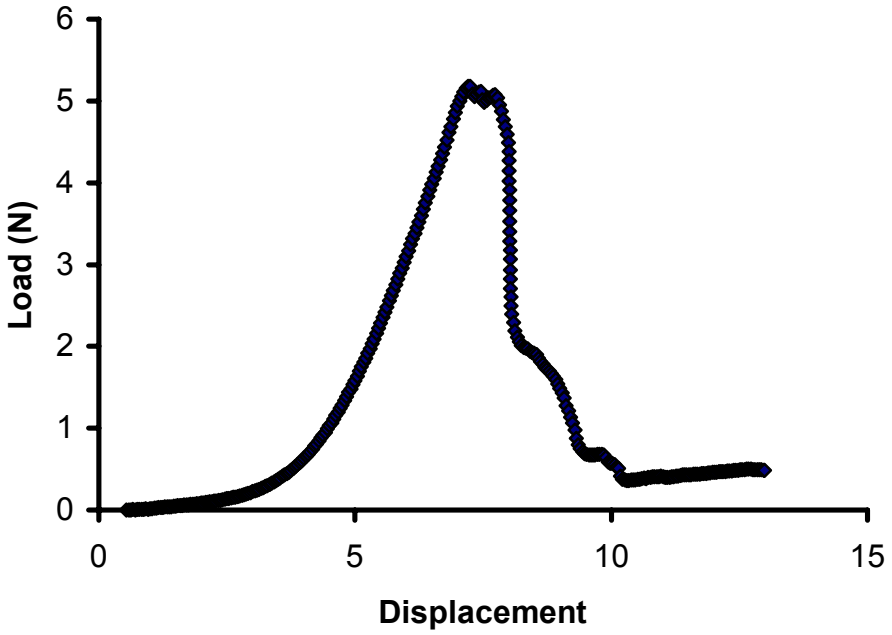


Fig. 4.6: Sample tracing of load displacement of a 5 day Control scar. X-axis represents displacement (mm) and Y-axis is the load to failure (N).

4.3 Collagen content

Hydroxyproline, which is the main component of collagen, is quantified in the scars of all groups of animals. The total hydroxyproline was quantified with reference to the standard straight-line obtained by plotting various known concentrations of the compound (Fig. 4.7). The samples of scars at earlier days consisted mainly of the granulation tissue. Any scab formed was discarded before preserving the tissue. Care was taken not to involve the sleeve of normal skin at the margins. The method was repeatable and consistent readings were obtained through out the procedure. The two time points day 5 and day 10 were chosen as they represent the points of peak collagen deposition and remodeling (section 1.4.2). The hydroxyproline content is expressed in terms of microgram per mg of the wet weight of the scar tissue. As hydroxyproline is the indicator of collagen content henceforth the terms hydroxyproline and collagen will be used as synonyms.

4.3.1 Effects of GSNO, SNAP and GSH on collagen content

The collagen content of control scars was 0.856 ± 0.104 μg/mg tissue at 5 days post-wounding and 0.436 ± 0.07 μg/mg after 10 days of wounding (Fig. 4.8). This is similar to the previously reported observations that the collagen deposition decreases after 5-7 days of healing (Zeitz.M, 1978). The collagen deposition characteristics of the GSNO treated animals were similar to that of the control group, but the collagen content was higher by 52% on the 5^{th} post-wounding day and 47% higher on the tenth post-wounding day (Table 4.3). The SNAP treated group had significantly lower collagen content than the control and GSNO groups on the 5^{th} post-wounding day ($p<0.05$). By the tenth post-wounding day no

significant differences were observed in the collagen content of the SNAP group. The collagen content of the GSH treated scars was lower by 76% on the 5th post-wounding day compared to the control group ($p<0.05$) and by the 10th day, it reaches control levels.

4.3.2 Effects of AG on collagen content

The NOS inhibitor, AG treated group had collagen content lower than the control and GSNO treated scars on the 5th post-wounding day ($p<0.05$). By the tenth post-wounding day collagen content was significantly lower than the GSNO group ($p<0.05$).

Group	5 Days	10 Days
Control	0.856 ± 0.10	0.436 ±0.07
GSNO	1.38 ±0.23 [a,c,d,e]	0.83 ±0.17 [a]
SNAP	0.459 ±0.16 [a]	0.561 ±0.07
GSH	0.204 ±0.04 [a]	0.65 ±0.15
AG	0.474 ±0.08 [a]	0.484 ±0.13 [b]

Table 4.3: Collagen content of scars. The collagen content (μg/mg) in the scars treated with GSNO, SNAP, GSH and AG are shown in this table. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) GSH and e) AG. Note that the comparisons were made between the groups of the same day

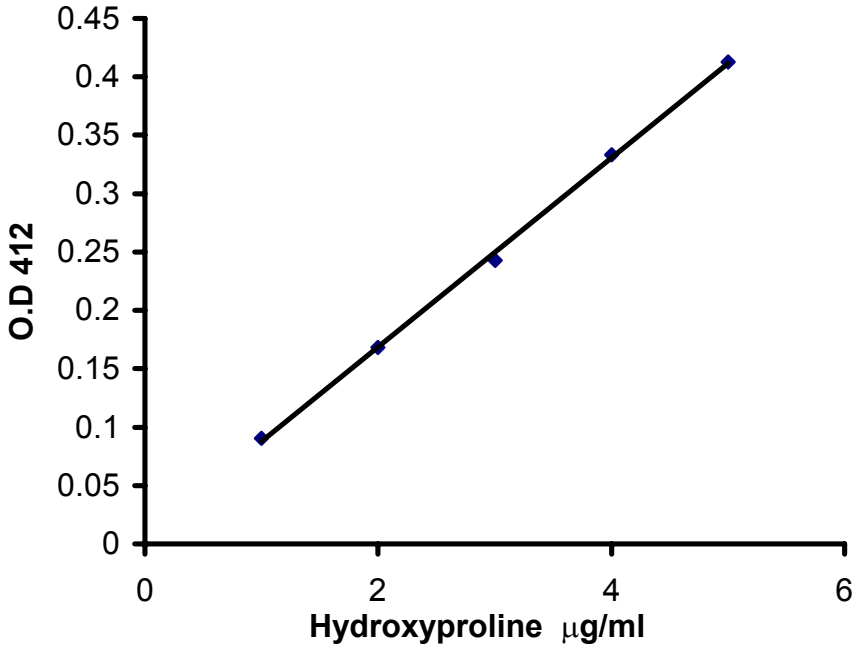


Fig. 4.7: Standard straight line graph of hydroxyproline assay. X-axis corresponds to the hydroxyproline concentrations and the Y-axis corresponds to optical density at 412nm. Each point on the line corresponds to an average of two readings.

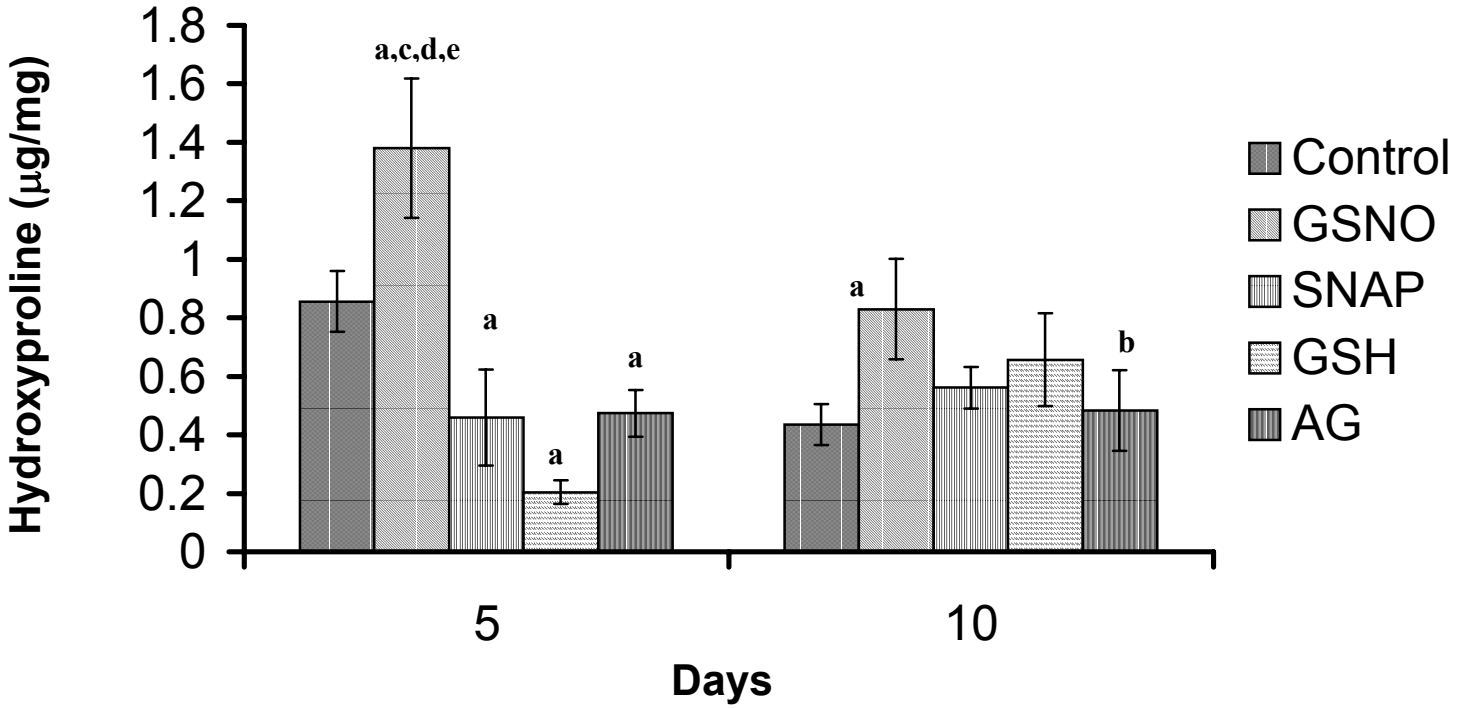


Fig. 4.8: Hydroxyproline concentration of scars treated with GSNO, SNAP, GSH and AG. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) GSH and e) AG. Note that the comparisons were made between the groups of the same day and error bars correspond to SEM.

4.4 Tissue protein content

The protein content of each tissue was measured before conducting MMP activity assay, total nitrite and total glutathione content in the scars (Fig. 4.9). The values obtained by these assays are normalized to the protein content of the corresponding sample. Similarly the plasma nitrite and nitrate is also normalized to the protein content. The protein concentration of the scars varied between 2-3 mg/ml whereas that of plasma was 15-20 mg/ml.

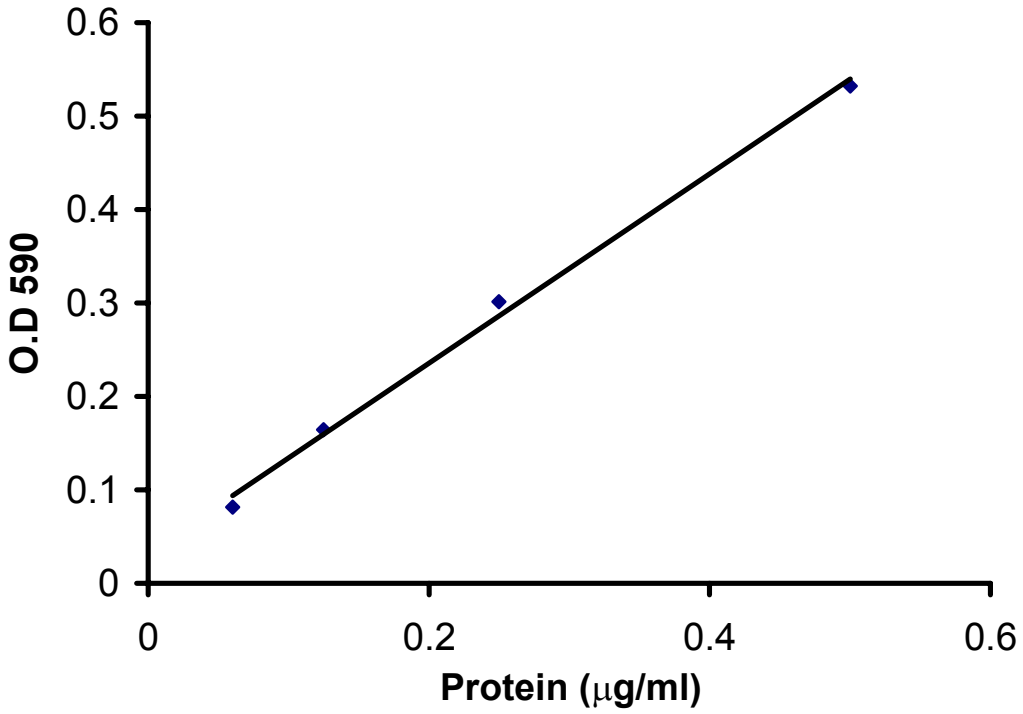


Fig. 4.9: Standard straight line graph for protein estimation. This graph is used in determining protein concentration in scar tissues. Each point represents an average of two readings at 590 nm absorbance. X-axis represents protein concentrations and Y-axis is the optical density (O.D).

4.5 Gelatinase Activity

Gelatinases are expressed in high concentrations during wound healing. The gelatinase (A&B) activity was determined in the scars of animals treated with NO donors and inhibitor. The Chemicon Gelatinase activity kit provides an accurate and repeatable procedure to determine the activity.

4.5.1 Effects of GSNO, SNAP and GSH on wound gelatinase activity

The gelatinase activity in the control scars was 10.38 ± 3.07, units/mg(u/mg) during the third day, 80.55 ±9.57 u/mg on the fifth day, 20.12 ± 4.99 u/mg on the 7^{th} day and 32.63 ± 3.44 on the 10^{th} post-wounding day (Table 4.4, Fig. 4.10). This suggests that there is a very short period (five days) of increased activity of the gelatinases following injury. This is consistent with the previously reported studies by Chia Soon (2000) that the action of gelatinase is transient. The activity of the GSNO treated scars was similar to that of the control scars. At the tenth post-wounding day gelatinase activity in the GSNO treated group is significantly lower than the control scars ($p < 0.05$). SNAP had lower activity during the 5^{th} post-wounding day compared to control and GSNO ($p<0.05$). On the 10^{th} post-wounding day SNAP treated scars had lower gelatinase activity compared to control group. Gelatinase activity was decreased in GSH treated scars on the 5^{th} and the tenth day compared to control and was lower than the GSNO group on the 5^{th} post-wounding day ($p<0.05$).

4.5.2 Effects of AG on wound gelatinase activity

AG treatment increased the scar gelatinase activity significantly high on the third post-wounding day, after which the activity decreased. The activity in the AG treated scars was significantly higher than the control, GSNO, SNAP and GSH treated scars on the third post-wounding day. The activity significantly decreased by the 5th post-wounding day compared to control, GSNO, SNAP and GSH. The activity of the AG treated scars on the 7th and 10th post-wounding days is not significantly different compared to the control group.

Group	Day 3	Day 5	Day 7	Day 10
Control	10.38 ± 3.07	80.55 ±9.57	20.12 ± 4.99	32.63 ±3.44
GSNO	23.33 ±4.15	88.70 ± 9.3 ^{c,d,e}	7.23 ±3.01	18.14 ±3.25 ^{a}
SNAP	14.7 ± 4.27	10.84 ±2.24 ^{a}	17.34 ± 4.5	13.9 ±4.94 ^{a}
GSH	22.82 ±5.29	25.7 ±3.05 ^{a}	18.15 ± 5.69	16.12 ± 2.29 ^{a}
AG	44.49 ± 11.85 ^{a,b,c,d}	26.4 ±9.14 ^{a}	16.84 ±1.6	27.7 ± 8.21

Table 4.4: Gelatinase activity of the scars. The gelatinase activity (u/mg protein) of the scars treated with GSNO, SNAP, AG and GSH at 3, 5, 7 and 10 days post-wounding are shown in this table. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) GSH and e) AG. Note that the comparisons were made between the groups of the same day.

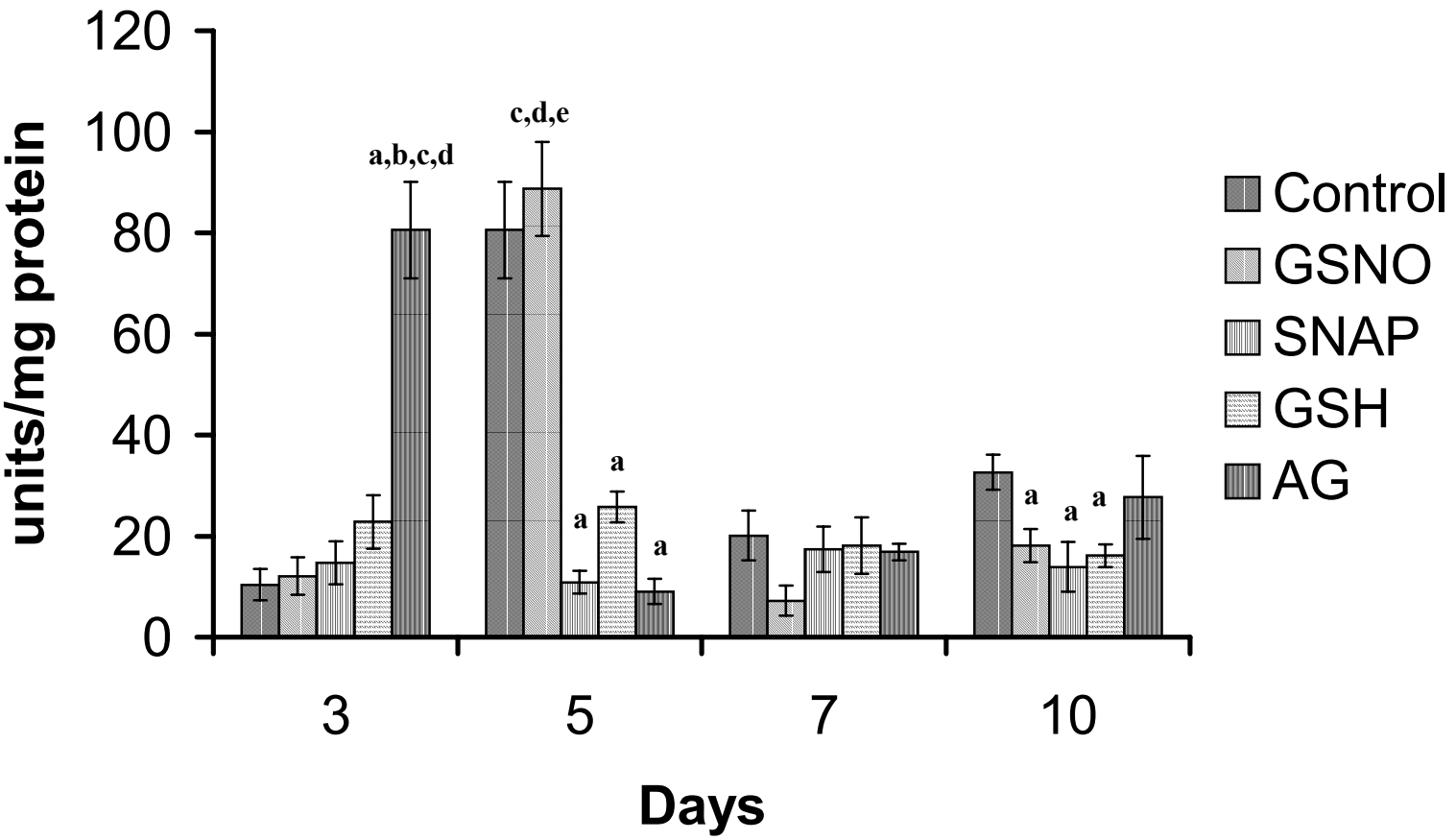


Fig. 4.10: Gelatinase activity in the scars of animals treated with GSNO, SNAP, AG and GSH. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) GSH and e) AG. Note that the comparisons were made between the groups of the same day and error bars correspond to SEM

4.6 Nitrite and nitrate (total nitrite) content

The effects of NO donor and inhibitor on the scar and plasma nitrite and nitrate were measured by the Griess method.

4.6.1 Scar nitrite and nitrate content

The effect of the NO donors and inhibitor scar nitrite content was measured at 3, 5, 7 and 10 days in all the groups.

4.6.1.1 Effects of GSNO, SNAP and GSH on scar nitrite content

The total nitrite was measured in the scars of animals treated with the above-mentioned drugs. The nitrate in each scar was further reduced to nitrite by aspergillus reductase and the total nitrite was measured at 545nm (Fig. 4.11). The nitrite content in the control scars on the 3^{rd}, 5^{th}, 7^{th} and 10^{th} post-wounding days were 28.60 ± 13.71, 30.61 ± 13.63, 24.08 ± 7.74 and 38.81 ± 3.37 μM/mg respectively (Fig. 4.12). The previous reports regarding the time course variation of NO in wound healing suggest that there is a peak by the 3^{rd} post-operative day and later the nitrite and nitrate decreases (Richard H. Lee, 2001). The variation in observation could be due to the change in the tissue model. The previous report was on the subcutaneous sponges placed in the scar area, whereas in this report the nitrite and nitrate are directly measured form the scar homogenate. Nitrite and nitrate being terminal metabolic byproducts of nitric oxide tend to accumulate in the tissue. By three days, nitrite content of the GSNO treated scars was significantly higher than the control SNAP, GSH and AG treated group (Table 4.5). The rest of the time points showed no difference in nitrite and nitrate levels. SNAP showed no

significant change in scar nitrite content. The GSH group had no significant changes on the scar nitrite and nitrate content.

4.6.1.2 Effects of AG on scar nitrite content

The scar nitrite content of AG treated scars showed a trend towards decreased values. No statistical significance was seen as the variation in each group is very high. AG scars had significantly lower nitrite content on the 3rd post-wounding day compared to GSNO group. On the 5th day AG group nitrite values were significantly lower than the SNAP group ($p<0.05$). By the tenth post-wounding day the scar nitrite content was lower than the control and GSNO treated group.

Group	Day 3	Day 5	Day 7	Day 10
Control	28.6±13.71	30.61±13.63	24.08±7.74	38.81±3.37
GSNO	63.88±20.77 [a,c,d,e]	32.9±3.60	24.53±1.24	42.31±13.29
SNAP	23.71±3.47	47.61±8.74	36.99±6.3	33.56±8.12
GSH	11.70±1.88	20.97±7.88	21.38±3.72	19.38±1.33
AG	7.68±1.28	11.50±5.67 [c]	26.45±15.09	12.14±2.14 [a,b]

Table 4.5: Nitrite and Nitrate content of the scars. The total nitrite content of the scars treated with GSNO, SNAP, AG and GSH at 3, 5, 7 and 10 days post-wounding are shown in this table. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) GSH and e) AG. Note that the comparisons were made between the groups of the same day.

4.6.2 Plasma nitrite content

Following the administration of NO donors and iNOS inhibitors, the changes in the plasma nitrite and nitrate were monitored. The increase in the plasma nitrite was taken as a reference for the harvesting of the tissues. Therefore the time at which the plasma nitrite and nitrate increase was noted and the scar specimens were harvested accordingly to observe the maximum response following treatment. This was done by reducing the nitrate to nitrite by aspergillus reductase enzyme and adding the Griess reagents (Section 3.2.6.1).

4.6.2.1 Effects of GSNO and SNAP on the plasma nitrite

The plasma nitrite was measured at 1, 3 and 5 hours following administration of GSNO and SNAP. The plasma nitrite at various hours was compared with control group. The mean plasma nitrite of all the control animals was 0.498 ±0.084 (Fig. 4.13). GSNO increased plasma nitrite by 3 hours after which it returned to normal (table 4.6). SNAP significantly increased the plasma nitrite content at 3rd and 5th hour after injection. The administration of GSNO in our study was much higher compared to the previous reports (Stefania, 1999). SNAP on the other hand increased the plasma nitrite significantly beginning from the first hour.

4.6.2.2 Effects of AG on plasma nitrite

The plasma nitrite concentrations did not vary following AG administration. Previous studies have suggested higher dose administration of AG (Michael Schaffer, 1999). In our study, high doses were administered initially but the effects of higher doses of AG are detrimental as it causes hypertension.

Group	1 hour	3 hours	5 hours
GSNO	0.428±0.02	0.679±0.04 [a,d]	0.496±0.057
SNAP	0.815±0.2 [a,b,d]	1.44±0.281 [a,b,d]	0.62±0.068 [a,b,d]
AG	0.316±0.071	0.372±0.04	0.39±0.052

Table 4.6: Nitrite and Nitrate content of the plasma. The total nitrite content of the plasma treated with GSNO, SNAP and AG at 1, 3 and 5 hours after the initial injection of the drug are shown in this table. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) AG. Note that the comparisons were made between the groups of the same day and error bars correspond to SEM.

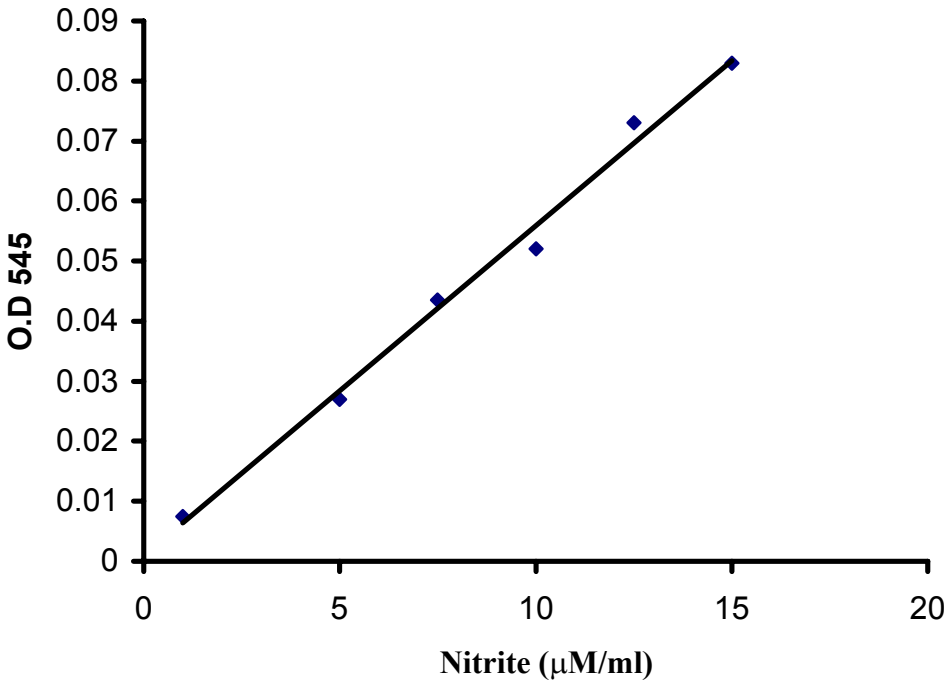


Fig 4.11: Standard straight line graph of nitrite concentrations. The standards are prepared by known concentrations of sodium nitrite ranging from 2.5– 15 μM/ml. The concentrations are measured at an absorbance of 545nm. Each point represents an average of two readings

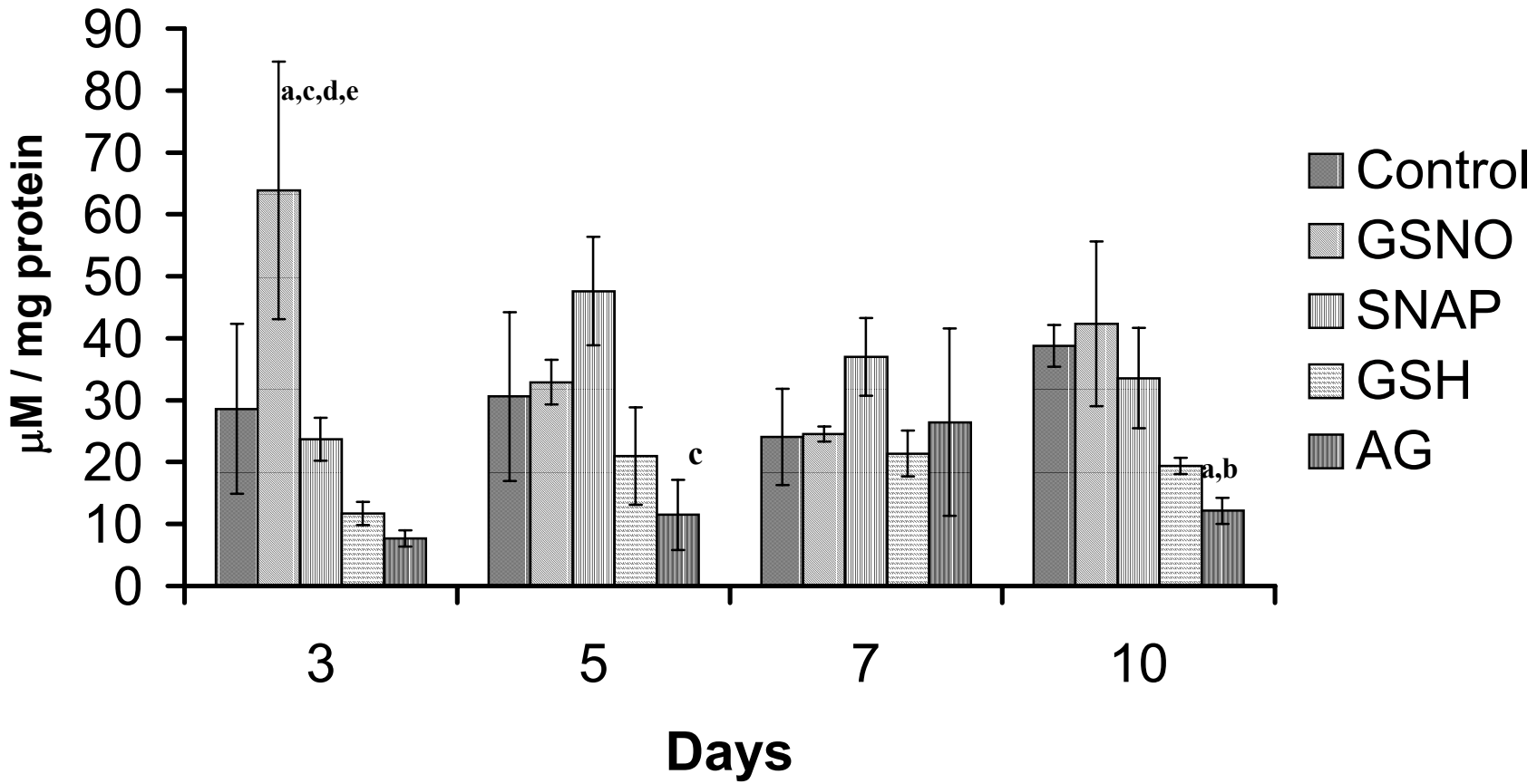


Fig. 4.12: Nitrite content of scar samples obtained at 3, 5, 7 and 10 days post-wounding. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) GSH and e) AG. Note that the comparisons were made between the groups of the same day and error bars correspond to SEM.

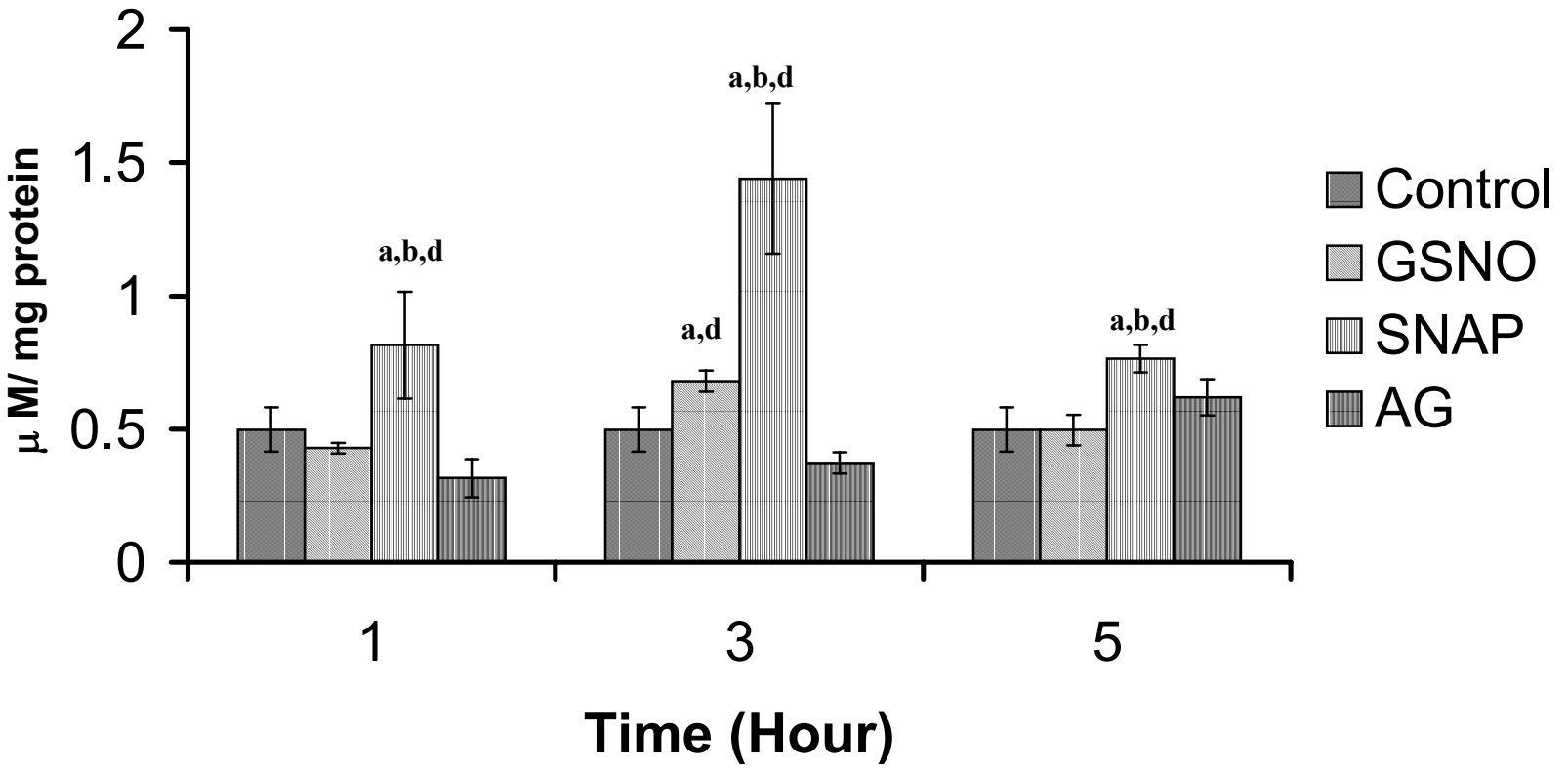


Fig. 4.13: Plasma nitrite concentration following the administration of GSNO, SNAP and AG (n=6). The plasma samples were taken 1, 3 and 5 hours after the administration of the drugs. Error bars correspond to SEM and the statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP and d) AG. Note that the comparisons were made between the groups of the same day and error bars correspond to SEM.

4.7 Glutathione content

The drugs administered interact with the tissue glutathione. The cellular glutathione acts as a major sink for most of the nitrites and nitrates formed in the cell. Therefore the variation in the levels of glutathione in the scar was measured. Scar GSSG levels were measured using the Cayman Glutathione assay kit. The results were compared with the standard straight line obtained by known concentrations of GSSG standards (Fig. 4.14). The GSSG content of each scar was normalized to the protein content of the scar.

4.7.1 Effects of GSNO, SNAP and GSH on scar glutathione content

The glutathione content of the control scars was 19.61±4.17, 18.34±1.81, 16.32±2.8 and 27.48±3.59 μM/mg protein on the 3rd, 5th, 7th and 10th post-wounding day (Table 4.7, Fig. 4.15). Therefore the GSSG content was constantly maintained throughout these time points. The GSSG content of GSNO group decreased significantly on the 10th post-wounding day compared to control ($p<0.05$). SNAP treated scars had significantly lower GSSG levels on 3rd, 5th, 7th and 10th compared to control, GSNO and GSH groups. GSH treated group had significantly higher GSSG than the GSNO group on the 7th and 10th post-wounding day.

4.7.2 Effects of AG on scar glutathione content

Scar GSSG content in the AG treated animals were relatively lower compared to the Control and GSNO group on the 3rd and 5th day post-wounding ($p<0.05$). The SNAP

treated group had significantly lower GSSG content than the AG group at 3rd, 5th, 7th and 10th post-wounding days.

Group	Day 3	Day 5	Day 7	Day 10
Control	19.61±4.17	18.34±1.81	16.32±2.8	27.48±3.59
GSNO	17.90±2.19 [c]	21.57±4.92 [c,d,e]	11.84±1.51	17.17±2.95 [b]
SNAP	3.49±0.647 [a]	2.16±0.862 [a]	2.86±0.726 [a,b]	3.88±1.14 [a]
GSH	14.2±1.05 [b]	13.15±0.938 [b]	22.22±6.09 [b]	26.69±3.07 [b]
AG	11.01±1.48 [a,b]	10.77±1.74 [b]	13.94±0.505 [b]	21.26±1.5 [b]

Table 4.7: Glutathione (GSSG) content of the scars. The glutathione content of the scars treated with GSNO, SNAP, AG and GSH at 3, 5, 7 and 10 days post-wounding are shown in this table. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) GSH and e) AG. Note that the comparisons were made between the groups of the same day.

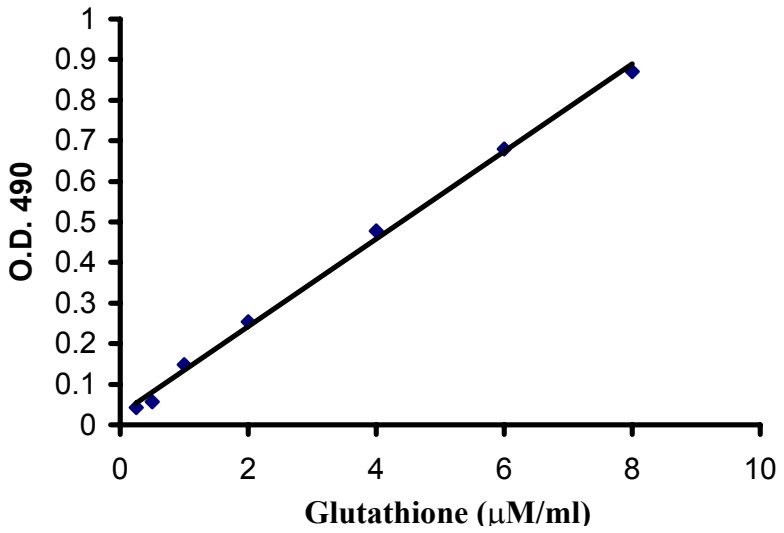


Fig 4.14: Standard straight line graph of glutathione determined by Cayman Glutathione assay kit. The graph represents the oxidized form of glutathione (GSSG). The readings are an average of two independent samples. The concentration of glutathione is read as an absorbance at 490 nm. X-axis represents the concentration of GSSG standard and Y-axis represents absorbance at 490 nm

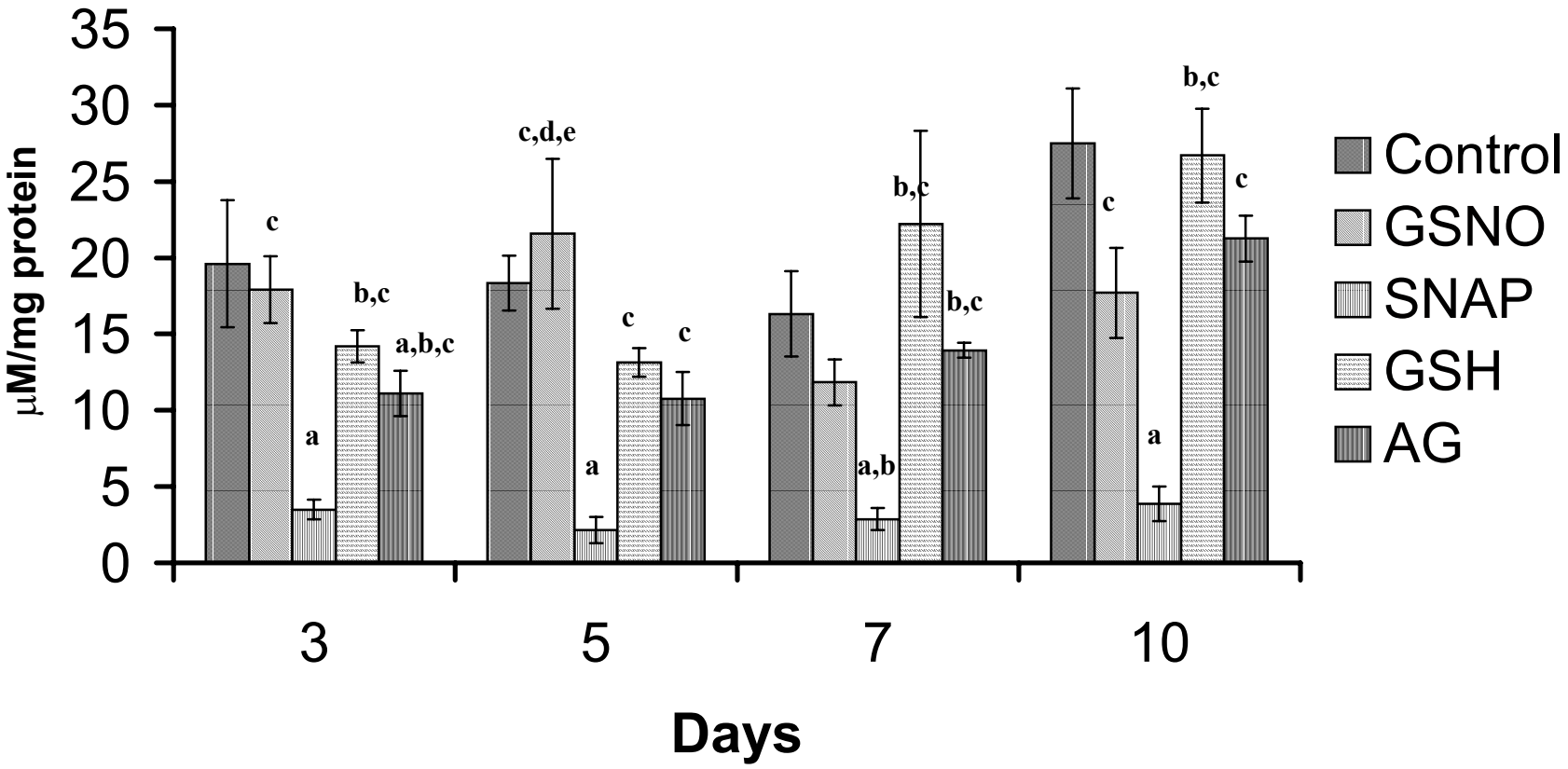
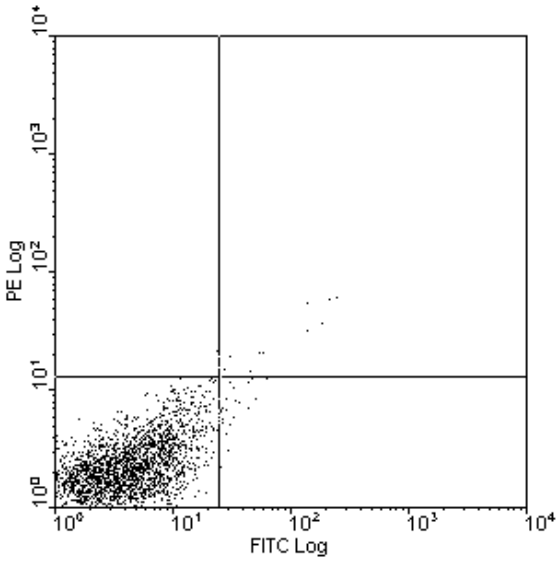


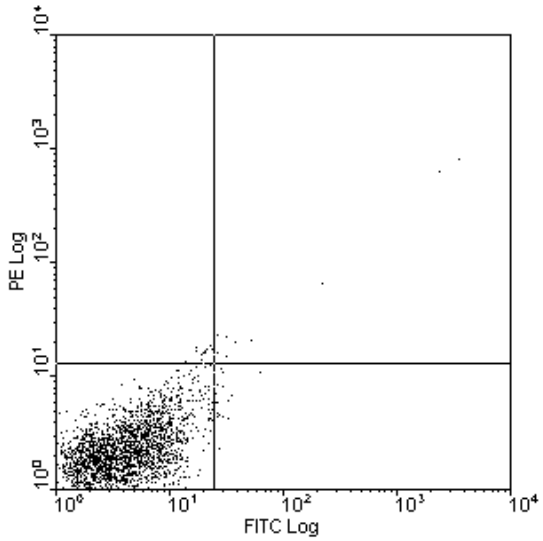
Fig. 4.15: Total glutathione concentration of scars at 3, 5, 7 and 10 days post-wounding. Results are mean ± SEM (n=6). The statistical significance $p<0.05$ is in comparison with a) control, b) GSNO, c) SNAP, d) GSH and e) AG. Note that the comparisons were made between the groups of the same day and error bars correspond to SEM.

4.8 Peritoneal macrophage flow cytometry

Nitric oxide is involved in the modulation of inflammatory responses, immune regulation and host defense. Therefore it is essential to examine the effect of NO modulating drugs on immune cells. As the NO donors and inhibitors were administered intra-peritoneally, the effects of these drugs on the expression of surface markers of peritoneal immune cells were studied. The MHC Class I, Class II and ICAM-1 markers are expressed on most of the peritoneal immune cells. To study the effect of these cells, single colour flow cytometry was conducted on the cellular extract obtained from the peritoneal lavage. The results obtained by flow cytometry are analysed using WinMDI version 2.8. Mean percentage of the population of the cells that stained positive was compared with the IgG isotype control (Fig. 4.16). All the peritoneal lavages were carried out in the rats after 3 hours of drug administration, which corresponds to the time point of maximum response i.e., increased plasma nitrite.



A



B

Fig. 4.16: Dot-Plot representation obtained by plotting the A) unstained peritoneal cells (negative control) and B) IgG isotype control. The analysis is done by plotting the log of FITC fluorescence in the X-axis and PE in the Y-axis. The gating is done based on the IgG readings.

4.8.1 Effects of GSNO, SNAP and AG on MHC Class I surface marker

MHC Class I marker is expressed by most of the nucleated cells. Expression of MHC class I is important in the recognition of self and non-self antigens.

4.8.1.1 Effects of GSNO and SNAP on MHC Class I marker

The expression of Class I marker was seen in 26.74 % of the peritoneal cells in the control group (Fig 4.17). The animals treated with GSNO had 22.35 % positive population and that of SNAP was 23.68 %. Neither GSNO nor SNAP showed any significant effect on the expression of Class I surface marker.

4.8.1.2 Effects of AG on MHC Class I marker

The expression of Class I surface marker was seen on 16.56 % of peritoneal cells in the AG treated group. Though the AG group had lower percentage of cells staining positive, no statistical significance were observed. Aminoguanidine has previously been studied to inhibit Class I marker in preventing pot-transfusion development of antidonor antibodies (Bang A, 1996). But in this study no significant difference was observed.

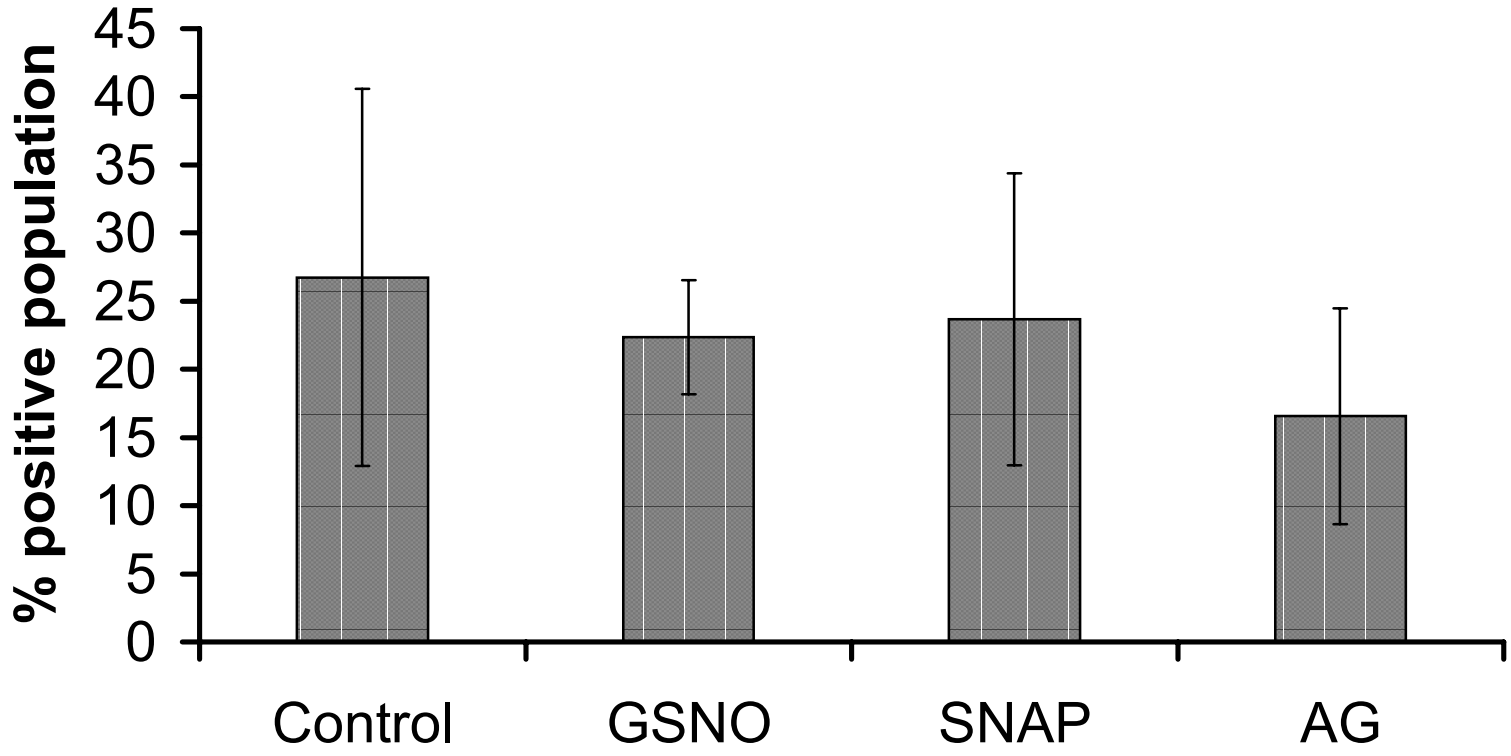


Fig. 4.17: Expression of MHC Class I surface markers on the peritoneal cells. The mean percentage of cells staining positive for the Class I surface marker are represented in each group (n=6). Error bars correspond to SEM. Y-axis represents the percentage of positively stained population.

4.8.2 Effects of GSNO, SNAP and AG on MHC Class II surface marker

The effect of NO donors on the expression of MHC Class II antigens was investigated in the peritoneal cells. Class II markers are expressed on the surface of macrophages, B-cells and dendritic cells. Expression of Class II molecules indicates the immune cell activation.

4.8.2.1 Effects of GSNO and SNAP on MHC Class II marker

There was significant increase in the peritoneal cells staining positive in animals treated with SNAP ($p<0.05$). GSNO treated group showed no significant changes compared to the control group (Fig. 4.18). Our observations are similar to the previous reports, where GSNO does not affect rat splenocyte MHC Class II expression (Badovinac V, 2000). The effects of SNAP in our study are in contrary to the previous observations where SNAP administration led to decrease in expression of Class II surface marker (Lu L, 1996).

4.8.2.2 Effects of AG on Class II marker

The expression of Class II surface marker in the AG treated group was significantly lower than that of the SNAP treated animals. In previous studies, expression of MHC Class II surface marker was reduced by AG and N-methyl-L-arginine (Ross R, 1998). This is similar to our observation that administration of AG decreases mean positive population of Class II markers in the peritoneum.

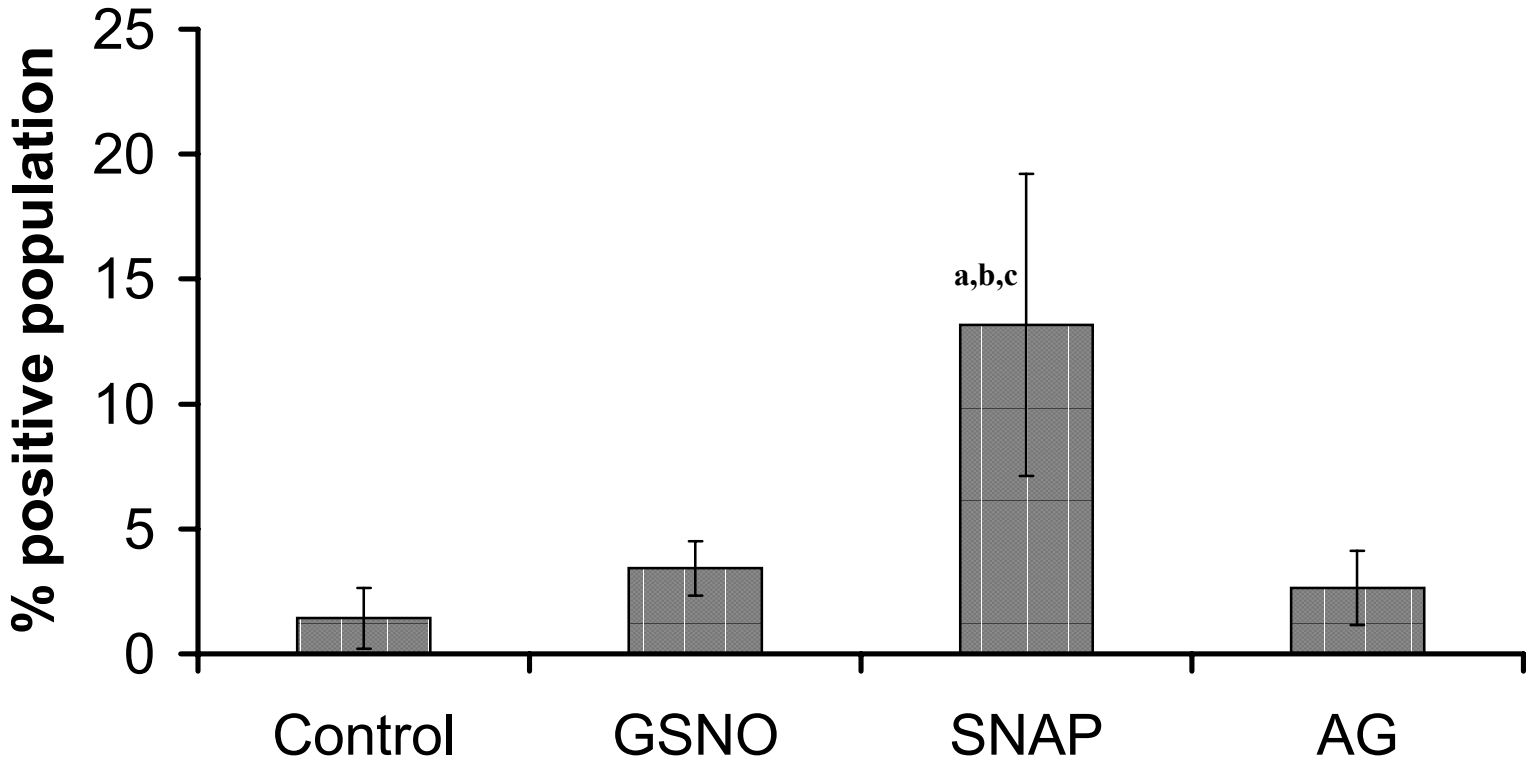


Fig. 4.18: Expression of MHC Class II surface markers on the peritoneal cells. The mean percentage of cells staining positive for the Class II surface marker are represented in each group (n=6). Error bars correspond to SEM. Y-axis represents the percentage of positively stained population. a, b and c are the statistical significance ($p<0.05$) compared to control, GSNO and AG respectively.

4.8.3 Effects of GSNO, SNAP and AG on ICAM-1 surface marker

The expression of ICAM-1 (CD54), which plays a significant role in the adhesion of lymphocytes to endothelial cells and regulates immune cell function in the peritoneum (Patarroyo M, 1994). The effect of NO donors and inhibitors was studied on the expression of ICAM-1 markers on the immune cells of the peritoneum.

4.8.3.1 Effects of GSNO and SNAP on ICAM-1 surface marker

The ICAM-1 was expressed in 33.1 % of the peritoneal cells in the control group. The GSNO group had 7.47 % positive staining cells and that of SNAP group was 4.28% (Fig. 4.19). Previous studies have shown that SNAP attenuates the upregulation of ICAM-1 expression in rat endothelial cells (Christian Kupatt, 1997) and GSNO decreases the expression of ICAM-1 in human skin. There was significant decrease in the expression of ICAM-1 markers in the GSNO and SNAP treated group.

4.8.3.2 Effects of AG on ICAM-1 expression

AG treated animals had 8.02% positive cells for ICAM-1. This was significantly lower compared to the control group ($p<0.05$). NO inhibitors are known to increase leukocyte adhesion (Christian Kupatt, 1997 & Benedetta Bussolati, 2002). Following AG administration, ICAM-1 expression decreased in our experimental setup. In a separate study conducted by Seijiro Kado et al (2001), it was shown that administration of AG, suppressed the expression of ICAM –1 in endothelial cells.

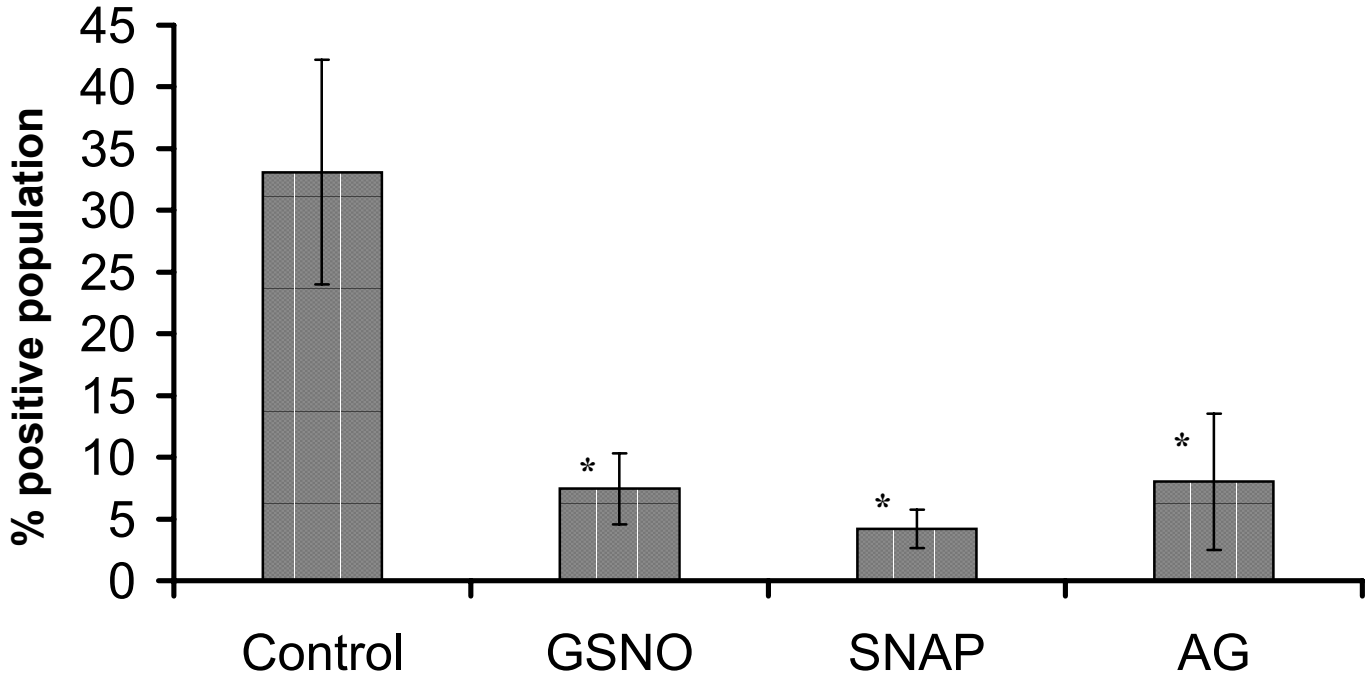
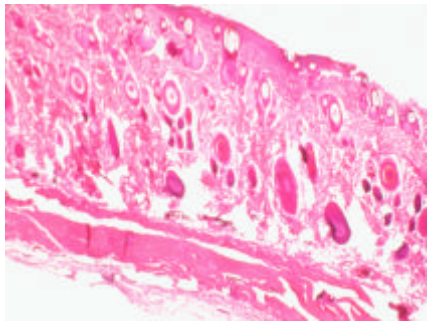


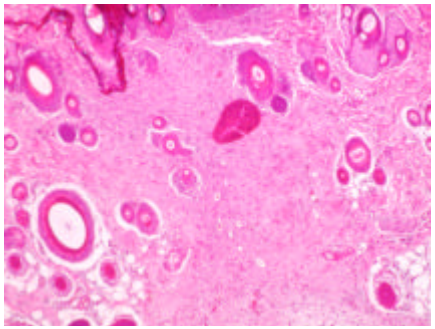
Fig. 4.19: Expression of ICAM-1 surface markers on the peritoneal cells. The mean percentage of cells staining positive for the ICAM-1 surface marker are represented in each group (n=6). Error bars correspond to SEM. Y-axis represents the percentage of positively stained population. * is the statistical significance ($p<0.05$).

4.9 Histology

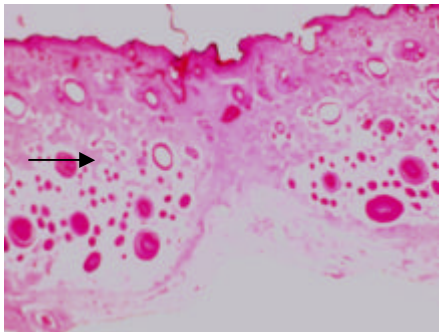
Gross examination of the scars did not show any significant changes under light microscopy (Fig. 4.20). Histologic examination of the scars from one or more animal from each group was done at different time points. The scar tissue consisted of fibroblasts and dense collagen bundles. Blood vessels were dilated, with margination and extravasation of leukocytes. The surface of the wound was covered with a crust composed of desiccated, degenerate leukocytes, fibrin and proteinacious debris. Subjacent to this was a matrix with edematous connective tissue, fibroblasts, fibrin, proteinacious debris and leukocytes. Neovascular networks and proliferating fibroblasts created a granulation tissue bed. Epidermis at the cut edges became hyperplastic. Migration of the epidermal tongue undermined the scab, which consisted of a desiccated surface crust with subjacent layer of intermediate scab matrix consisting of proteinaceous debris, fluid, leukocytes, fibrin and fibroblasts but no recognizable capillaries. Migration of the epidermis continued until the defect was bridged. Granulation tissue continued to proliferate upwardly until the wound bed was filled, and then began its maturation into fibrous connective tissue of the eventual scar. Fibroblasts began forming rows parallel to the surface to the wound. After the defect was bridged and filled in capillaries began to recede. Neutrophils were present within the wound bed in lessening numbers.



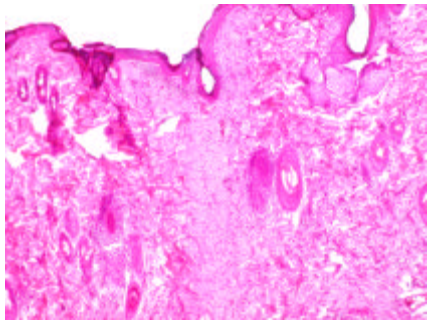
A



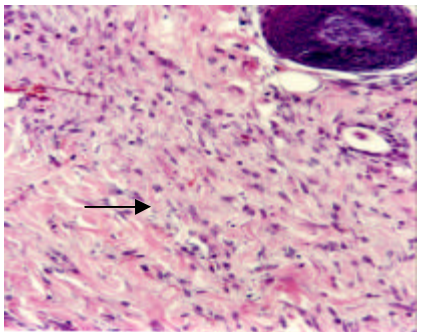
B



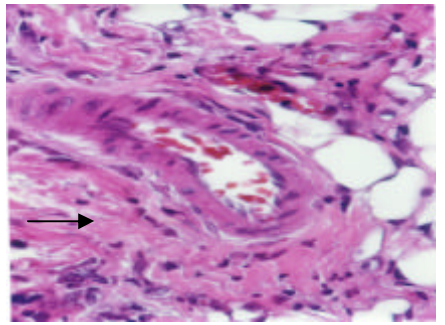
C



D



E

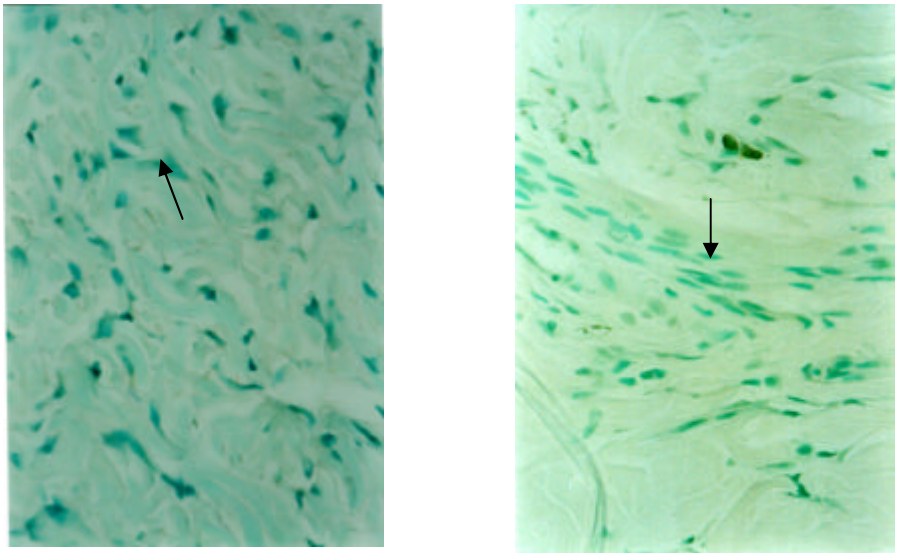


F

Fig. 4.20: Haemotoxylin and Eosin staining of normal skin and scars. A) normal skin (20X), B) scar (20X), C) normal scar (10X), D) Scar with fibrous tissue (20X), E) close view (40X) of fibrous tissue, dense fibroblasts are seen (arrow), F) close view of a capillary in the scar tissue (40X).

4.9.1 Immunohistochemistry

Immunohistochemistry of MMP2 and MMP9 were done in animals treated with GSNO and AG. eNOS and iNOS isoenzymes were localized using monoclonal antibodies. The slides were compared with negative controls in which the primary antibody was omitted.



A **B**

Fig. 4.21: Negative controls used in the staining. Scar tissues (A&B) without the primary antibody serve as negative control. This picture shows the close view of the dermis with dense fibroblasts (40X).

4.9.1.1 Immunohistochemistry of MMP2 & MMP9

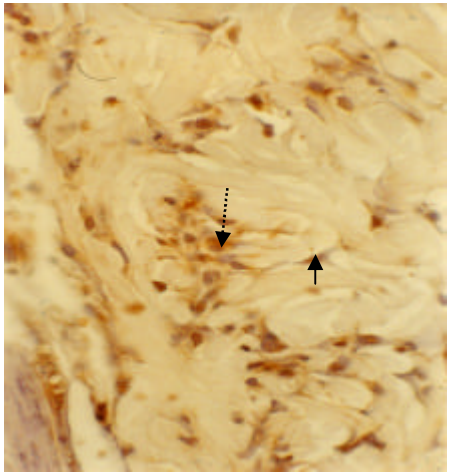
In order to evaluate the effects of GSNO and AG on the protein expression of Gelatinases, localization was done by immunohistochemistry on the GSNO treated scars. The scoring method as previously described by Moochhala S (1996) was adopted in this study. The results were analyzed by Kruskal-wallis test for multiple comparisons of non-parametric data. The median score of staining of each group was compared.

4.9.1.1.1 Effects of GSNO on MMP2 expression in scars

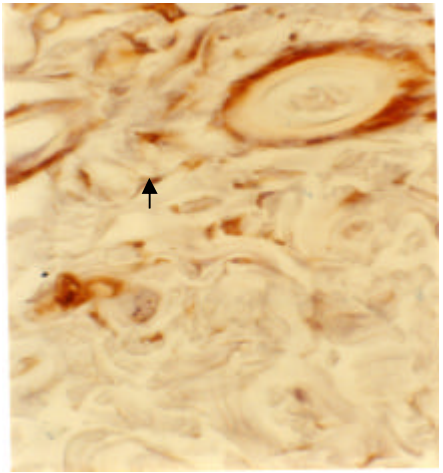
MMP2 immunostaining was done on skin sections at 3, 5, 7 and 10 days in the control and GSNO treated scars (Fig. 4.22). The expression of gelatinase A was seen in most of the cells in the dermis and the epidermis. In the control scars, expression of MMP2 was significantly higher at 3 and 5 days post-wounding the median score was 4, following which it decreases by day 7. By day 10 it reaches a median score of 1. Similarly the Gelatinase A expression in GSNO treated scars was lower in the treated scars. Only on the seventh post-operative day the GSNO treated scar showed marginally higher expression of MMP2 than the control scars. But there was no statistical significance seen in these various scars at 3, 5, 7 and 10 days post-wounding. Note that the expression of the MMP2 is seen in fibroblasts and epithelial cells. No changes are see in the inflammatory infiltrate in the GSNO treated and control animals.

4.9.1.1.2 Effects of AG on MMP2 expression in scars

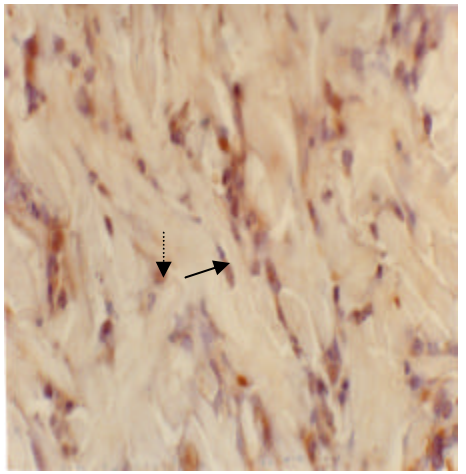
The expression of Gelatinase A was seen in most of the cells in the dermis and the epidermis. There was no significant difference in the scoring of the scars stained for gelatinase-A. No significant histological changes were observed in the scars treated with AG.



A



B



C

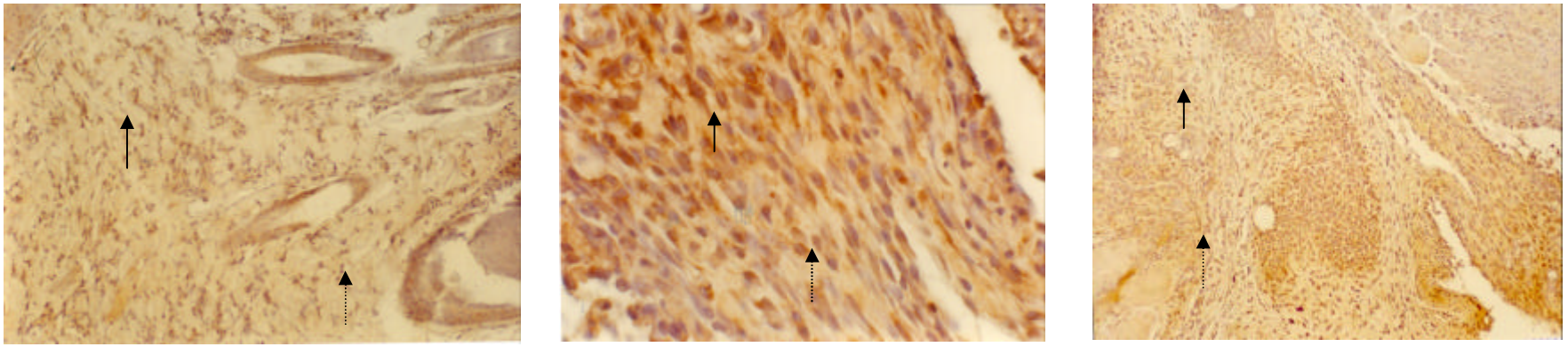
Fig. 4.22: Immunohistochemistry of MMP2 enzymes in scars treated with A) Saline (control) B) GSNO and C) AG. The expression of MMP2 is observed in fibroblasts (arrow) inflammatory infiltrates (dotted arrow). All the microscopic observations were done at 20X magnification.

4.9.1.1.3 Effects of GSNO on MMP9 expression in scars

Immunohistochemistry of MMP9 (Fig. 4.23) in the scars at various time points revealed that there is no significant change in the normal samples at all time points. The expression decreases by 5th post-operative day and again returns to levels similar to the 3rd day. Treatment with GSNO increases the protein expression significantly on day 5 compared to the normal, and the rest of the time points there is no significant difference. The major effect of MMP9 in wound healing is mainly on the epithelialisation, The immunostaining of Gelatinase B shows increased expression by fibroblasts and inflammatory cells.

4.9.1.1.4 Effects of AG on MMP9 expression of scars

Immunostaining of gelatinase-B on AG treated scars revealed no significant difference in the protein expression. The dermal fibroblasts and infiltrated polymorphs showed positive staining of this antibody. Scoring of these sections revealed no significant difference compared to GSNO treated or control scars.



A **B** **C**

Fig. 4.23: Immunohistochemistry of MMP9 enzymes in scars treated with A) Saline (20X) B) GSNO (20X)and C) AG (10X). The expression of MMP9 is observed in fibroblasts (arrow) inflammatory infiltrates (dotted arrow).

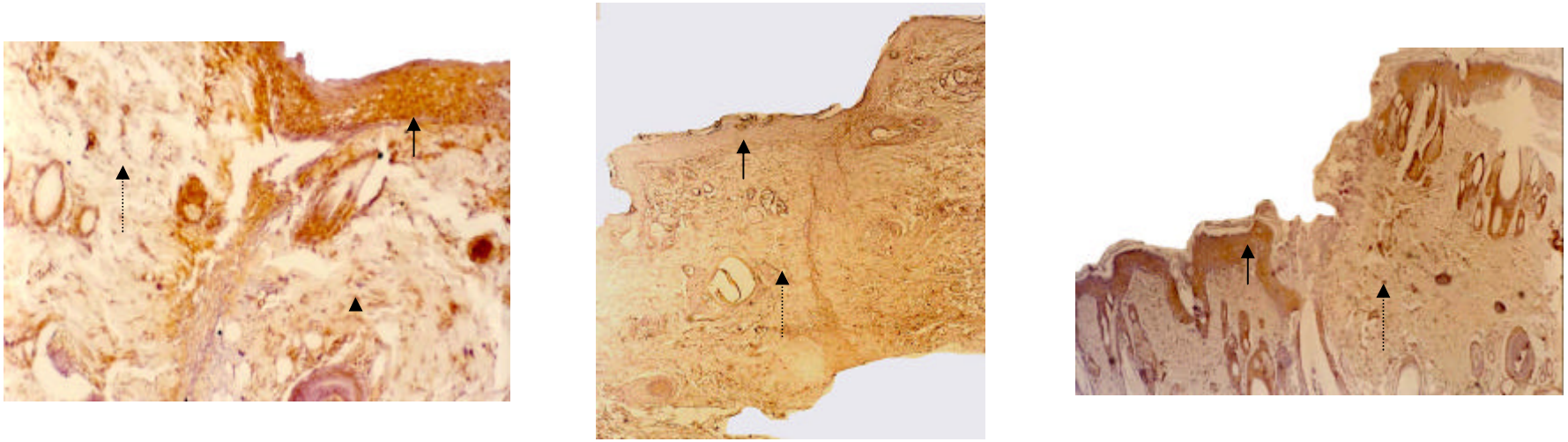
4.9.1.2 Immunohistochemistry of NOS isoenzymes

4.9.1.2.1 Effects of GSNO on eNOS expression in scars

All blood vessel endothelium within wound beds stained strongly positive for eNOS, illustrating the location and density of capillaries. eNOS also stained positive for the epidermal cells more towards the upper layers. Non-wounded skin also showed positive eNOS staining. Immunostaining (Fig. 4.24) was done on the scars at different time points but no significant difference was observed between the groups. There was no noticeable effect of GSNO on the expression of eNOS. The drug does not seem to have any effect on the expression of eNOS isoenzyme. The staining of eNOS revealed that the enzyme was also present in normal uninjured skin.

4.9.1.2.2 Effects of AG on eNOS expression in scars

Immunostaining of AG did not have histologically visible effect on the eNOS expression. The protein was expressed in the epidermal layer and few dermal fibroblasts. No statistical difference was observed.



A **B** **C**

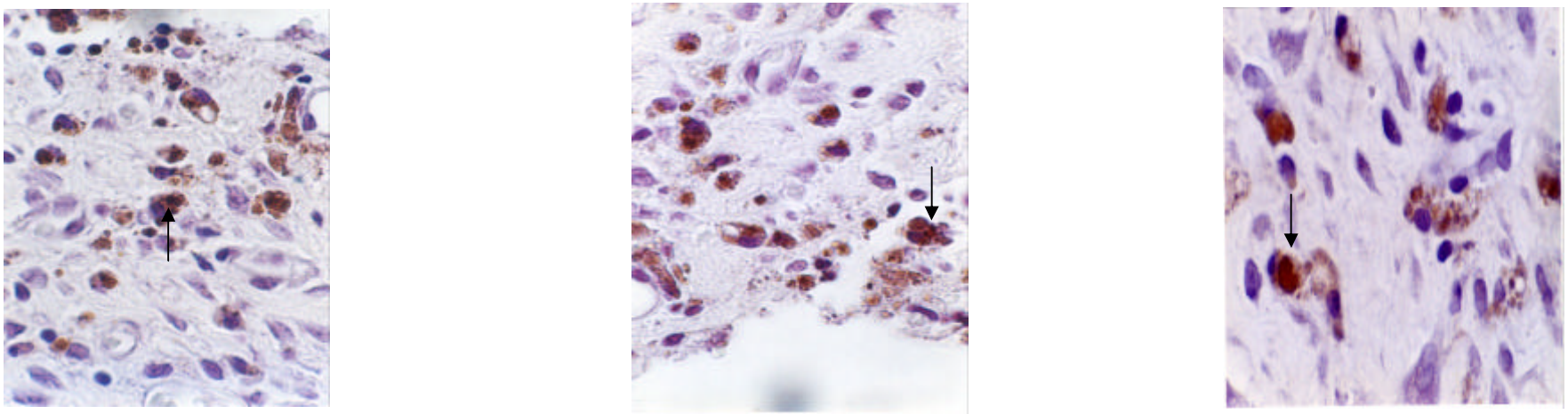
Fig. 4.24: Immunohistochemistry of eNOS enzymes in scars treated with A) Saline (control) B) GSNO and C) AG. The expression of eNOS is observed in keratinocytes, fibroblasts (arrow) and inflammatory infiltrates (dotted arrow). All the microscopic observations were done at 20X magnification

4.9.1.2.3 Effects of GSNO on iNOS expression in scars

Care was taken to clearly examine the slides and to score them. Positive staining of iNOS was not significant and was occasionally seen in a few sections (Fig. 4.25). As the staining was insignificant, scoring could not be done. Most of the specimens even did not have positive staining. The iNOS staining is very much limited to cells such as the polymorphs and macrophage like cells in the scar environment.

4.9.1.2.4 Effects of AG on iNOS expression in scars

No significant difference was seen between treatment groups in immunohistochemical staining pattern for iNOS isoform. AG is a competitive inhibitor of NOS that competes with L-arginine. Therefore it will not affect the expression of the enzyme. The expression of iNOS enzyme was not observed in most of the control, GSNO or AG treated scars. The effects of inhibition are better understood by the analysis of the end products of iNOS pathway i.e nitrite and nitrate.



A **B** **C**

Fig. 4.25: Immunohistochemistry of iNOS enzymes in scars treated with A) Saline (control) B) GSNO and C) AG. The expression of iNOS is observed in inflammatory infiltrates (arrow). All the microscopic observations were done at 20X magnification

Chapter 5

Discussion

5.1 Experimental design

Nitric oxide and glutathione are highly reactive molecules that are known to regulate important cellular functions. In this study we have examined the effects of GSNO, a nitrosothiol which is a well-known NO donor. This compound also produces glutathione upon cleavage. The effects of this drug were compared to another nitrosothiol NO donor, SNAP and glutathione. An iNOS specific inhibitor, AG was adopted to study the effects of NO inhibition on wound healing.

5.1.1 Rate of wound contraction

Wound contraction represents a complex and well co-ordinated event, which involves processes such as migration of keratinocytes and fibroblasts, deposition of matrix tissue, interaction of cells and cytokines leading to proper healing. A square wound model was adopted to compare the effects of a NO donor, GSNO and an iNOS specific inhibitor, AG on wound contraction. The results are interpreted as rate of wound contraction.

5.1.2 Tensile strength

Biomechanical strength is a key factor in determining the final outcome of healing. One of the most crucial phases in wound healing is the progressive increase in biomechanical strength of the tissue resulting from the formation and turnover of granulation tissue. Although, the rat represents one of the most common models used in the study of dermal

wound healing, no detailed study has been performed to correlate the biomechanical and biochemical changes occurring in the rat dermis during normal healing. Standardized protocols were developed for use in a detailed investigation into the biomechanical and biochemical properties of a dermal wound healing model in the rat. The methodology described allowed for the simultaneous and reproducible measurement of tensile strength and collagen content.

5.1.3 Collagen content

In the present study we have examined the effects of GSNO on the collagen content in a rat wound model. In order to attribute the effects of GSNO to it's NO or GSH components, we have used SNAP and GSH to serve as controls.

5.1.4 Gelatinase activity

Using Fluorescent substrate degradation method and immunohistochemistry we studied the effect of NO donors on wound gelatinase expression. During wound healing in rat skin, six MMP genes are expressed (ST1, ST3, GelA, GelB, Col3, MT1-MMP). Among these six MMP genes ST1, GelB, and Col3 are mainly expressed between days 1 and 5 after cutaneous incision, while the three others, MT1-MMP, GelA, and ST3, continued to be expressed at high levels until day 7. High levels of GelA were observed in the wound stroma, particularly on day 5 after cutaneous incision when both the MT1-MMP and GelA RNAs attained their highest expression levels. This observation suggests that GelA activated by MT1-MMP contributes to the remodeling processes

implicated in the restoration of connective tissue. In this study, the expression patterns of gelatinase A and gelatinase B were investigated in rodent cutaneous wound samples. Collagen substrate degradation is a relevant method for investigating the activity of MMPs and, since most MMPs degrade their specific substrates. Due to the general lack of immunohistochemical antibodies detecting only the activated MMP form, a combination of activity and immunolocalization would suggest the differential effect of the NO donors and inhibitors on the level of activity of the enzyme.

5.1.5 Glutathione content

The scar wound glutathione content was measured by the Cayman's glutathione assay kit. In this method the sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 or 414 nm provides an accurate estimation of GSH in the sample. The administration of GSNO and SNAP had varied responses on wound glutathione status.

5.2 Effects of NO donors and inhibitor on wound healing

5.2.1 Effects of GSNO on wound healing

The control wounds healed between 12-14 days post wounding. During the first two days there was an increase in the wound area as a result of traction of the surrounding tissue and loose attachment to the underlying connective tissue. Administration of GSNO improves the rate of wound contraction by 46% compared to controls (saline treated animals). This implies GSNO improves keratinocyte and fibroblast proliferation and migration and enhances collagen deposition, which form the center stage of wound healing. Early wound closure decreases the possibilities of wound infection and other related complications such as development of large scars due to delayed healing. In vitro studies of the effects of GSNO on keratinocytes have shown that it promotes proliferation and differentiation of the cells (Frank S, 1999). Previous studies pertaining to NO releasing drugs were either only on the collagen content or strength of the scar (Witte M.B, 2002; Shi H. P, 2002) whereas we have clearly studied the effects on rate of wound contraction as well. The fact that GSNO improves the load to failure and stiffness shows that it enhances the total wound strength, thus giving a stronger and stiffer scar. To measure the biomechanical properties of skin a standard protocol previously established by Paul RG et al (1997) has been adopted. The improvement in the biomechanical properties and rate of wound contraction indicate that GSNO not only enhances the speed of healing

but also the quality of the wound. This is the first report where the complete outcome of healing in the form of wound contraction and strength of the scar following the administration of a NO donor has been studied. To completely understand the underlying mechanism of improvement in wound healing we quantified the hydroxyproline content, which is a direct measure of the collagen content. Our experiments show that treatment of wounds with GSNO resulted in increased collagen content. The control scars exhibit increased deposition of collagen by the fifth day and at later time points the collagen deposition is lower. The collagen deposition in the GSNO treated scars followed the same pattern as in control scars, i.e. it increases by day 5 and decreases by day 10, but was significantly higher. This implies that GSNO facilitates physiological reparative tissue deposition.

The exact mechanism by which NO enhances collagen deposition is not known. Witte M. B. et al (1996) demonstrated that NO enhances collagen synthesis, most likely at a posttranscriptional level. As wound healing progresses, increased arginase activity produces an environment favorable for fibroblast replication and collagen production (Witte M. B, 2002). Contrary to our findings Arti Shukla et al (1999) have reported a decrease in wound collagen synthesis following sodium nitroprusside (SNP) administration. The release of NO by SNP is negligible. It has been shown by David A. Wink et al (1996) that, compared to many NO donors such as GSNO and SNAP, the release of NO by SNP is insignificant. Therefore the decreased wound collagen deposition following SNP could be a result of the release of more cytotoxic byproducts such as cyanide,

which inhibits iron moiety. Moreover the topical application of SNP results in the release of small quantities of NO, which would diffuse into the external atmosphere than entering the wound environment. The present experiments demonstrate that administration of GSNO increases collagen deposition. The effects of GSNO were studied on the protein expression of gelatinases. Immunohistochemical localization revealed that there was no significant change following administration of GSNO and AG. This may be because the drug might act on the activation mechanisms of the extracellular enzymes. We clearly demonstrated the differences in the GSNO treated and the rest of the groups such as SNAP, AG and GSH groups. MMP activity in wound healing is vital for most of the physiological processes, such as epithelialisation, debridement, infiltration of inflammatory and parenchymal cells (Velimatti kahari, 1997). The above-mentioned processes occur mainly during the phase of inflammation (up-to 3 days) and phase of ECM deposition and re-epithelialisation (up-to 10 days). The activity of MMP is not affected in the initial phase. This indicates that GSNO does not affect the vital physiological processes such as debridement, keratinocyte migration and dissolution of clot, which are chiefly controlled by MMP. Though it has been found that GSNO inhibits MMP activity in platelets and in turn inhibits platelet aggregation the effect is not to such an extent to affect healing. Debridement, inflammation and epithelialisation were all maintained as it was observed that the wounds healed without any delay.

In our study we have observed an increase in the collagen content without any interference of MMP activity. Alteration of this vital process would result in aberrant healing. Any wound enhancing agent necessarily has to have no effect on these enzymes. Certain reports regarding inhibition of MMP activity by NO have been carried out in an in-vitro set-up (Marek W. Radomski, 1992). The MMP inhibitory effect in an in vivo model may not be as pronounced as in vitro as the NO donor compounds undergo various intermediary metabolism pathways. Optimal wound healing involves appropriate deposition of collagen, improved activity of gelatinases and regaining of original strength. This when compared to GSNO in our study revealed that there was no inhibition of MMP activity. There are other similar studies especially regarding the vascular endothelium that inhibition of MMP release by NO is one of the pathways in preventing the occurrence of atherosclerosis. Our report suggests that enhanced collagen deposition, uninhibited MMP activity and thiol status favor enhanced wound healing. The mechanisms of MMP regulation by various RSNO's are yet to be studied in detail. The facilitatory and the regulatory effects of GSNO on wound healing following its biotransformation in the wound environment may provide possible targets for further research in the enhancement of wound healing. The drugs administered interact with the tissue glutathione. The cellular glutathione acts as a major sink for most of the nitrites and nitrates formed in the cell (for details see section 5.6). Therefore the variation in the levels of glutathione in the scar was measured. The control scars had similar GSSG at all time points of

wound healing, with a significant increase by day 10. This could be mainly due to accumulation of the anti-oxidants by the time all the free radicals are neutralized. Similar results are also seen in the GSH treated scars. GSNO treated scars had comparable GSSG levels with the control scars. The total nitrite was measured in the scars of animals treated with the above-mentioned drugs. The nitrate was further reduced to nitrite by aspergillus reductase and the total nitrite was measured at 540nm. By three days, nitrite content of the GSNO treated scars was 55% more than the control group. The rest of the time points showed no difference in nitrite and nitrate levels. SNAP showed no significant change in scar nitrite content. The variation in plasma nitrite was monitored following the administration of the NO donors GSNO and SNAP. This is important as it gives a clear picture of the systemic nitrite variation in the body following NO donor administration. Our results show that GSNO increases nitrite in the plasma by 3 hours of administration and then returns to control levels by the fifth hour.

5.2.2 Effects of SNAP on wound healing

The rats were treated with equimolar doses of SNAP compared to nitrosoglutathione. The effects were examined on the biomechanical properties, collagen content, gelatinase activity and the glutathione levels. Load to failure and stiffness showed no significant difference compared to controls. Collagen content was significantly lower than the control and GSNO groups on the 5^{th} post-wounding day ($p<0.05$) but by the tenth

post-wounding day no significant differences were observed. This compound has been shown to restore the collagen secretion in iNOS knock-out fibroblasts. But in an intact animal where SNAP undergoes intermediary metabolism and constant interaction with other cells and may exert its action in a manner different from that in an in vitro set up. through other pathways. The gelatinase activity was lower in the SNAP treated scars. In a separate set of experiments, Maria Angeles Martinez et al (2001) have demonstrated that SNAP inhibited MMP-2 activity through the activation of cGMP in platelets. This inturn inhibits platetlet aggregation. Therefore MMP-2 is an important enzyme that promotes fibrinogen stimulated platelet aggregation and NO from SNAP inhibits this vital physiological process. Tatsuya Okamoto et al (2002) have demonstrated that SNAP suppress MMP-9 induction by preventing NF-κB activation through the inhibition of nuclear translocation and/or DNA binding. Though the gelatinase activity decreased there was no changes in the collagen content. The SNAP treated scars had significantly lower levels of glutathione. It was found that SNAP lowers activity of glutathione reductase which in turn leads to decreased GSSG levels (Tsuneko Fujii, 2000; Kang KW, 2002). In wound healing it becomes more complicated to clearly infer the effect of SNAP on tissue oxidative status as it involves interaction of parenchymal and immune cellswhich are rich in anti-oxidants. In this study no significant change in scar nitrite content was observed following SNAP administration.

5.2.3 Effects of GSH on wound healing

The biomechanical testing of the GSH treated scars revealed lower stiffness than the control, SNAP and GSNO groups at the tenth day. The collagen content was lower by 76% on the 5th post-wounding day compared to the control group ($p<0.05$) and by the 10th day, it reached control levels. Collagen synthesis has been shown to improve following administration of certain anti-oxidant precursors such as VitC, Rexofelast (VitE related compound). GSH has been found to inhibit collagen synthesis in certain tissues such as the airway epithelium, (Hui Jung Kim, 2002) hepatocytes (Shanthi Wasser, 2001) and mesangial cells (Mark A. Catherwood, 2002). Thus the effect of GSNO depends on the tissue. Suresh C. Tyagi et al (1993) have also demonstrated that reduced GSH reduces the transcription of MMP expression and fibroblast proliferation in normal cardiac fibroblasts. Gelatinase activity was decreased in GSH treated scars on the 5th and the tenth day compared to control and was lower than the GSNO group on the 5th post-wounding day ($p<0.05$). MMP regulation occurs at the level of gene transcription and on activation of pro-MMPs. Various stimuli, including growth factors, cytokines, chemical agents (phorbol esters), and mechanical stress, induce MMP gene expression. The MMP-9 promoter region contains nuclear factor-κB, activator protein-1, stimulatory protein-1, and phorbol ester–responsive elements (Sato H, 1993). Previous findings indicating that nuclear factor-κB and activator protein-1 are redox

sensitive offer a potential mechanism by which oxidative stress may regulate MMP-9 transcription and activity (Li N, 1999). Another possible mechanism for the activation of MMP activity is posttranslational modification by ROS.

5.2.4 Effects of AG on wound healing

The iNOS inhibitor AG did not decrease the rate of wound contraction significantly but a trend towards a low rate of wound contraction was seen. The results were not statistically significant. As AG is also an inhibitor of advanced glycation end products (AGE), collagen cross-linking is inhibited and this inturn may result in decreased rate of wound contraction. But in this study no significant inhibition in the rate of wound contraction was noticed.

Experiments that have been performed in animals, patients and healthy volunteers confirm that dietary or intravenous L-arginine supplementation enhances both wound breaking strength and collagen deposition (Adrian Barbul 1985, Witte MB 2002). In our study the iNOS specific inhibitor AG inhibited the deposition of collagen in the scar significantly.

In our experiments we observed that administration of AG increased the gelatinase activity in the initial 3 days following healing. Later by the fifth day AG lowered the scar gelatinases activity. There was no significant difference in the gelatinase activity on the seventh and tenth post-wounding days.

In our study AG did not alter the antioxidant status in the wound environment. The observation of the changes of glutathione depends on the tissue that is studied. It is seen that in liver the glutathione levels are increased following administration of AG while GSSG levels of kidney are reduced (Kurshid Alam, 2001; Dobashi K, 2001). The exact mechanisms of glutathione regulation by iNOS inhibitors are not yet clearly understood.

AG administration lowered the scar nitrite content on the 3rd, 5th and 10th postwounding day. There was no significant difference on the 7th postwounding day. This is contradictory to some previous studies where administration of AG in high doses is necessary to achieve reduction in scar nitrite content (Michael Schaffer, 1999).

The administration of AG did not decrease plasma nitrite and nitrate content. When higher doses are administered to the rats, immediate effects such as altered behavior and restlessness are seen. In a separate report Hans Oxlund et al (1996) have observed the decrease in wound collagen at a dose of 125mg/kg body weight. As there are varied opinions and observations of the effects of AG on plasma nitrite, scar nitrite and scar collagen, it is important to measure all the related parameters.

5.3 Summary of discussion

The present experiments demonstrate that administration of GSNO increases collagen deposition. The collagen deposition in the GSNO treated scars followed the same pattern as in control scars, i.e. it

increases by day 5 and decreases by day 10, but was significantly higher. This implies that GSNO facilitates physiological reparative tissue deposition. NO has been associated with modulating collagen synthesis in the scar (Michael Schaeffer, 1996). Fibroblasts isolated from rat wounds were shown to synthesize and release NO in vitro. Moreover, NO regulates collagen synthesis by the wound fibroblasts in an autocrine fashion (Michael Schaffer, 1997). Inhibition of NO production using S-methylisothiouronium (S-MITU) was associated with attenuation of wound tensile strength and significantly diminished hydroxyproline content after wounding (Michael Schaffer, 1999). The increase in collagen content may be attributed to increased stimulation of the collagen synthesis or increased proliferation of fibroblasts (Ming Du, 1997; Maria B Witte, 2000). The proliferative effect of GSNO is varied and depends on the target cells. For e.g.: Balb/c3T3 (clone A31-1-1) fibroblasts (Ming Du, 1997) show increased proliferation whereas NIH 3T3 cells show induction of apoptosis when exposed to GSNO (Tzyh-Chyuan Hour, 1999). The exact mechanism by which NO enhances collagen deposition is not clearly understood. Interpretation of the breakdown of the nitrosothiols becomes more complicated and varied in cells and tissues as factors such as the thiols & superoxide, react with GSNO and produce many other byproducts (Schmidt K, 1997). To understand the breakdown of GSNO, some reports have suggested that a different breakdown mechanism of GSNO called GSNO lyase exists in the cells (Michael P Gordge, 1998). But, the main effect of GSNO could be due to the cellular bio-transformation of this compound, which gives

GSSG and other products such as nitrite, nitrate and peroxynitrate. The extracellular breakdown of GSNO and the cellular uptake of the NO released by this substance have been linked to the activity of membrane protein disulfide isomerase (PDI). This enzyme catalyzes NO release from extracellular S-nitrosothiols, which accumulates in the membrane and reacts with O2 to produce N_2O_3. Intracellular thiols may then be nitrosated by N_2O_3 at the membrane-cytosol interface (Niroshan Ramachandran, 2001). Similarly the effect of GSNO on the thiols has been studied and found that it has an inhibitory effect on the crystalline glutathione reductase (GR), but not on the GR levels of intact cells (Becker K, 1995). Therefore, the effects of GSNO are controlled mainly by the type of the cells involved in its metabolism and the redox state of these cells. GSNO increased plasma nitrite concentration 3 hours following administration, which further returned to control levels by 5 hours.

Nitrosothiols are found to provide protection against peroxide mediated cytotoxicity whereas the other NO donor compounds such as SIN-1 has been shown to have no beneficial effects (David A Wink, 1996). The administration of GSH itself doesn't serve any benefit, as the peptide is not easily taken up by the cells. It has to be broken into its component aminoacids and then taken into the cells. This is followed by intracellular synthesis of GSH (Meister A, 1993). Cellular GSH appears to inhibit the early signaling events leading to iNOS expression and suggest that the control of iNOS induction in hepatocytes is sensitive to the thiol redox status of the cell (Tirmenstein MA, 2000).

MMP activity in wound healing is vital for most of the physiological processes, for e.g. epithelialisation, debridement, infiltration of inflammatory and parenchymal cells (Chia Soon, 2000). The above-mentioned processes occur mainly during the phase of inflammation (up-to 3 days) and phase of ECM deposition and re-epithelialisation (up-to 10 days). The activity of MMP is not affected in the initial phase. This indicates that GSNO does not affect the vital physiological process i.e. MMP activity, which in turn regulates dissolution of clot, debridement and keratinocyte migration. Though it has been found that GSNO inhibits MMP activity in platelets and in turn inhibits platelet aggregation the effect is not to such an extent to affect healing. Debridement, inflammation and epithelialisation were all maintained as it was observed that the wounds healed without any delay. GSH inhibited the activity of MMP and this was similar to previous observations (Gundumi Aravinda Upadhya, 2000). Suresh C. Tyagi et al (1996) have also demonstrated that reduced GSH reduces the transcription of MMP expression and fibroblast proliferation in normal cardiac fibroblasts. This is the first report describing the effects of nitrosothiols on wound collagen and MMP activity. In our study we have observed an increase in the collagen content without any interference of MMP activity. Alteration of this vital process would result in aberrant healing. Any wound enhancing agent necessarily has to have no effect on these enzymes. Certain reports regarding inhibition of MMP activity by NO have been carried out in an in-vitro set-up (Paul Jurasz, 2001). The MMP inhibitory effect in an in vivo model may not be as profound as in

an in vitro model, as the NO donor compounds undergo various intermediary metabolisms which require the presence certain other cells. Our report suggests that enhanced collagen deposition, uninhibited MMP activity and thiol status favor enhanced wound healing. The mechanisms of MMP regulation by various RSNOs are yet to be studied in detail. The facilitatory and the regulatory effects of GSNO on wound healing following its biotransformation in the wound environment may provide possible targets for further research in the enhancement of wound healing

5.4 General discussion

Nitric oxide produced in the cells may function in non-specific host defense during wound healing. This presumably occurs because nitric oxide either alone or in combination with other reactive oxygen species is cytotoxic (Moncada 1991). Nitric oxide is also important in regulating cell proliferation (keratinocyte and fibroblast) in the wound environment. The cytotoxic effects of NO are either due to complex formation with the iron-sulphur moiety of proteins, resulting in inhibition of the proteins or activation of metabolic pathways. The binding of nitric oxide to the heme-containing guanylate cyclase activates this enzyme resulting in increased intracellular levels of cGMP an important regulator of cell growth and differentiation (Moncada 1991). Nitric oxide also inhibits ribonucleotide reductase (Kwon N.S 1990) as well as mitochondrial respiration and DNA synthesis, events that control cellular proliferation and are important in non-specific host defense. The

effect of NO on cells is considered biphasic, as lower concentrations are protective against oxidative killing and higher doses enhance oxidative killing (Mahesh.S.Joshi et al, 1999). The clear variation of responses of GSNO and SNAP can be understood based on the above observations that SNAP caused an increased oxidative damage at higher concentration by depleting intracellular GSH. The beneficial effect of GSNO can be explained by its breakdown into GSH and NO, thus providing an efficient and well-balanced antioxidant and a signal transduction molecule. Therefore it is important to modulate/ control this equilibrium so as to achieve appropriate wound healing. These redox mechanisms are well known to regulate inflammatory responses in various other tissues and as well as in many dermatidis. We therefore hypothesized that agents, which affect the equilibrium, can also alter wound healing. Because of the differences in investigative conditions e.g. choice of experimental animal, mode of administration of the drugs and healing time studied, it is difficult to compare the results. In conclusion these results demonstrate that the administration of nitrosoglutathione has beneficial effects on wound healing compared to SNAP, which is a NO donor of the same group. Administration of glutathione alone does not enhance healing. The net biological effects of GSNO may therefore depend upon the complex interactions of various resident and immune cells and mediators as well as on the extracellular matrix.

Chapter 6

Conclusions & Future directions

6.1 Conclusions

Normal wounds heal completely in a period of 12-14 days. The Rate of wound healing calculated by measuring length and breadth of clearly defined wounds with proper edges is a repeatable and comparable method. This is limited to experimental models, as in clinical situations the wound volume varies and the borders are not clear-cut. Anyhow this method can be adopted in examining the effects of pharmacological agents in an animal model. In this study administration of nitrosoglutathione increased the rate of wound healing. Aminoguanidine, an iNOS inhibitor did not affect wound healing either positively or negatively.

To compare the effects of GSNO on wound healing, animals were treated with GSH and another NO donor belonging to the same class of compounds. The effect on wound healing was quantified by measuring collagen content (hydroxyproline), material property (load & stiffness), gelatinase activity and scar glutathione concentrations. Our results revealed that neither SNAP (a NO donor) nor GSH (an antioxidant) showed any significant improvement of quantitative parameters of wound healing. GSNO improved strength and collagen content of the scars. SNAP & GSH significantly decreased the strength, collagen content and MMP activity in the scars. The plasma nitrite and nitrate increased, following GSNO & SNAP treatment. In the GSNO group plasma nitrite increased only till the 3rd hour following which it returned to normal. SNAP showed increased plasma nitrite for 5 hours post-injection. SNAP significantly decreased the wound GSH content beginning from the 3rd post-operative day, lasting till the 10th day. AG mainly had

the following effects: 1) rate of wound contraction was not altered 2) lowered collagen content 3) increased gelatinase activity by the third post-wounding day and 4) scar nitrite and nitrate were significantly lowered.

The effects of injection of NO donors and inhibitors on the peritoneum were monitored by the expression of immune cell markers. MHC class I had no difference. SNAP increased expression of class II markers. ICAM was found to be lower in the GSNO, SNAP and AG treated groups. The variation in the expression of peritoneal cells markers is not significant as to prove that i.p. administration of these drugs did not alter immunity. The NOS isoenzyme localization by immunohistochemical methods revealed no significant change in the treatment groups.

Thus, our study shows that GSNO improves wound healing by improving collagen content and material property of the scar. This can be hypothesized due to the combined effects of NO and GSH component of this compound. The various mechanisms, which are regulated by accumulation of intracellular GSNO, its breakdown and effects on a cell physiology, is an interesting area for further research.

6.2 Future directions

NO is a key regulator of many biochemical pathways which regulate tissue responses to injury. It is of interest to further look into the effect of nitric oxide in the following areas of wound healing and tissue repair.

1. **Elucidate novel mechanisms by which NO and GSH influence the response of a cell to injury. In a tissue such as skin, the effects of NO and GSH interaction are complex as there are numerous immune cells along with resident parenchymal cells.**
2. **The start signals for cell proliferation and differentiation are to be identified and the pharmacological manipulation of this process holds a high potential for drug discovery.**
3. **The clinical applications of drugs that alter the redox pathway are numerous. Clinical trials on such drugs in ischaemic ulcers, non-healing wounds and pressure sores need to be conducted. NO donors with anti-microbial and vasodilatory effects are potential candidates in this aspect.**
4. **It would be interesting to identify an ideal NO donor, which has prolonged half-life and augments repair by increasing the keratinocyte proliferation without inhibiting the inflammatory pathways such as activation of NF-κB.**
5. **The efficacy of topical nitric oxide donors in conditions such as diabetic ulcers, infected wounds and in ischaemic ulcers needs to be studied.**

Because of their wide range of effects, RSNOs have great potential as therapeutic agents. They may be particularly useful alternatives to organic nitrates, as they do

not lead to the development of tolerance. However, interactions with copper, thiols, ascorbic acid and other reducing agents may influence their stability and ability to act as useful therapeutic agents. As yet RSNOs have only been used intravenously in animal studies and small clinical trials. Further study of their pharmacology is warranted, particularly to determine whether oral preparations might be available in the future.

Chapter 7

References

Abraham J. A., Whang J. L., Tumolo A., et al. Human basic fibroblast growth factor: nucleotide sequence and genomic organization". EMBO Journal, 1986, 5:2523-2528.

Adam J. Singer and Richard A. F. Clark. "Cutaneous wound healing". The New England Journal of Medicine, 1999, Vol 341(10):738-746.

Adrian Barbul, Rhonda S Fishel, Seiichi Schimazu, Hannah L Wasserkrug, Norman N Yoshimura, Robert C Tao and Gershon Efron. "Intravenous Hyperalimentation With High Arginine Levels Improves Wound Healing and Immune Function". Journal Surg Res, 1985, 38:328-334.

Adrian Barbul, Spiro A Lazarou, David T Efron, Hana L Wasser Krugh and Gershon Efron. "Arginine enhances wound healing and lymphocyte immune responses in humans". Surgery, 1990, 108:331-337.

Agren M. S. "Gelatinase activity during wound healing." British Journal of Dermatology, 1994, 131: 636-640.

Agren M. S., Jorgensen L. N., Andersen M., Viljanto J. and Gottrup F. "Matrix metalloproteinase 9 level predicts optimal collagen deposition during early wound repair In humans" British Journal of Surgery, 1998, 85:68-71.

Aki Hirayama, Alberto A. Noronha-Dutra, Michael P. Gordge, Guy H. Neild and John S. Hothersall. "S-Nitrosothiols are stored by platelets and released during Platelet-Neutrophil interactions" Nitric Oxide: Biology and Chemistry, 1999, Vol. 3, No. 2:95-104.

Akiko Okada, Catherine Tomasetto, Yves Lutz, Jean-Pierre Bellocq, Marie-Christine Rio and Paul Basset. "Expression of matrix metalloproteinases during rat skin wound healing: Evidence that membrane type-1 matrix metalloproteinase is a stromal activator of pro-gelatinase A" Journal of Cell Biology, Vol. 137, No. 1, 1997: 67-77.

Alain P. Gobert, Philippe Vincendeau, Djavad Mossalayi and Bernard Veyret "Mechanism of Extracellular Thiol Nitrosylation by N_2O_3 produced by activated macrophages" Nitric Oxide: Biology and Chemistry, 1999, Vol. 3, No. 6:467-472.

Alain Simeon, Frederique Monier, Herve Emonard, Philippe Gillery, Philippe Birembaut, William Hornebeck and Francois-Xavier Maquart. "Expression and Activation of matrix metalloproteinases in wounds: Modulation by the Tripeptide-Copper Complex Glycly-L-Histidyl-L-Lysine-Cu^{2+}". J. Invest Dermatol., 1999, 112:957-964.

Albina J. E., Mills C. D., Adrian Barbul, et al. Arginine metabolism in wounds. Am J Physiol, 1988, 254:E459-E467.

Alexander C. M., Howard E. W., Bissell M. J. and Werb Z. "Rescue of mammary epithelial cell apoptosis and entactin degradation by a tissue inhibitor of metalloprotei- nases-1 transgene". Journal Cell Biol., 1996, 135:1669-1677.

Anand Ramamurthi and Randy S. Lewis "Measurement and Modeling of Nitric Oxide Release Rates for Nitric Oxide Donors" Chem. Res. Toxicol., 1997, 10:408-413.

Anders E Ulland, Jeffery D.Shearer, Cynthia Coulter and Michael D.Caldwell Altered wound Arginine Metabolism by Corticosterone and Retinoic acid. J Surg Res 1997, 70:84-88.

Anderson J. E., Mitchell C. M., MeGeachic J. K., Grounds M. D. "The time course of basic fibroblast growth factor expression in crush-injured skeletal muscles of SJL/J and BALB/e mice". Exp Cell Res., 1995, 216:325-334.

Andonegui G., Trevani A. S., Gamberale R., Carreras M. C., Poderoso J. J., Giordano M. and Geffner J. R. "Effect of Nitric Oxide donors on oxygen-dependent cytotoxic responses mediated by neutrophils" J. Immunology, 1999, 162:2922-2930.

Ann F. Haas, Jon W. Wong, Christine K. Iwahashi, Barry Halliwell, Carroll E. Cross and Paul A. Davis. "Redox regulation of wound healing? NF-κB Activation in cultured human keratinocytes upon wounding and the effect of low energy HeNe irradiation" Free Radical Biology & Medicine, 1998, Vol. 25, No. 9:998-1005.

Ann Schwentker, Yoram Vodovotz, Richard Weller and Timothy R. Billiar. "Nitric Oxide and wound repair: role of cytokines?" Nitric Oxide Biology and Chemistry, 2002, 7:1-10.

Anne H. Cross, Thomas P. Misko, Robin F. Lin, William F. Hickey, John L. Trotter, and Ronald G. Tilton. "Aminoguanidine, an inhibitor of inducible Nitric Oxide Synthase, Ameliorates experimental autoimmune encephalomyelitis in SJL Mice" J. Clin. Invest., 1994,93:2684-2690.

Anne Quirinia and Andrus Viidik. "Diclofenac and indomethacin influence the healing of normal and ischaemic incisional wounds in skin". Scand J Plast Reconstr Hand Surg, 1997, 31:213-219.

Annette B. Wysocki. "Wound Measurement". Intl. Journal of Dermatol., 1996, Vol. 35, No. 2:82-91.

Antonella Zampolli, Giuseppina Basta, Guido Lazzerini, Martin Feelisch and Raffaele De Caterina. "Inhibition of endothelial cell activation by Nitric Oxide donors". The Journal of Pharmacology and Experimental Therapeutics, 2000, 295(2):818-823.

Antonelli-Orlidge A, Saunders K. B., Smith S. R., D'Amore P. A. "An activated form of TGF-β3 is produced by cocultures of endothelial cells and pericytes". Proc Natl Acad Sci USA 1989, 86:4544-4548.

Arsi Broberg and Jyrki Heino "Integrin α2β1-Dependent contraction of floating collagen gels and induction of collagenase are inhibited by Tyrosine Kinase inhibitors." Experimental Cell Research, 1996, 228:29-35.

Arthur J Chu, Jai K Prasad. "Up regulation by Human recombinant transforming growth factor β-1 of collagen Production in Cultured Dermal Fibroblasts Is mediated by the inhibition of Nitric Oxide Signaling". Journal Am. Coll. Surg., 1999, 188:271-280.

Arti Shukla, Anamika Mohan Rasik and Gyanendra Kumar Patnaik "Depletion of reduced Glutathione, Ascorbic acid, Vitamin E and Antioxidants Defence Enzymes in a healing cutaneous wound" Free Rad. Res., 1997, Vol. 26:93-101.

Arti Shukla, Anamika M. Rasik and Ravi Shankar. "Nitric Oxide inhibits wound collagen synthesis". Mol Cell Biochem, 1999, 200: 27-33.

Asha Jacob, Jeffery D. Molkentin, Albert Smolenski, Suzanne M Lohmann and Najma Begum. "Insulin inhibits PDGF directed VSMC migration via NO/cGMP increase of MKP-1 and its activation of MAPKs". Am J Physiol Cell Physiol, 2002, 283:C704-C713.

Ashvin Thambyah, Palanniappan Thiagarajan and James C.H. Goh. "Biomechanical study on the effect of twisted human patellar tendon". Clinical Biomechanics, 2000, Volume 15, Issue 10: 756-760.

Atif D. Agaiby and Mary Dyson. "Immuno-inflammatory cell dynamics during cutaneous wound healing". Journal Anat., 1999, 195:531-542.

Aygenc E, Celikkanat S, Bilgili H, Aksaray F, Orhun S, Kaymakci M, Ozdem C. "Pentoxifylline effects on acute and late complications after radiotherapy in rabbit Otolaryngol Head Neck". Surg 2001, 124(6):669-673.

Badovinac V, Trajkovic V, Mostarica-Stojkovic M. "Nitric oxide promotes growth and major histocompatibility complex-unrestricted cytotoxicity of interleukin-2-activated rat lymphocytes". Scand J Immunol 2000, 52(1):62-70.

Bailey A. J., Sims T.J., Le Lous M. and Bazin S. "Collagen polymorphism in experimental granulation tissue". Biochemical and Biophysical Research Communications, 1975, Vol. 66, No. 4:1160-1164.

Baird A., Esch F., Mormede P., et al. "Molecular characterization of fibroblast growth factor: distribution and biological activities in various tissues". Recent Prog Horm Res, 1986, 42:143-205.

Bang A, Speck E. R., Blanchette V. S., Freedman J, Semple J. W. "Recipient humoral immunity against leukoreduced allogeneic platelets is suppressed by aminoguanidine, a selective inhibitor of inducible nitric oxide synthase". Blood, 1996, 88(8):2959-2966.

Bar R. S., Boes M., Booth B. A., Dake B. L., Henley S., Hart M. N. "The effects of platelet-derived growth factor in cultured microvessel endothelial cells". Endo- crinology, 1989, 124:1841-1848.

Barbara Munz, Stefan Frank, Griseldis Hu Bner, Eydfinnur Olsen, Sabine Werner "A novel type of glutathione peroxidase: expression and regulation during wound repair" Biochem. Journal, 1997, 326:579-585.

Barrandon Y and Green H. "Cell migration is essential for sustained growth of keratinocyte colonies: the roles of transforming growth factor – alpha and epidermal growth factor". Cell, 1987, 50(7):1131-1137.

Battegay E. J., Rupp J, lruela-Arispe L, Sage E. H., Pech M. "PDGF-BB modulates endothelial proliferation and angiogenesis in vitro via PDGF Preceptors". J Cell Biol 1994, 125:917-928.

Bauer J. A. "Hydroxocobalamins as Biologically compatible Donors of Nitric Oxide implicated in the accelaration of Wound healing". Medical Hypotheses, 1998, 51:65-67.

Becker, Ming GUI and R. Heiner Schirmer. "Inhibition of human glutathione reductase by S-nitrosoglutathione". Biochem., 1995, 234:472-478.

Beer H. D., Fassler R and Werner S. "Glucocorticoid-regulated gene expression during cutaneous wound repair". Vitam Horm 2000, 59:217-239.

Behrendtsen O., Alexander C. M. and Werb Z. "Metalloproteinases mediate extracellular matrix degradation by cells from mouse blastocyst outgrowths". Development, 1992, 114, 2:447-456.

Bekyarova G, Yankova T, Galunska B. "Increased antioxidant capacity, suppression of free radical damage and erythrocyte aggrerability after combined application of alpha-tocopherol and FC-43 perfluorocarbon emulsion in early postburn period in rats". Artif Cells Blood Substit Immobil Biotechnol, 1996, 24(6): 629-641.

Belinda Adamson B.S, Daniel Schwarz, Patty Klugston, Robert Gilmont, Larry Perry, R. N. Jack Fisher, William Lindblad and Riley Rees. "Delayed Repair: The role of glutathione in a rat incisional wound model" Journal of surgical research, 1996, 62:159-164.

Bell E., Ivarsson B. and Merril C. "Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vivo". Proc. Natl. Acad. Sci. USA, 1979, 76:1274-1278.

Benedetta Bussolati, Filippo Mariano, Massimiliano Migliori, and Giovanni Camussi. "Nitric oxide/platelet activating factor cross-talk in mesangial cells modulates the interaction with leukocytes". Kidney Int., 2002, 62(4): 1322-1331.

Benedich A, Philips M, Tnngerdy R. P. "Antioxidant vitamins and their function in immune responses". Eds., Plenum Press, New York, NY, 1990:33.

Benedict C. Nwomeh, Hui-Xiu Liang, Kelman Cohen I. and Dorne R. Yager. "MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers." Journal of Surgical Research, 1999, 81: 189-195.

Benedict C. Nwomeh, Hui-Xiu Liang, Robert F. Dielgelmann, Kelman Cohen I. and Dorne R. Yager "Dynamics of the matrix metalloproteinases MMP-1 and MMP-8 in acute open human dermal wounds" Wound Rep. Reg., 1998, 6:127-134.

Bentz H, Morris N. P., Murray L. W., Sakai L. Y., Hollister D. W. and Burgeson R. E. "Isolation and partial characterization of a new human collagen with an extended triple-helical structural domain". Proc. Natl. Acad. Sci. USA, 1983, 80:3168-3172.

Bereha Khodr and Zeinab Khalil "Modulation of inflammation by reactive oxygen species: Implications for aging and tissue repair" Free Radical Biology & Medicine, 2001, Vol. 30, No. 1:1-8.

Bergmann U., Tuuttila A., Stetler-Stevenson W. G. and Tryggvason K. "Autolytic activation of recombinant human 72 kilodalton type IV collagenase". Biochemistry, 1995, 34:2819-2825.

Berk B. C., Alexander R. W., Brock T. A., Gimbrone M. A., Webb R. C. "Vasoconstriction: a new activity for platelet-derived growth factor". Science 1986, 232 :87-90.

Bernd Mayer, Silvia Pfeiffer, Astrid Schrammel, Doris Koesling, Kurt Schmidt and Friedrich Brunner. "New Pathway of Nitric Oxide/Cyclic GMP signaling involving S-Nitrosoglutathione" Journal of Biological Chemistry, 1998, Vol. 273, No. 6:3264-3270.

Bhora F. Y., Dunkin B. J., Batzri S., et al. Effect of growth factors on cell proliferation and epithelialization in human skin. Journal Surg Res 1995, 59:236-244.

Birk D. E., Zycband E. I., Winkelmann D. A., Trelstad R. L. "Collagen fibrillogenesis in situ. Discontinuous segmental assembly in extracellular compartments". Ann N Y Acad Sci., 1990, 580:176-194.

Bissett D. L, Chatterjee R and Hannon D. P. "Photoprotective effect of superoxide-scavenging antioxidants against ultraviolet radiation-induced chronic skin damage in the hairless mouse". Photodermatol. Photoimmunol. Photomed., 1990, 7:56.

Bohm F, Edge R, McGarvey D. J and Truscott T.G. "β-Carotene with vitamins E and C offers synergistic cell protection against NO_x. FEBS Lett., 1998, 436: 387-389.

Border W. A., Noble N. A. "Transforming growth factor beta in tissue fibrosis". N Engi J Med 1994, 331:1286-1292.

Border W. A., Okuda S, Languino L. R., Sporn M. B., Ruoslahti E. "Suppression of experimental glomerulonephritis by antiserum against transforming growth factor-beta 1". Nature 1990, 346:371-374.

Boutard V, Havouis R, Fouqueray B, Philippe C, Moulinoux JP and Baud L. "Transforming growth factor-beta stimulates arginase activity in macrophages. Implications for the regulation of macrophage cytotoxicity". Journal Immunol, 1995,15,155(4):2077-2084.

Bowen-Pope D. F., Malpass T. W., Foster D. M., Ross R. "Platelet-derived growth factor in vivo: levels, activity, and rate of clearance". Blood 1984, 64:458-469.

Bradley P. Pickett, Lawrence P. A. Burgess, George H. Livermore, Thomas L. Tzikas and Jafar Vossoughi. "Tensile strength vs healing time for wounds closed under tension". Arch. Otolaryngol. Head Neck Surg. 1996, 122:565-568.

Bredt D. S, Hwang P. M, Glatt C. E, Lowenstein C, Reed R. R and Snyder S. H. "Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase". Nature 1991, 351(6329):714-718.

Brian K. Pilcher, Jo Ann Dumin, Barry D. Sudbeck, Stephen M. Krane, Howard G. Welgus and William C. Parks. "The activity of collagenase-1 is required for keratinocyte migration on a Type 1 collagen matrix". The Journal of Cell Biology, 1997, Vol. 137, No. 6, 1445-1457.

Brinckerhoff C. E., Sirum-Connolly K. L., Karmilowiez M. J. and Auble D. "Expression of stromelysin and stromelysin-2 in rabbit and human fibroblasts". Matrix Suppl., 1992, 1:165-175.

Broadley K. N., Aquino A. M., Woodward S. C., et al. "Monospecific antibodies implicate basic fibroblast growth factor in normal wound repair". Lab Invest, 1989, 61:571-575.

Brown G. L., Curtsinger L. J., White M, Mitchell R. O., Pietsch J, Nordquist R, von Fraunhofer A, Schultz G. S. "Acceleration of tensile strength of incisions treated with EGF and TGF-beta". Ann Surg, 1988, 208(6):788-794.

Brown L. F., Yeo K. T., Berse B, et al. "Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing". J Exp Med 1992, 9:1375-1379.

Bruns R. R., Press W., Engvall E., Timpl R., Gross J. "Type VI collagen in extracellular, 100-nm periodic filaments and fibrils: identification by immunoelectron microscopy". Journal Cell Biol., 1986, 103(2):393-404.

Bull H. A., Hothersall J, Chowdhury N et al. "Neuropeptides induce release of Nitric oxide from human dermal microvascular endothelial cells". J Invest Dermatol 1996, 106: 655-660.

Calof A. L., Campanero M. R., O'Rear J. J., Yurchenco P. D. and Lander A. D. "Domain-specific activation of neuronal migration and neurite outgrowth-promoting activities of laminin". Neuron, 1994, 13:117-130.

Campochlaro P. A., Hackett S. F., Vinores S. A., et al. Platelet-derived growth factor is an autocrine growth stimulator in retinal pigmented epithelial cells. J Cell Sci 1994, 107:2459-2469.

Cao J., Sato H., Takino T. and Seiki M. "The C-terminal region of membrane type matrix metalloproteinase is a functional transmembrane domain required for pro-gelatinase A activation". Journal Biol. Chem., 1995, 270:801-805.

Cao M , Westerhausen-Larson A, Niyibizi C., Kavalkovich K, Georgescu H. I., Rizzo C. F., Hebda P. A., Stefanovic M and Evans C. H. "Nitric Oxide Inhibits The Synthesis of Type 2 Collagen Without altering Col2A1 mRNA abundance: prolyl hydroxylase as a possible Target". Biochemistry 1997, 324: 305-310.

Carmelict P, Ferriera V, Brier G, et al. Abnormal blood vessel development and lethality in embryoes lacking a single VEGF allele. Nature 1996, 380:435-439.

Carrel A. and Hartmann A. "Cicatrization of wounds. I. The relation between the size of a wound and the rate of its cicatrisation." Journal Exp. Med., 1916, 24:429-450.

Chamberlain J., M. Shah and M. W. J. Ferguson. "The effect of suramin on healing adult rodent dermal wounds". J. Anat., 1995, 186:87-96.

Charity C. Fox, Scott D. Jewell and Caroline C. Whitacre. "Rat peritoneal mast cells present antigen to a PPD-specific T cell line". Cellular Immunology, 1994, 158, 253-264.

Charles J. Doillon, Michael G. Dunn, Edward Bender and Frederick H. Silver. "Collagen Fiber formation in repair tissue: Development of strength and toughness". Collagen Res. Rel., 1985, Vol. 5 :481-492.

Chia Soon, William W. Shaw, Xinli Zhang, Michael T. Longaker, Eric W. Howard and Kang Ting. "Differential Expression of matrix metalloproteinases and their tissue-derived inhibitors in cutaneous wound repair" Plast. Reconst. Surg., 2000, 105:638-647.

Christiaan Leeuwenburgh and Li Li Ji "Glutathone and Glutathione Ethyl Ester Supplementation of Mice alter Glutathione Homeostasis during exercise" J. Nutr., 1998, 128:2420-2426.

Christian Kupatt, Christian Weber, Dieter A. Wolf, Bernhard F. Becker, Thomas W. Smith1 and Ralph A. Kelly. "Nitric Oxide Attenuates Reoxygenation induced ICAM-1 Expression in Coronary Microvascular Endothelium: Role of NFκB". J Mol Cell Cardiol 1997, 29:2599–2609.

Christian Wetzler, Heiko Kampfer, Josef Pfeilschifter and Stefan Frank. "Keratinocyte-derived chemotactic cytokines: Expressional modulation by nitric oxide in vitro and during cutaneous wound repair in vivo". Biochem. Biophy. Res Comm., 2000, 274(3): 689-696.

Christopher M. Howard, Daniel J. Sexton and Bulent Mutus "S-Nitrosoglutathione/Glutathione Disulphide/Cu^{2+} - Dependent Stimulation of L-arginine Transport in Human Platelets" Thrombosis Research, 1998, 91:113-120.

Chu A. J., Prasad J. K. "Up-regulation by human recombinant transforming growth factor beta-1 of collagen production in cultured dermal fibroblasts is mediated by the inhibition of nitric oxide signaling". Journal Am Coll Surg., 1999, 188(3):271-280.

Chuang C. Chiueh and Pekka Rauhala. "The redox pathway of S-nitrosoglutathione, glutathione and nitric oxide in cell to neuron communications" Free Rad. Res., 1999, Vol. 31, 641-650.

Circolo A., Welgus H. G., Pierce G. F., Kramer J., Strunk R. C. "Differential regulation of the expression of proteinases/antiproteinases in fibroblasts. Effects of interleukin-1 and platelet-derived growth factor". Journal Biol Chem., 1991, 266(19):12283-12288.

Clark R. A. F. "The human neutrophil respiratory burst oxidase". Journal Inf. Dis., 1990, 161:1140.

Clark R. A. F., Folkvord J. M. and Wertz R. L. "Fibronectin as well as other extracellular matrix proteins, mediate human keratinocyte adherence". Journal Invest. Dermatol., 1985, 85:373-378.

Clark R. A. F., Lanigan J. M., DellaPelle P., Manseau E., Dvorak H. F. and Colvin R. B. "Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound reepithelialization". Journal Invest. Dermatol., 1982, 79:264-269.

Clark R. A. F., Quinn J. H., Winn H. J., Lanigan J. M., DellaPelle P. and Colvin R. B. "Fibronectin is produced by blood vessels in response to injury". Journal Expr. Med., 1982, 156:646-651.

Clark R. A. F., Winn H. J., Dvorak H. F. and Colvin R. B. " Fibronectin beneath reepithelialization epidermis in vivo: Sources and significance". Journal Invest. Dermatol., 1983, (Suppl.), 80:26S-30S.

Clauss M, Gerlach M, Gerlach H, et al. "Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoa- gulant activity, and promotes monocyte migration". J Exp Med 1990, 172:1535-1545.

Clore J. N., Cohen I. K. and Diegelmann R. F. "Quantitation of collagen types I and III during wound healing in rat skin". Proc. Soc. Exp. Biol. Med., 1979, 161:337-340.

Connolly D. T., Heuvelman D. M., Nelson R, et al. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest 1989, 84:1470-1478.

Corcoran M. L., Stetler-Stevenson W. G., Brown P. D. and Wahl L.M. "Interleukin 4 inhibition of prostaglandin E2 synthesis blocks interstitial collagenase and 92-kDa type IV collagenase/gelatinase production by human monocytes". Journal Biol. Chem., 1992, 267, 1:515-519.

Craig E. Thomas and Victor Darley-Usmar. "Forum on therapeutic applications of reactive oxygen and nitrogen species in human disease". Free Radical Biology & Medicine, 2000, 28(10), 1449-1450.

Cross A. R. and Jones O. T. G. "Enzymic mechanisms of superoxide production". Biochem. Biophys. Acta., 1991, 1057:281.

Cross S. E., Naylor I. L., Coleman R. A. and Teo T. C. "An experimental model to investigate the dynamics of wound contraction". British Journal of Plastic Surgery, 1995, 48:189-197.

Dalibor Gergel and Arthur I. Cederbaum. "Interaction of Nitric Oxide with 2-Thio-5-nitrobenzoic Acid: Implications for the Determination of Free Sulfhydryl Groups by Ellman's Reagent" Archives of Biochemistry and Biophysics, 1997, Vol. 347, No. 2: 282-288.

Damore P. A., Brown R.H.,Jr, Ku P-T, et al. Elevated basic fibroblast growth factor in the serum of patients with Duchenne muscular dystrophy". Ann Neurol., 1994, 35:362-365.

Daniela Bruch-Gerharz, Thomas Ruzicka and Victoria Kolb-Bachofen. "Nitric Oxide in human skin: Current status and future prospects". Journal Invest Dermatol, 1998, 110:1-7.

Daniela, Bruch-gerharz, Thomas Ruzicka and Victoria Kolb-Bachofenn. "Nitric Oxide in human skin: current status and future prospects". J. Invest Derm., 1998, 110:1-7.

Darr D and Fridovich I. "Free radicals in cutaneous biology". Journal Invest. Dermatol., 1994, 102:671.

D'Arnore P.A., Smith S. R. "Growth factor effects on cells of the vascular wall: a survey". Growth Factors, 1993, 8:61-75.

David A. Wink, John A. Cook, Roberto Pacelli, William DeGRaff, Janet Gamson, James Liebmann, Murali C. Krishna and James B. Mitchell. "The effect of various Nitric Oxide-Donor Agents on Hydrogen Peroxide-Mediated Toxicity: A Direct correlation between Nitric Oxide formation and protection" Archives of Biochemistry and Biophysics, 1996, Vol. 331, No. 2:241-248.

David Jourd Heuil, Katarina Hallen, Martin Feelisch and Matthew B. Grisham. "Dynamic state of S-nitrosothiols in human plasma and whole blood" Free Radical Biology & Medicine, 2000, Vol.28, No. 3:409-417.

David Jourd'heuil, Christie T. Mai, F. Stephen Laroux, David A. Wink and Matthew B. Grisham. "The reaction of S-nitrosoglutathione with superoxide" Biochemical and Biophysical Research Communications, 1998, 244:525-530.

David R. Knighton, Gregg D. Phillips and Vance D. Fiegel. "Wound healing angiogenesis: Indirect stimulation by basic fibroblast growth factor". The Journal of Trauma, 1990, Vol. 30, No. 12: S134-S143.

David T. Efron, Daniel Most and Adrian Barbul. "Role of Nitric Oxide in wound healing". Curr Opin Clin Nutr Metab Care, 2000, 3:197-204.

Davidson J. M., Broadley K. N. "Manipulation of the wound-healing process with basic fibroblast growth factor". Arm NY Acad Sci 1991,638:306-315.

Debbie Hege Svendsrud, Thrina Loennechen and Jan-Olof Winberg. "Effect of adenosine analogues on the expression of matrix metalloproteinases and their inhibitors from human dermal fibroblasts" Biochem. Pharmacol, 1997, 53: 1511-1520.

Degim Z, Celebi N, Sayan H, Babul A, Erdogan D, Take G. "An investigation on skin wound healing in mice with a taurine-chitosan gel formulation". Amino Acids 2002,22(2):187-198.

Detwiler T. C. and Feinman R.D. "Kinetics of the thrombin-induced release of calcium by platelets". Biochemistry, 1973, 12:282-289.

Deuel T.F., Senior R.M., Huang J.S., Griffin G.L. "Chemotaxis of monocytes and neutrophils to platelet-derived growth factor". J Clin Invest 1982, 69:1046-1049.

DiCorleto P. E., Bowen-Pope D. F. "Cultured endothelial cells produce a platelet- derived growth factor-like protein". Proc Natl Acad Sci USA, 1983, 80:1919-1923.

Diegelmann R. F., Bryson G. R., Flood L. C. and Graham M. F. "A microassay to quantitate collagen synthesis by cells in culture". Analytical Biochemistry, 1990, 186(2):296-300.

Diegelmann R. F., Rothkopf L. C. and Cohen I. K. "Measurement of collagen biosynthesis during wound healing". J. Surg. Res., 1975, 19 :239-243.

Dlugosz A. A., Cheng C, Denning M. F., Dempsey P. J., Coffey R. J. Jr and Yuspa S. H. "Keratinocyte growth factor receptor ligands induce transforming growth factor alpha expression and activate the epidermal growth factor receptor signaling pathway in cultured epidermal keratinocytes". Cell Growth Differ, 1994, 5(12):1283-1292.

Douglas T. Cromack, Beatriz Porras-Reyes and Thomas A. Mustoe. "Current concepts in wound healing: Growth factor and macrophage interaction". Advances in Understanding Trauma and Burn Injury, 1990, Vol. 30, No. 12:S129-133.

Douglas T. Cromack, Beatriz Porras-Reyes and Thomas A. Mustoe. "Current concepts in wound healing: Growth factor and macrophage interaction". The Journal of Trauma, 1990, Vol. 30, No. 12:S129-S133.

Doxey D. L., Ng M. C., Dill R. E., lacopino A. M. "Platelet-derived growth factor levels in wounds of diabetic rats". Life Sci 1995, 57:1111-1123.

Du M, Islam M, Lin L, et al. "Promotion of proliferation of murine balb/C3T3 fibroblasts mediated by nitric oxide at lower concentrations". Biochem. Mol Bio Int 1997, 41:625-631.

Eleonora Ford, Martin N. Hughes and Peter Wardman. "Kinetics of the reactions of Nitrogen Dioxide with glutathione, cysteine and uric acid at physiological pH." Free Radical Biology & Medicine, 2002, Vol. 32, No. 12:1314-1323.

Epstein J. H. "The pathological effects of light on the skin". Free Rad Biol, 1977, 3:219.

Etsuro Sato, Sekiya Koyama, Sharon L. Camhi, Dan K. Nelson and Richard A. Robbins. "Reactive oxygen and nitrogen metabolites modulate fibronectin-induced fibroblast migration in vitro" Free Radical Biology & Medicine, 2001, vol. 30 No. 1: 22-29.

Ewa Karna, Jerzy Palka, Slawomir Wolezynski. "Doxycycline-induced inhibition of prolidase activity in human skin fibroblasts and its involvement in impaired collagen biosynthesis" European Journal of Pharmacology, 2001, 430: 25-31.

Fajardo L. F., Prionas S. D., Kwan H. H., Kowalski J, Allison A. C. "Transforming growth factor-beta- 1 induces angiogenesis in vivo with a threshold pattern". Lab Invest 1996, 74:600-608.

Felix Broche and Jose M. Tellado. "Defense mechanisms of the peritoneal cavity". Curr Opin Crit Care 2001, 7:105-116.

Ferrara N, Carver-Moore K, Chen H, et al. "Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene". Nature 1996, 380:439-442.

Ferrara N, Henzel W. J. "Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells". Biochem Biophys Res Commun 1989, 161:851-855.

Ferrell B. A., Josephson K., Norvid P., Alcorn H. "Pressure ulcers among patients admitted to home care". Journal Am. Geriatr. Soc., 2000, 48(9), 1042-1047.

Finklestein S. P., Apostolides P. J., Caday C. G., Prosser J., Philips M. F., Klagsbrun M. "Increased basic fibroblast growth factor (BFGF) iminunoreactivity at the site of focal brain wounds". Brain Res, 1988, 460:253-259.

Flynn J. M., Shelton R. M. and Landini G. "Investigation of primary cell-biomaterial interactions using silver nitrate staining of nucleolar organizing regions". Biomaterials, 2002, 23:19-26.

Fong G. H, Rossant J, Gertsenstein M, Breitman M. L. "Role of the flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium". Nature 1995, 376:66-68.

Forstermann U, Closs E. I, Pollock J. S, Nakane M, Schwarz P, Gath I, Kleinert H. "Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions". Hypertension, 1994, 23(6 Pt 2):1121-1131.

Fortenberry J. D., Owens M.L., Chen N. X and Brown L.A.S. "S-nitrosoglutathione inhibits TNF-α-induced NFκB activation in neutrophils" Inflamm. Res., 2001, 50:089-095.

Frank J Thornton, Michael R Schaffer, Maria B Witte, Lyle L. Moldawer, Sally L. D. Mackay, Amer Abouhamez B. S., Cynthia L Tannahill and Adrian Barbul. "Enhanced Collagen Accumulation Following Direct Transfection of The Inducible Nitric Oxide Synthase Gene in Cutaneous Wounds". Biophys Biochem Res Commun., 1998, 246:654-659.

Frank S, Hubner G, Breier G, Longaker M. T., Greenhalgh D. G., Werner S. "Regulation of vascular endothelial growth factor expression in cultured keratinocytes". J Biol Chem 1995, 270:12607-12613.

Frank S, Stallmeyer B, Kampfer H, Kolb N, Pfeilschifter J. "Nitric oxide triggers enhanced induction of vascular endothelial growth factor expression in cultured keratinocytes (HaCaT) and during cutaneous wound repair". FASEB Journal, 1999 13(14):2002-14.

Frisch S. M., Reich R., Collier I. E., Genrich L. T., Martin G. and Goldberg G. I. "Adenovirus E1A represses protease gene expression and inhibits metastasis of human tumor cells". Oncogene, 1990, 5:75-83.

Fuchs J and Milbradt R. "Antioxidant inhibition of skin inflammation induced by reactive oxidants: evaluation of the redox couple dihydrolipoate/lipoate". Skin Pharmacol., 1994, 7: 278.

Fuchs J and Packer L. "Ultraviolet irradiation and the skin antioxidant system". Photodermatol. Photoimmunol. And Photomed., 1990, 7: 90.

Fukai N., Apte S. S., Olsen B. R. Nonfibrillar collagens. Methods Enzymol., 1994, 245:3-28.

G. Kesava Reddy and Chukuka S. Enwemeka. "A Simplified method of the analysis of hydroxyproline in biological tissues". Clinical Biochemistry, 1996, Vol. 29, No. 3:225-229.

Gabbiani G., Chapponier C., and Huttner I. "Cytoplasmic filaments and gap junctions in epithelial cells and myofibroblasts during wound healing". Journal Cell Biology, 1978, 76:561-568.

Gabbiani G., Hirschel B. J., Ryan G. B., Statkov P. R. and Majno G. "Granulation tissue as a contractile organ. A study of structure and function". Journal Exp. Med., 1972, 135:719-734.

Gabbiani G., Ryan G. B. and Majno G. "Presence of modified fibroblastsin granulation tissue and their possible role in wound contraction". Experentia, 1971, 27:549-550.

Galeano M, Torre V, Deodato B, Campo G. M, Colonna M, Sturiale A, Squadrito F, Cavallari V, Cucinotta D, Buemi M, Altavilla D. "Raxofelast, a hydrophilic vitamin E – like anti-oxidant stimulates wound healing in genetically diabetic mice". Surgery 2001 Apr;129(4):467-77

Garry J. Southan and Csaba Szabo "Selective Pharmacological inhibition of distinct Nitric Oxide synthase isoforms" Biochemical Pharmacology, 1996, Vol. 51:383-394.

Gauss-Muller V., Kleinman H. K., Martin G. R. and Schiffman E. "Role of attachment factors and attractants in fibroblast chemotaxis". Journal Lab. Clin. Med., 1980, 96:1071-1080.

Gay S, Viljanto J, Raekallio J and Penttinnen R. "Collagen types in early phases of wound healing in children". Acta. Chir. Scand. 1978, 144:205-211.

Geisler S., Lichtinghagen R., Boker K.H.W., Veh R. W. "Differential distribution of five members of the matrix metalloproteinase family and one inhibitor (TIMP-1) in human liver and skin" Cell Tissue Res., 1997, 289: 173-183.

George A. C. Murell, Daniel Jang and Riley J Williams. "Nitric Oxide Activates Metalloproteinase Enzymes In articular Cartilage". Biochem Biophys Res Commun., 1995, 206(1):15-21.

George P. Stricklin, Liying Li and Lillian B. Nanney. "Localization of mRNAs representing interstitial collagenase, 72-kDa gelatinase, and TIMP in healing porcine burn wounds" J Invest Dermatol., 1994, 103:352-358.

George P. Stricklin, Liying Li, Vesna Jancic, Barbara A. Wenczak and Lilian B. Nanney. "Localization of mRNAs representing collagenase and TIMP in sections of healing human burn wounds" Am. J Pathol 1993, 143: 1657-1666.

Gipson I. K., Grill S. M., Spurr S. J. and Brennan S. J. "Hemidesmosome formation in vitro". Journal Cell Biol., 1983, 97:849-857.

Giustizieri M. L., Albanesi C, Scarponi C, De Pita O, Girolomoni G. "Nitric oxide donors suppress chemokine production by keratinocytes in vitro and in vivo". Am J Pathol 2002, 161(4):1409-1418.

Goldstein S, Czapski G, Lind J and Merenyi G. "Tyrosine nitration by simultaneous generation of NO and O_2^- under physiological conditions. How the radicals do the job. Journal Biol. Chem., 2000, 275:3031-3036.

Gospadarowiez D, Abrahain J. A., Schilling J. "Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculostel- late cells". Proc Natl Acad Sci USA 1989, 86:7311-7315.

Griffits C. E. M., Barker J. N. W. N, Kunkel S. and Nickoloff B. J. "Modulation of leukocyte adhesion molecules, a T-cell chemotaxin(IL-8) and a regulatory cytokine (TNF-α) in allergic contact dermititis (rhus dermatitis)". Br. Journal Dermatol, 1991, 124:519.

Grillo H. C. "Derivation of fibroblasts in the healing wound". Arch. Surg., 1964, 88:218-224.

Grillo H. C. "Origin of fibroblasts in wound healing: An autoradiographic study of inhibition of cellular proliferation by local X-irradiation". Ann. Surg., 1963, 157:453-467.

Grimm M, Spiecker M, De Caterina R, Shin W. S., Liao J. K. "Inhibition of major histocompatibility complex class II gene transcription by nitric oxide and antioxidants". J Biol Chem., 2002, 277(29):26460-26467.

Grotendorst G. R. "Can collagen metabolism be controlled?". Journal Trauma, 1984, 24(9 Suppl):S49-54.

Grotendorst G. R., Chang T., Seppa H. E., Kleinman H. K., Martin O. R. "Platelet- derived growth factor is a chemoattractant for vascular smooth muscle cells". J Cell Physiol 1982, 1 13:261-266.

Grotendorst G. R., Martin G. R., Pencev D., Sodek J. and Harvey A. K. "Stimulation of granulation tissue formation by platelet derived growth factor in normal and diabetic rats". Journal Clin. Invest., 1985, 76:2323-2329.

Gundumi Aravinda Upadhya and Steven M. Strasberg "Glutathione, Lactobionate and Histidine: Cryptic Inhibitors of matrix metalloproteinases contained in University of Wisconsin and Histidine/Tryptophan/Ketoglutarate Liver preservation solutions" Hepatology, 2000, 31:1115-1122.

Han Ping Shi, David T. Efron, Daniel Most and Adrian Barbul. "The role of iNOS in wound healing". Surgery, 2001, 130:225-229.

Han Ping Shi, Rhonda S. Fishel, David T. Efron, Jeremy Z. Williams, Matthew H. Fishel and Adrian Barbul. "Effect of supplemental ornithine on wound healing" Journal of Surgical Research, 2000, 106:299-302.

Hans Oxlund, Henrik Christensen, Mikkel Seyer-Hansen and Troels T. Andreassen. "Collagen deposition and mechanical strength of colon anastomoses and skin incisional wounds of rats". Journal of Surgical Research, 1996, 66:25-30.

Hardwick J. B. J., Tucker A.T., Wilks M., Johnston A. and Benjamin N. "A novel method for the delivery of nitric oxide therapy to the skin of human subjects using a semi-permeable membrane" Clinical Science, 2001, 100:395-400.

Harper J. R., Spiro R. C., Gaarde W. A., et al. Role of transforming growth factor beta and decorin in controlling fibrosis. Methods Enzymol 1994, 245:241-254.

Harris E. D., and Krane S. M. "Collagenases". N. Engl. J. Med., 1974, 291: 652-661.

Hasty K. A., Hibbs M. S., Kang A. H. and Mainardi C. L. "Heterogeneity among human collagenases demonstrated by monoclonal antibody that selectively recognizes and inhibits human neutrophil collagenase". Journal Exp. Med., 1984, 159:1455-1463.

Hasty K. A., Hibbs M. S., Kang A. H. and Mainardi C. L. "Secreted forms of human neutrophil collagenase". Journal Biol. Chem., 1986, 261:5645-5650.

Hazel Sutherland, Roba Khundkar, Olga Zolle, Anne McArdle, Alec W.M.Simpson, Jonathan C. Jarvis and Stanley Salmons. "A fluorescence-based method for measuring Nitric Oxide in extracts of skeletal muscle". Nitric Oxide: Biology and Chemistry, 2001, 5:475-481.

Heck D. E., Laskin D. L., Gardner C. R., etal. "Epidermal Growth factor suppresses nitric oxide and Hydrogen peroxide production by keratinocytes. Potential role of Nitric oxide in the regulation of wound healing". Journal Biol Chem., 1992, 267: 21277-21280.

Heike Steiling, Barbara Munz, Sabine Werner and Maria Brauchle "Different types of ROS-Scavenging enzymes are expressed during cutaneous wound repair" Experimental Cell Research, 1999, 247:484-494.

Heiko Kampfer, Nicole Kolb, Markus Manderscheid, Christian Wetzler, Josef Pfeilschifter and Stefan Frank. "Macrophage-Derived Heme-Oxygenase-1: Expression, Regulation and possible functions in skin repair". Molecular Medicine, 2001, 7(7):488-498.

Heimark R. L., Twardzik D. R., Schwartz S. M. "Inhibition of endothelial cell regeneration by type-beta transforming growth factor from platelets". Science 1986, 233:1078-1080.

Heughan C., Hunt T. K.. "Some aspects of wound healing research: a review". Can J Surg., 1975, 18(2):118-126.

Higashiyama S, Abraham J. A., Klagsbrun M. "Heparin-binding EOF-like growth factor stimulation of smooth muscle cell migration: dependence of interactions with cell surface heparan sulfate". J Cell Biol 1993, 122:933-940.

Higashiyama M, Hashimoto K, Takada A, Fujita K, Kido K and Yoshikawa K. "The role of growth factor in wound healing". Journal Dermatol 1992, 19(11):676-679.

Hong Zeng, Netanya Y. Spencer and Neil Hogg. "Metabolism of S-nitrosoglutathione by endothelial cells". Am J Physiol Heart Circ Physiol., 2001, 281: H432-H439.

Hoppenreijs V. P., Pels E., Vrensen G. F., Treffers W. F. "Basic fibroblast growth factor stimulates corneas endothelial cell growth and endothelial wound healing of human corneas". Invest Ophthalmol Vis Sci., 1994, 35 :931-944.

Huang J., Olsen T., Huang S. "The role of growth factors in tissue repair I". The molecular and cellular biology of wound repair, Eds. Plenum Press, 1988;243-251.

Hugo P. Monteiro, Jasmine Gruia-Gray, Tereza M.S. Peranovich, Laura C. Barbosa De Oliviera and Arnold Stern. "Nitric Oxide stimulates Tyrosine Phosphorylation of focal adhesion kinase, SRC kinase and Mitogen-activated protein kinases in murine fibroblasts" Free Radical Biology & Medicine, 2000, Vol. 28, No. 2:174-182.

Huhtala P., Humphries M. J., McCarthy J. B., Tremble P. M., Werb Z. and Damsky C. H. "Cooperative signalling by alpha 5 beta 1 and alpha 4 beta 1 integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin". Journal Cell Biology, 1995, 129, 3, 867-879.

Hui Jung Kim, Xiangde Liu, Hangjun Wang, Tadashi Kohyama, Tetsu Kobayashi, Fu-Qiang Wen, Debra J. Romberger, Shinji Abe, William MacNee, Irfan Rahman, and Stephen I. Rennard. "Glutathione prevents inhibition of fibroblast-mediated collagen gel contraction by cigarette smoke". Am J Physiol Lung Cell Mol Physiol, 2002, 283: L409-L417.

Ignarro L. J, Murad F. Eds. "cGMP signaling through cAMP and cGMP dependent Protein kinases In : Nitric oxide ,Biochemistry , Molecular Biology and therapeutic Implications". San Diego: Academic Press. Inc, 1995, 305-322.

Ignotz R. A. and Massague J. "Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into extracellular matrix". Journal Biol. Chem., 1986, 261:4337-4345.

Istvan Arany, Mirian M. Brysk, Henry Brysk and Stephen K. Tyring. "Regulation of inducible Nitric Oxide Synthase mRNA levels by differentiation and Cytokines in Human Keratinocytes". Biochem and Biophys Res Commun 1996, 220:618-622.

James N. Smith and Tara P. Dasgupta. "Kinetics and Mechanism of the Decomposition of S-Nitrosoglutathione by L-Ascorbic Acid and Copper Ions in aqueous solution to produce Nitric Oxide". Nitric Oxide:Biology and Chemistry, 2000, Vol. 4, No. 1:57-66.

James Varani, Bill Burmeister, Robert G. Stirin, Susan B. Shollenberger, Dennis R. Inman, Suzanne E.G. Fligiel, Douglas F. Gibbs and Kent Johnson. "Expression of Serine proteinases and metalloproteinases in organ-cultured human skin" Am. J Pathol 1994, 145: 561-573.

Janusz Marcinkiewicz "Nitric Oxide and antimicrobial activity of reactive oxygen intermediates" Immunopharmacology, 1997, 37:35-41.

Jeanette R. Hill, John A. Corbett, Guim Kwon, Connie A. Marshall and Michael L. McDaniel. "Nitric Oxide Regulates Interleukin 1 Bioactivity Released from Murine Macrophages, 1996, Volume 271, Number 37, Issue of September 13:22672-22678.

Jeffry D. Shearer, John R. Richards, Charles D. Mills and Michelle D. Caldwell. "Differential regulation of macrophage arginine metabolism: a proposed role in wound healing". AM. J. Physiol., 1997, 272:E181-E190.

Jens J. Thiele, Christina Schroeter, Sherry N. Hsieh, Maurizio Podda, Lester Packer. "The Antioxidant Network of the stratum corneum" Curr. Prob. Dermatol., 2001, Vol. 29:26-42.

Jochen Thur, Roswitha Nischt, Thomas Krieg and Nicolas Hunzelmann. "Quantitative analysis of α1(1) procollagen transcripts in vivo by competitive polymerase chain reaction". Matrix Biology, 1996, Vol. 15:49-52.

Jonathan S Reichner, Adriana J Mezarovs, Claudine A Louis, William L Henry jr, Balduino Mostro Fransescio, Barbara-anne martin and jorge E Albina. "Molecular and metabolic evidence for the restricted expression of inducible nitric oxide synthase in healing wounds". Am. J. Pathol 1999, 154:1097-1104.

Jorge E. Albina, Charles D Mills, William L Henry Jr and Michael D Caldwell. "Temporal expression of Different pathways of L-Arginine Metabolism In healing wounds". The Journal Of Immunology, 1990, Vol 144(10): 3877-3880.

Joseph A. Bauer, Weisun Rao, Daniel J. Smith. "Evaluation of Linear Polyethyleneimine / nitric oxide adduct on wound repair: Therapy versus toxicity". Wound Repair and Regen 1998, 6: 569- 577.

Joseph A. Knight. "Review: Free Radicals, Antioxidants and the Immune systems" Annals of Clinical & Laboratory Science, 2000, Vol. 30, No. 2:145-158.

Jude E. B., Boulton A. J., Ferguson M. W., Appleton I. "The role of nitric oxide synthase isoforms and arginase in the pathogenesis of diabetic foot ulcers: possible modulatory effects by transforming growth factor beta 1". Diabetologia, 1999, 42(6):748-757.

Justus Benrath, Manfred Zimmermann and Frank Gillardon. "Substance P and nitric oxide mediate wound healing of ultraviolet photodamaged rat skin: evidence for an effect of nitric oxide on keratinocyte proliferation". Neuroscience Letters, 1995, Volume 200, Issue 1 : 17-20.

Kaija Inkinen, Hilkka Turakainen, Henrik Wolff, Laura Ravanti, Veli-Matti Kahari and Juhani Ahonen. "Expression and activity of matrix metalloproteinase-2 and –9 in experimental granulation tissue." APMIS, 2000, 108:318-328.

Kale Juva and Darwin J. Prockop. "Modified procedure for the assay of H^3- or C^{14}- labeled hydroxyproline". Analytical Biochemistry, 1966, 15:77-83.

Kanegasaki S. "Active oxygen generating system in immune cells". Jap. J. Clin. Pathol., 1992, 40(4):392.

Kang K. W, Choi S. H and Kim S. G. "Peroxynitrite activates NF-E2-related factor 2/antioxidant response element through the pathway of phosphatidylinositol 3-kinase: The role of nitric oxide synthase in rat glutathione S-transferase A2 induction". Nitric Oxide Biol Chem, 2002, 7(4):244-253.

Karola Vorauer-Uhl, Eckhard Fürnschlief, Andreas Wagner, Boris Ferko and Hermann Katinger. "Topically applied liposome encapsulated superoxide dismutase reduces postburn wound size and edema formation". Eur J Pharm Sci, 2001, 14(1):63-67.

Kate A. Scott, Edward J. Wood, Eric H. Karran. "A matrix metalloproteinase inhibitor which prevents fibroblast-mediated collagen lattice contraction" FEBS Letters 1998, 441:137-140.

Kazhuhiro Sasaki, Takako Hattori, Takuo Fujisawa, Kojiro Takahashi, Hajime Inoue and Masaharu Takigawa. "Nitric Oxide Mediates Interleukin -1 - Induced Gene Expression of Matrix Metalloproteinases and Basic Fibroblast Growth Factor in rabbit Articular Chondrocytes". Journal Biochem., 1998, 123: 431-439.

Kei Kuroda, Hiroshi Shinkai. "Differential regulations of matrix metalloproteinases and tissue inhibitors of metalloproteinases in dermal fibroblasts by dibutyril cyclic AMP" Journal of dermatological science, 1997, 15:95-100.

Kelman Cohen I and Bruce A. Mast. "Models of wound healing". The Journal of Trauma, 1990, Vol. 30, No. 12:S149-S155.

Khouri R, Hong S, Deune E, et al. De novo generation of permanent neovascularized soft tissue appendages by platelet-derived growth factor. J Clin Invest 1994, 94:1757-1763.

Kim K. J., Li B, Winer J, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo. Nature 1993, 362:841-844.

Kirk S. J., M Hurson, M. C. Regan, D. R. Holt, H. L. Wasserkrug and G Efron. "Arginine enhances wound healing and lymphocyte immune function in elderly human beings". Surgery, 114:155-160.

Kiyohara Y, Nishiguchi K, Komada F, Iwakawa S, Hirai M, Okumura K. "Cytoprotective effects of epidermal growth factor (EGF) ointment containing nafamostat, a protease inhibitor, on tissue damage at burn sites in rats". Biol Pharm Bull, 1993, 16(11):1146-1149.

Klagsbrun M. "Mediators of angiogenesis: the biological significance of basic fibroblast growth factor (bFGF)-heparin and heparan sulfate interactions". Cancer Biol 1992, 3:81-87.

Knighton D. R., Hunt T. K., Thakral K. K., Goodson W. H. 3rd. "Role of platelets and fibrin in the healing sequence: an in vivo study of angiogenesis and collagen synthesis". Ann Surg 1982,196(4):379-388.

Kokushi Yamasaki, Howard D. J. Edington, Carol McClosky, Edith Tzeng, Alina Lizonowa, Imre kovezdi, David L sted and Timothy R Billiar. "Reversal of impaired wound repair in iNOS- deficient mice by topical adino viral mediated iNOS Gene transfer". Journal clin. Invest 1998, Vol. 101:967-971.

Kolb Bachofen, Fehsel K, Michel G et al. Epidermal keratinocyte expression of inducible nitric oxide synthase in skin lesions of Psoriasis Vulgaris. Lancet, 1994, 344 : 139.

Kostyk S. K., D'Amore P.A., Herman Inn, Wagner J. A. "Optic nerve injury alters basic fibroblast growth factor localization in the retina and optic tract". Journal Neurosei, 1994, 14:1441-1449.

Krawczyk W. S. "A pattern of epidermal cell migration during wound healing". Journal Cell Biology, 1971, 49:247-263.

Kristyn S. Bohl Masters, Joseph Leibovich S., Paula Belem, Jennifer L. West and Laura A. Poole-Warren. "Effects of nitric oxide releasing poly(vinyl alcohol) hydrogel dressings on dermal wound healing in diabetic mice". Wound Rep Reg, 2002, 10:286-294.

Ku P., Damore P. "Regulation of basic fibroblast growth factor (BFGF) gene and protein expression following its release from sublethally injured cells". Journal Cell Biochem., 1995, 58:328-343.

Kubicka U, Olszewski W. L., Tarnowski W, Bielecki K, Ziolkowska A, Wierzbicki Z. "Normal human immune peritoneal cells: subpopulations and functional characteristics". Scand J Immunol 1996, 44(2):157-163.

Kukovetz W. R., Holzmann S. and Schmidt K. "Cellular mechanisms of action of therapeutic nitric oxide donors" European Heart Journal, 1991, 12 (Supplement E):16-24.

Kurkinen M., Vaheri A., Roberts P. J., Stenman S. "Sequential appearance of fibronectin and collagen in experimental granulation tissue". Lab Invest., 1980, 43(1):47-51.

Khurshid Alam, Mahmoud N. Nagi, Othman A. Al-Shabanah, Abdullah M. Al-Bekairi. "Beneficial effect of nitric oxide synthase inhibitor on hepatotoxicity induced by allyl alcohol". Journal of biochemical and molecular toxicology, 2001, 15(6): 317-321.

Kurt Schmidt, Wolfgang Desch, Peter Klatt, Walter R. Kukovetz, Bernd Mayer. "Release of nitric oxide from donors with known half-life: a mathematical model for calculating nitric oxide concentrations in aerobic solutions" Naunyn-Schmiederg's Arch Pharmacol, 1997, 355:457-462.

Kuzuya M., Asai T., Kanda S., Maeda K., etal. « Glycation cross-links inhibit matrix metalloproteinase-2 activation in vascular smooth muscle cells cultured on collagen lattice » Diabetologia, 2001, 44:433-436.

Kwon N. S, Nathan C. F, Gilker C, Griffith O. W, Matthews D. E, Stuehr D. J. "L-citrulline production from L-arginine by macrophage nitric oxide synthase. The ureido oxygen derives from dioxygen". J. Biol Chem., 1990, 265(23): 13442-13445.

Laurence Besseau, Bernard Coulomb, Corrine Lebreton-Decoster and Marie-Madeleine Giraud-Gille. "Production of ordered collagen matrices for three-dimensional cell culture". Biomaterials, 2002, 23:27-36.

Laurent Pascual-Le Tallec, Carla Korwin-Zmijowska, Monique Adolphe. « Effects of simulated solar radiation on type I and type III collagens, collegenase (MMP-1) and stromelysin (MMP-3) gene expression in human dermal fibroblasts cultured in collagen gels » Journal of Photochemistry and Photobiology B : Biology 1998, 42 : 226-232.

Lawrence J. Dahm, Paula S. Samiec, John W. Eley, Elaine W. Flagg, Ralph J. Coates and Dean P. Jones. "Utilization of oral Glutathione". Free Radicals: From Basic Science to Medicine, 1993: 506-522.

Leek R., Harris A., Lewis C "Cytokine networks in solid human tumors: regulation of angiogenesis". Journal Leukoe Biol., 1994, 56:423-455.

Leibovich S. J. and Ross R. "The role of the macrophage in wound repair: A study with hydrocortisone and antimacrophage serum". Am. J. Pathol., 1975, 78:71-100.

Leif R. Lund, John Romer, Thomas H. Bugge, Boye S. Nielsen, etal. "Functional overlap between two classes of matrix-degrading proteases in wound healing" The EMBO journal, 1999, Vol. 18, No. 17: 4645-4656.

Lepisto J, Peltonen J, Vaha-Kreula M, Ninikoski J, Laato M. "Platelet-derived growth factor isoforms PDGF-AA, -AB and -BB exert specific effects on collagen gene expression and raitotic activity of cultured human wound fibroblasts". Biochem Biophys Res Commun 1995, 209:393-399.

Leppert D., Waubant E., Galardy R., Bunnett N. W. and Hauser S. L. "T Cell gelatinases mediate basement membrane transmigration in vitro". Journal Immunol., 1995, 154, 9:4379-4389.

Letterio J, Geiser A, Kulkarni A, Roche N, Sporn M, Roberts A. "Maternal rescue of transforming growth factor-01 null mice". Science 1994, 264:1936-1938.

Leung D. W., Cachlanes G, Kuang W. J., Goeddel D. V., Ferrara N. "Vascular endothelial growth factor is a secreted angiogenic miltogen". Science 1989, 246:1306-1309.

Levene C. K and Bates C. J. "The effect of hypoxia on collagen synthesis in cultured 3T6 fibroblasts and its relationship to the mode of action of ascorbate". Biochim. Biophys. Acta, 1976, 444: 446-452.

Levenson S. M., Geever E. F., Crowley L. V., Oates J. F. III., Berard C. W. and Rosen H. "The healing of rat skin wounds". Ann. Surg., 1965, 161:293-308.

Levi H. C. Makala, Yoshifumi Nishikawa, Takenori Kamada, Hiroshi Suzuki, Xuenan Xuan, Ikuo Igarashi and Hideyuki Nagasawa. "Comparison of the accessory activity of murine peritoneal cavity macrophage derived dendritic cells and peritoneal cavity macrophages In a mixed lymphocyte reaction". Journal Vet. Med. Sci., 2001, 63(12):1271-1277.

Li N and Karin M. "Is NF-κ B the sensor of oxidative stress?" FASEB J., 1999, 13:1137–1143.

Li V., Folkerth R., Watanabe H., Yu C., Rupnick M., Barnes P., Scott R., et al. "Microvessel count and cerebrospinal fluid basic fibroblast growth factor in children with brain tumors". Lancet, 1994,344:82-86.

Lidia Mazur "Radioprotective effects of the thiols GSH and WR-2721 against X-ray-induction of micronuclei in erythroblasts" Mutation Research, 2000, 468:27-33.

Lillian S. Ashcroft, Michael A. Horan, Sarah E. Herrick, Roy W. Tarnuzzer, Gregory S. Schultz, Mark W. J. Ferguson. "Age-related differences in the temporal and spatial regulation of matrix metalloproteinases(MMPs) in normal skin and acute cutaneous wounds of healthy humans" Cell Tissue Res., 1997, 290:581-591.

Lindblom L, Cassuto J, Yregard L, Mattsson U, Tarnow P, Sinclair R. "Importance of nitric oxide in the regulation of burn oedema, proteinuria and urine output". Burns 2000, 26(1):13-17.

Lindner V., Reidy M. A. "Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor". Proc Natl Acad Sci USA, 1991, 88:3739-3743.

Linsenmayer T. F., Chen Q. A., Gibney E., Gordon M. K., Marchant J. K., Mayne R, Schmid T.M. "Collagen types IX and X in the developing chick tibiotarsus: analyses of mRNAs and proteins". Development, 1991, 111(1):191-196.

Liotta L. A., Abe S., Robey P. G. and Martin G. R. "Preferential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor". Proc. Natl. Acad. Sci. USA, 1979, 76:2268-2272.

Louis J. Ignarro, Jon M. Fukuto, Jeanette M. Griscavage, Norma E. Rogers and Russell E. Byrns. "Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate : Comparison with enzymatically formed nitric oxide from L-arginine". Proc. Natl. Acad. Sci. USA, 1993, Vol. 90: 8103-8107.

Lowenstein C. J., Dinerman J. L., Snyder S. H. "Nitric Oxide: a physiologic messenger". Ann intern Med., 1994, 120:227-237.

Lu L, Bonham C. A., Chambers F. G., Watkins S. C., Hoffman R. A., Simmons R. L., Thomson A. W. "Induction of nitric oxide synthase in mouse dendritic cells by IFN-gamma, endotoxin, and interaction with allogeneic T cells: nitric oxide production is associated with dendritic cell apoptosis". Journal Immunol., 1996,157(8):3577-3586.

Lund J. N., Scholefield J. H. "Glyceryl trinitrate is an effective treatment for anal fissure". Dis Colon Rectum, 1997, 40(4):468-470.

Madden J. W. and Peacock E. E. "Studies on the biology of collagen during wound healing. I. Rate of collagen synthesis and deposition in cutaneous wounds of the rat". Surgery, 1968, 64:288-294.

Madlener M. "Differential expression of matrix metalloproteinases and their physiological inhibitors in acute murine skin wounds" Arch Dermatol Res., 1998, 290:S24-S29.

Magnus S. Agren, Carolyn J. Taplin, J. Frederick Woessner, Jr., William H. Eaglstein and Patricia M. Mertz. "Collagenase in Wound Healing: Effect of wound age and type" Journal Invest Dermatology 1992, 99:709-714.

Mahesh S. Joshi, Julie L. Ponthier and Jack R. Lancaster, JR. "Cellular Antioxidant and Pro-oxidant Actions of Nitric Oxide". Free Radical Biology & Medicine, 1999, Vol. 27, Nos. 11/12:1357-1366.

Manuel Lois, Lou Ann S. Brown, I. Marc Moss, Jesse Roman and David M. Guidot. "Ethanol ingestion increases activation of matrix metalloproteinases in rat lungs during acute endotoxemia" AM J Respir Crit Care Med, 1999, 160:1354-1360.

Marc S. Rendell, Mark L. Johnson, Denae Smith, David Finney, Christopher Capp, Rebecca Lammers and Scott Lancaster. "Skin blood flow response in the rat model of wound healing: Expression of vasoactive factors". Journal of Surgical Research, 2002, 107:18-26.

Marek W. Radomski, Daryl D. Rees, Alberto Dutra and Salvador Moncada. "S-nitrosoglutathione inhibits platelet activation in vitro and in vivo" J. Pharmacol., 1992, 107:745-749.

Maria Agelli and Sharon M. Wahl. "Collagen production by fibroblasts". Methods in Enzymology, 1998, Vol. 163:642-656.

Maria Angeles Martinez, Eduardo Salas, Anna Radomski, Marek W. Radomski. "Matrix metalloproteinase-2 in platelet adhesion to fibrinogen: interactions with nitric oxide" Med Sci Monit, 2001,7(4):646-651.

Maria B. Witte and Adrian Barbul. "Role of Nitric Oxide in wound repair". Am. J. Surg., 2002, 183(4):406-412.

Maria B. Witte, Frank J. Thornton, Teruo Kiyama, David T. Efron, Gregory S. Schulz, Lyle L. Moldawer and Adrian Barbul. "Metalloproteinase inhibitors and wound healing: A novel enhancer of wound strength". Surgery, 1998, 124: 464-70.

Maria Laura Giustizieri, Cristina Albanesi, Claudia Scarponi, Ornella De Pita and Giampiero Girolomoni. "Nitric Oxide donors suppress chemokine production by keratinocytes in vitro and in vivo". Am. J. Pathol, 2002, 161:1409-1418.

Marianne Madlener, William C. Parks and Sabrina Werner. "Matrix metalloproteinases (MMPs) and their physiological inhibitors (TIMPs) are differentially expressed during excisional skin wound repair" Experimental Cell Research, 1998, 242:201-210.

Mariarosaria Galeano, Valerio Torre, Barbara Deodata, Giuseppe M. Campo, Michele Colonna, Alessio Sturiale, Francesco Squadrito, Vittorio Cavallari, Domenico Cucinotta, Michele Buemi and Domenica Altavilla. "Raxofelast, a hydrophilic vitamin E-like antioxidant, stimulates wound healing in genetically diabetic mice". Surgery, 2001, 129:467-477.

Marie-Laure Ferrier, Sophie Combet, Mieke Van Landschoot, Maria S. Stoenoiu, Yvette Cnops, Norbert Lameire and Olivier Devuyst. Inhibition of nitric oxide synthase reverses changes in peritoneal permeability in a rat model of acute peritonitis. Kidney International, 2001, Vol. 60:2343-2350.

Marja Makela, Hannu Larjava, Emma Pirila, Paivi Maisi, Tuula Salo, Timo Sorsa and Veli-Jukka Uitto. « Matrix metalloproteinase 2 (Gelatinase A) is related to migration of keratinocytes ». Experimental Cell Res. 1999, 251:67-78.

Mark A. Catherwood, Lesley A. Powell, Paul Anderson, Dorothy Mcmaster, Peter C. Sharpe and Elisabeth R. "Trimble Glucose-induced oxidative stress in mesangial cells". Kidney International, 2002, Volume 61 Issue 2: 599.

Mark Steffen, Theresa M. Sarkela, Anna a. Gybina, Terry W. Steele, Nathaniel J. Trasseth, Douglas Kuehl and Cecilia Giulivi. "Metabolism of S-nitrosoglutathione in intact mitochondria" Biochem., Journal, 2001, 356:395-402.

Marletta M. A, Hurshman A. R and Rusche K. M. "Catalysis by nitric oxide synthase". Curr Opin Chem Biol., 1998, 2(5):656-663.

Martin Feelisch "The use of Nitric Oxide donors in pharmacological studies" Naunyn-Schmiederg's Arch Pharmacol, 1998, 358:113-122.

Meister A. "Approaches to the therapy of glutathione deficiency" Free Radicals: From Basic Science to Medicine, 1993:489-503.

MeNell P. L., Muthukrishnan L., Warder E., D'Ainore P.A. "Growth factors are released by mechanically wounded endothelial cells". Journal Cell Biol., 1989, 109:811-822.

Mertz P. M., DeWitt D. L., Stetler-Stevenson W. G. and Wahl L.M. "Interleukin 10 supression of monocyte prostaglandin H synthase-2. Mechanism of inhibition of prostaglandin-dependent matrix metalloproteinase production". Journal Biol. Chem., 1994, 269, 33:21322-21329.

Merwin J. R., Anderson J. M., Kocher 0, Van Itallic C. M., Madri J. A. "Trans- forming growth factor beta, modulates extracellular matrix organization and cell-cell Functional complex formation during in vivo angiogenesis". J Cell Physiol 1990, 142:117-128.

Michael Kirsch, Hans-Gert Korth, Reiner Sustmann and Herbert de Groot. "The Pathobiochemistry of Nitrogen Dioxide". Biol. Chem., 2002, Vol. 383:389-399.

Michael P. Gordge, Paolo Addis, Alberto A. Noronha-Dutra and John S. Hothersall " Cell-Mediated Biotransformation of S-Nitrosoglutathione". Biochemical Pharmacology, 1998, Vol. 55:657-665.

Michael R Schaffer, Nico Fuchs, Barbara Proksch, Martina Bongartz, Thomas Beiter and Horst – Dieter Becker. "Tacrolimus impairs wound healing", Transplantation 1998, 65(6): 813-818.

Michael R Schaffer, Philip A Efron, Frank J Thornton, Karin Klingel, Steven S Gross and Adrian Barbul. "Nitric Oxide an Autocrine Regulator of Wound Fibroblast Synthetic Function". Journal Immun, 1997, 158:2357-2381.

Michael R Schaffer, Udaya Tantri, Francis J Thronton and Adrian Barbul. "Inhibitons of nitric oxide synthesis in wounds: pharmacology and effect on accumulation of collagen in wounds in mice". Eur.J.Sur 1999, 165:262-267.

Michael R Schaffer, Udaya Tantri, Gretchen M. Ahrendt, Hana L Wasser Krugh and Adrian Barbul. "Acute protein calory malnutrition impairs wound healing: a possible role of decreased nitric oxide synthesis". Journal AM. Coll. Surg 1997, 184:37-43.

Michael R Schaffer, Udaya Tantry, Phillip A Efron, Gretchen M Ahrendt, Francis J.Thronton and Adrian Barbul. "Diabetes –impaired healing and reduced wound nitric oxide synthesis: A possible pathophysiologic correlation". Surgery, 1997, 121:513-519.

Michael R. Schaffer, Udaya Tantry, Robert A. Van Wesep and Adrian Barbul. "Nitric Oxide Metabolism In Wounds". Journal Sur Res 1997, 71: 25-31.

Michael R. Schaffer, Wiebke Weimer, Susanne Wider, Christina Stulten, Martina Bongartz, Wilfried Budach and Horst-Dieter Becker. "Differential expression of inflammatory mediators in radiation-impaired wound healing." Journal of Surgical Research, 2002, 107:93-100.

Michelle R. Schaffer, Udaya Tantri, Steven S Gross, Hannah L Wasserkrug and Adrian Barbul. "Nitric Oxide regulates wound healing". Journal Surg. Res 1996, 63 :237-240.

Michael S. Crane, Richard Ollosson, Kevin P. Moore, Adriano G. Rossi, and Ian L. Megson. "Novel Role for Low Molecular Weight Plasma Thiols in Nitric Oxide-mediated Control of Platelet Function". Journal Biol. Chem., 2002, Vol. 277, Issue 49:46858-46863.

Mignatti P., Morimoto T., Rifkin D. B. "Basic fibroblast growth factor, a protein devoid of secretory signal sequence, is released by cells via a pathway independent of the endoplasmic reticulum-Golgi complex". Journal Cell Physiol., 1992, 151:81-93.

Millauer B, Shawver L. K., Risan W, Ullrich A. "Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant". Nature 1994, 367:576-579.

Miller J. W., Adamis A. P., Shima D. T., et al. Vascular permeability factor/ vascular endothelial cell growth factor is temporally and spatially correlated with ocular angiogenesis in a primate model. Am J Pathol 1994, 145:574-584.

Minamiyama Y., Takemura S. and Inoue M. "Effect of Thiol Status on Nitric Oxide Metabolism in the circulation" Archives of Biochemistry and Biophysics, 1997, Vol. 341, No. 1:186-192.

Misato Kashiba, Emiko Kasahara, Kuang C. Chien and Masayasu Inoue. "Fates and Vascular action of S-Nitrosoglutathione and related compounds in the circulation" Archives of Biochemistry and Biophysics, 1999, Vol. 363, No. 2:213-218.

Moncada S, Palmer R. M., Higgs E. A. "Nitric Oxide : Physiology, Pathophysiology and Pharmacology". Pharmacol Rev., 1991, 43:109-142.

Montesano R., Vassali J. D., Baird A., Guillemin R., Orci L. Basic fibroblast growth factor induces angiogenesis in vitro. Proe Natl Acad Sci USA, 1986, 83:7297-7301.

Montgomery A. M., Sabzevari H, and Reisfeld R. A.. "Production and regulation of gelatinase B by human T-cells. Biochem. Biophys. Acta., 1993, 1176, 3:265-268.

Moochhala S, Chhatwal V. J., Chan S. T., Ngoi S. S., Chia Y. W., Rauff A. "Nitric oxide synthase activity and expression in human colorectal cancer". Carcinogenesis, 1996, 17(5):1171-1174.

Most D, Efron D. T., Shi H. P., Tantry U. S., Barbul A. "Characterization of incisional wound healing in inducible nitric oxide synthase knockout mice". Surgery 2002, 132(5):866-876.

Murali S, Hardten D. R., DeMartelaere S, et al. "Effect of topically administered platelet-derived growth factor on corneal wound strength". Curr Eye Res 1994, 13:857-862.

Muscara M. N., McKnight W, Asfaha S, Wallace J. L. Wound collagen deposition in rats: effects of an NO-NSAID and a selective COX-2 inhibitor". Br.Journal Pharmacol, 2000,129(4):681-686.

Muthukrishnan L., Warder E., McNeil P.L. "Basic fibroblast growth factor is efficiently released from a cytosolic storage site through plasma membrane disruptions of endothelial cells". Journal Cell Physiol., 1991, 148:1-16.

Nakane M, Mitchell J, Forstermann U and Murad F. "Phosphorylation by calcium calmodulin-dependent protein kinase II and protein kinase C modulates the activity of nitric oxide synthase". Biochem Biophys Res Commun., 1991, 180(3): 1396-1402.

Nathan C. "Nitric Oxide as a secretory product of mammalian cells". FASEB J 1992, 6:3051-3064.

Neil Hogg "Biological chemistry and clinical potential of S-Nitrosothiols" Free Radical Biology & Medicine, 2000, Vol. 28, No. 10:1478-1486.

Neil Hogg "The Biochemistry and Physiology of S-Nitrosothiols" Annu. Rev. Pharmacol, Toxicol, 2002, 42:585-600.

Neil Hogg, Ravinder J. Singh, B. Kalyanaraman. "The role of glutathione in the transport and catabolism of nitric oxide". FEBS Letters, 1996, 382:223-228.

Nelly Blumenkrantz. "Automated triple assay for proline, hydroxyproline and hydroxylysine on one single sample". Clin. Biochem., 1980, 13(4):177-183.

Nemethy G, Scheraga H. A. "Stabilization of collagen fibrils by hydroxyproline". Biochemistry, 1986, 52:3184-3188.

Newman S. L., Henson J. E. and Henson P. M. "Phagocytosis of senescent neutrophils by human monocyte derived macrophages and rabbit inflammatory macrophages". Journal Exp. Med., 1982, 156:430-442.

Nguyen M., Watanabe H., Budson A. E., Richic J. P., Foiknian J. "Elevated levels of an anglogenic peptide, basic fibroblast growth factor, in urine of patients with a wide spectrum of cancers". Journal Natl Cancer lnst., 1993, 86:356-361.

Nicosia R., Nicosia S., Smith M. "Vascular endothelial growth factor, platelet- derived growth factor, and insulin-like growth factor-1 promote rat aortic angiogenesis in vitro". Am J Pathol 1994, 145:1023-1029.

Niroshan Ramachandran, Paul Root, Xing-Mai Jiang, Phillip J. Hogg and Bulent Mutus. "Mechanism of transfer of NO from extracellular S-Nitrosothiols into the cytosol by cell-surface protein disulfide isomerase". PNAS, 2001, Vol. 98, No. 17: 9539-9544.

Okada Y., Naka K., Kawamura K., Matsumoto T., Nakanishi I., Fujimoto N., Sato H. and Seiki M. "Localization of matrix metalloproteinase 9 (92-kilodalton gelatinase/type IV collagenase = gelatinase B) in osteoclasts: Implications for bone resorption". Lab. Invest., 1995, 72, 3:311-322.

O'Reilly M. A., Danielpour D, Roberts A. B., Sporn M. B. "Regulation of expression of transforming growth factor-beta 2 by transforming growth factor-beta isoforms is dependent upon cell type". Growth Factors, 1992, 6(3):193-201.

Osamu Nohara, Marianna Kulka, Rene E. Dery, Fiona L. Wills, Nadir S. Hirji, Mark Gilchrist and A. Dean Befus. "Regulation of CD8 expression in mast cells by exogenous or endogenous nitric oxide" The journal of Immunology, 2001, 167:5935-5939.

Osamu Ohgoda, Atsushi Sakai, Hiroyasu Koga, Kazuo Kanai, Tsuneo Miyazaki and Yoshimi Niwano. "Fibroblast-migration in a wound model of ascorbic acid-supplemented three-dimensional culture system: the effects of cytokines and malotilate, a new wound healing stimulant, on cell-migration". J Dermatol Sci., 1998, 17(2):123-131.

Outi Kosonen, Hannu Kankaanranta, Jukka Uotila, Eeva Moilanen. "Inhibition by nitric oxide-releasing compounds of E-selectin expression in and neutrophil adhesion to human endothelial cells". European Journal of Pharmacology, 2000, 394:149-156.

Overall C. M., Wrana J. L. and Sodek J. "Transcriptional and post-transcriptional regulation of 72-Da gelatinase/type IV collagenase by transforming growth factor-beta 1 in human fibroblasts. Comparisons with collagenase and tissue inhibitor of matrix metalloproteinase gene expression. Journal Biol. Chem., 1991, 266:14064-14071.

Padua R. R., Kardami E. "Increased basic fibroblast growth factor (BFGF) accumulation and distinct patterns of localization in isoproterenol-induced cardiomyocyte injury". Growth Factors, 1993,8:291-306.

Palmer R. M. J., Ashton D. S., Moncada S. "Vascular endothelial cells synthesize Nitric Oxide from L arginine". Nature 1988, 333:664-666.

Pandarinathan Chithra, G.B. Sajithlal and Gowri Chandrakasan. "Influence of Aloe Vera on collagen characteristics in healing dermal wounds in rats". Mol. Cell Biochem., 1998, 181:71-76.

Parsons S. L., Watson S. A., Brown P. D., Collins H. M., etal. "Matrix Metalloproteinases" British Journal of Surgery, 1997, 84:160-166.

Patarroyo M. "Adhesion molecules mediating recruitment of monocytes to inflamed tissue". Immunobiology 1994, 191(4-5):474-477.

Patricia A Craven, Rebecca K studer , Jill felder , Stephen Philips and Frederick R De Rubertis. "Nitric Oxide Inhibition of Transforming Growth Factor β and Collagen Synthesis in Mesangial Cells". Diabetes, 1997, 46:671-681.

Paul C. Lee, Melina R. Kibbe, Matthe J. Schuchert, Donna B. Stolz, Simon C. Watkins, Bartley P. Griffith, Timothy R. Billiar and Larry L. Shears, II. "Nitric Oxide induces angiogenesis and upregulates $\alpha_v\beta_3$ integrin expression on endothelial cells" Microvascular Research, 2000, 60:269-280.

Paul Jurasz, Grzegorz Sawicki, Marek Duszyk, Jolanta Sawicka, Carlos Miranda, Irvin Mayers and Marek W. Radomski. "Matrix metalloproteinase 2 in tumor cell-induced platelet aggregation: Regulation by Nitric Oxide". Cancer Research, 2001, 61:376-382.

Paul R. G., Tarlton J. F., Purslow P. P., Sims T. J., Watkins P., Marshall F., Ferguson M. J., Bailey A. J. "Biomechanical and Biochemical study of a standardized wound healing model". Int. J. Biochem. Cell Biol., 1997, Vol. 29, No. 1:211-220.

Peacock E. E. Jr. "Wound contraction and scar contracture". Plast Reconstr Surg, 1978, 62(4):600-602.

Pepper M. S., Ferrara N, Orei L, Montesano R. "Vascular endothelial growth factor (VEOF) induces plasminogen activators and plasminogen activator inhibitory in microvascular endothelial cells". Biochem Biophys Res Commun 1991, 181:902-906.

Peter Brenneisen , Jutta Wnk, L. Oliver Klotz, Meinhard Wlaschek, Karlis Briviba, Thomas Krieg, Helmut Sies and Karin Scharffetter-Kochanek. "Central role of ferrous/ferric iron in the ultraviolet B irradiation-mediated signaling pathway leading to increased interstitial collagenase (Matrix-degrading metalloprotease (MMP)-1) and stromelysin-1 (MMP-3) mRNA levels in cultured human dermal fibroblasts." Journal of Biological Chemistry, 1998, Vol. 273, No. 9:5279-5287.

Peter Brenneisen, Karlis Briviba, Meinhard Wlaschek, Jutta Wenk and Karin Scharffetter-Kochanek. "Hydrogen Peroxide(H_2O_2) increases the steady-state mRNA levels of collagenase/MMP-1 in human dermal fibroblasts" Free Radical Biology & Medicine, 1997, Vol. 22, No. 3:515-524.

Peters K. G., De Yries C, Williams L. T. "Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggests a role in endothelial differentiation and blood vessel growth". Proc Natl Acad Sel USA 1993, 90:8915-8919.

Petros A, Lamb G, Leone A, Moncada S, Bennett D and Vallance P. "Effects of a nitric oxide synthase inhibitor in humans with septic shock". Cardiovasc Res., 1994, 28(1):34-39.

Picardo M, Zompetta C, Marchese C, De Luca C, Faggioni A, Schmidt R. J and Santucci B. "Paraphenylene diamine, a contact allergen, induces oxidative stress and ICAM-1 expression in human keratinocytes". Br. J. Dermatol, 1992, 126:450.

Pierce E. A., Avery R. L., Foley E. D., Aicilo L. P., Smith L. E. H. "Vascular endothelial growth factor/vascular permeability factor expression in a mouse model of retinal neovascularization". Proc Natl Acad Sci USA 1995, 92:905-909.

Pierce G. F., Brown D, Mustoe T. A. "Quantitative analysis of inflammatory cell influx, procollagen type I synthesis, and collagen cross-linking in incisional wounds: influence of PDGF-BB and TGF-beta 1 therapy". J Lab Clin Med., 1991, 117(5):373-382.

Pierce G. F., Tarpley J. E., Ailman R. M., et al. Tissue repair processes in healing chronic pressure ulcers treated with recombinant platelet-derived growth factor BB. Am J Pathol 1994, 145:1399-1410.

Pierce G. F., Tarpley J. E., Tseng J, et al. Detection of platelet-derived growth factor (PDGF)-AA in actively healing human wounds treated with recombinant PDGF-BB and absence of PDOF in chronic nonhealing wounds. J Clin Invest 1995, 96:1336-1350.

Pierce G. F., Vande Berg J, Rudolph R, Tarpley J, Mustoe T. A. "Platelet-derived growth factor-BB and transforming growth factor beta 1 selectively modulate glycosaminoglycans, collagen, and myofibroblasts in excisional wounds". Am J Pathol., 1991, 138(3):629-646.

Pierre-Andre Becherel, M. Djavad Mossalayi, Fateh Quaaz, Liliane Le golf, Bernard Dugas, Nathalie Paul-Eugene, Camille Frances, Olivier Chosidow, Erich Kilchherr, Jean-Jacques Guillosson, Patrice Debre and Michel Arock. "Involvement of cyclic AMP and Nitric Oxide in immunoglobulin E-dependent activation of FCεRII/CD23^{+} Normal human keratinocytes" J. Clin. Invest., 1994:2275-2279.

Pipili-Synetos E, Sakkoula E, Haralabopoulos G, Andriopoulou P, Peristeris P, Maragoudakis M. E. "Evidence that nitric oxide is an endogenous antiangiogenic mediator". Br J Pharmacol, 1994, 111(3):894-902.

Prem P. Gogia. Ed. Clinical wound management. Slack Inc., 1995.

Qureshi A. A., Hosoi J, Xu S et al. Langerhans cells express inducible nitric oxide synthase and produce nitric oxide. J Invest Dermat., 1996, 107: 815-821.

Rafael Radi, Adriana Cassina and Roberto Hodara. "Nitric Oxide and peroxynitrite interactions with mitochondria". Biol. Chem., 2002, Vol. 383: 401-409.

Rajeeva Singh, Walter A. Blattler and Albert R. Collinson "An amplified assay for Thiols based on reactivation of Papain" Analytical Biochemistry, 1993, 213:49-56.

Richard H. Lee, David Efron, Udaya Tantry and Adrian Barbul. " Nitric Oxide in the Healing Wound : A Time-Course Study". Journal of Surgical Research, 2001, 101:104-108.

Richardson G and Benjamin N. "Potential therapeutic uses for S-nitrosothiols". Clinical Science, 2002, 102:99-105.

Rijian Wang, Aziz Gahary, You J. Shen, Paul G Scott and Edward E Tredget. "Human dermal Fibroblasts Produce Nitric Oxide and Express Both Constitutive and Inducible Nitric Oxide synthase isoforms". J. Invest Dermatol., 1996, 106:419-427.

Rijian Wang, Aziz Gahary, You J. Shen, Paul G. Scott and Edward E Tredget. "Nitric Oxide Synthase Expression and Nitric Oxide Production are Reduced in Hypertrophic Scar Tissue and Fibroblasts". Journal Invest Dermatol, 1997, 108: 438-444.

Riley S. Rees, David J.Smith, JR., Belinda Adamson, Michael IM and Daniel Hinshaw. "Oxidant stress: The role of the glutathione redox cycle in skin preconditioning" Journal of Surgical Research, 1995, 58:395-400.

Risau W., Drexler H., Mironov V., et al. Platelet-derived growth factor in angiogenic in vivo. Growth Factors 1992, 7:261-266.

Rita Paroni, Elena De Vecchi, Giuliana Cighetti, Cinzia Arcelloni, Isabella Fermo, Adalberto Grossi and Pierangelo Bonini. "HPLC with 0-Phthalaldehyde precolumn derivatization to measure total, oxidised and protein-bound glutathione in blood, plasma and tissue" Clin. Chem., 1995, 41/3:448-454.

Roberts A. B., McCune B. K., Sporn M. B. "TGF-beta: regulation of extracellular matrix". Kidney Int., 1992, 41(3): 557-559.

Rodney Mathews W. and Steven W. Kerr " Biological Activity of S-Nitrosothiols: The role of Nitric Oxide" The Journal of Pharmacology and Experimental Therapeutics, 1993, Vol. 267, No. 3:1529-1537.

Rosalind M. Hembry and Ehrlich H.P. "Immunolocalization of collagenase and tissue inhibitor of metalloproteinases(TIMP) in hypertrophic scar tissue". British Journal of Dermatology, 1986, 115: 409-420.

Ross R, Gillitzer C, Kleinz R, Schwing J, Kleinert H, Forstermann U, Reske-Kunz A. B. "Involvement of NO in contact hypersensitivity". Int Immunol., 1998,10(1):61-69.

Ross R., Raines E., Bowen-Pope D. "The biology of platelet-derived growth factor". Cell 1986, 46:155-169.

Rowe A., Farrell A. M and Bunker C. B. "Constitutive endothelial and inducible nitric oxide synthase in inflammatory dermatoses" British Journal of Dermatology, 1997, 136:18-23.

Ruben Zamora, Katelijne E. Matthys, Arnold G. Herman. "The protective role of thiols against nitric oxide-mediated cytotoxicity in murine macrophage J774 cells" European Journal of Pharmacology, 1997, 321:87-96.

Ruslana Bryk, Donald J. Wolff "Pharmacological modulation of nitric oxide synthesis by mechanism-based inactivators and related inhibitors" Pharmacology and Therapeutics, 1999, 84:157-178.

Rutherford R. B. and Ross R. "Platelet factors stimulate fibroblasts and smooth muscle cells quiescent to plasma serum to proliferate". Journal Cell Biol., 1976, 69: 196-203.

Ryan G. B. and Majno G. "Inflammation". Am. J. Pathology, 1977, 86(1):183-276.

Ryszard Dabrowski, Andrzej Marczynski and Piotr Styczynski. "The effects of the stimulation of histamine-forming capacity (HFC) and the inhibition of histamine catabolism on tensile strength and collagen content of skin wounds in the rats". Acta Physiol. Pol., 1983, 34(1):91-97.

Sakai L. Y., Keene D. R., Morris N. P., Burgeson R. E. "Type VII collagen is a major structural component of anchoring fibrils". Journal Cell Biol., 1986, 103(4):1577-1586.

Sato H, Kita M and Seiki M. "Src activates the expression of 92-kDa type IV collagenase gene through the AP-1 site and the GT box homologous to retinoblastoma control elements: a mechanism regulating gene expression independent of that by inflammatory cytokines". Journal Biol Chem., 1993, 268:23460-23468.

Sato N., Beitz J., Kato J, et al. "Platelet-derived growth factor indirectly stimulates angiogenesis in vitro". Am J Pathol 1993, 142:1119-1130.

Sato Y, Rifkin D. B. "Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-beta 1-like molecule by plasmin during co-culture". J Cell Biol 1989, 109:309-315.

Schneemann M, Schoedon G, Frei K, Schaffner A. "Immunovascular communication: activation and deactivation of murine endothelial cell nitric oxide synthase by cytokines". Immunol Lett 1993, 35(2): 159-162.

Schroder J. M. "Cytokine networks in skin". Jounal Invest Dermatol, 1995, 105:20s.

Seifert R. A., Schwartz S. M., Bowen-Pope D. F. "Developmentally regulated pro- duction of platelet-derived growth factor-like molecules". Nature 1984, 311:669-671.

Seijiro Kado, Toru Wakatsuki, Mikio Yamamoto, Naokazu Nagata. "Expression of intercellular adhesion molecule-1 induced by high glucose concentrations in human aortic endothelial cells". Life Sciences 2001, 68:727–737.

Seltzer J. L., Adams S. A., Grant G. A. and Eisen A. Z. "Purification and properties of a gelatin-specific neutral protease from human skin". Journal Biol. Chem., 1981, 256, 4662-4668.

Senger D. R., Gall! S. J., A.M. D, Perruzzi C. A., Harvey V. S., Dvorak H. F. "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid". Science 1993, 219.

Senger D. R., Van De Water L, Brown L. F., et al. "Vascular permeability factor (VPF, VEGF) in tumor biology". Cancer Metastasis Rev 1993, 12:303-324.

Seppa H. E. J., Grotendorst G. R., Seppa S. I., Schiffman E. and Martin G. R. "The platelet derived growth factor is a chemoattractant for fibroblasts". Journal Cell. Biol., 1982, 92:584-588.

Seppa H., Grotendorst 0., Seppa S., Schiffmann E., Martin G. "Platelet-derived growth factor is chemotactic for fibroblasts". J Cell Biol 1982, 92:584.

Sergei N. Belenky, Richard A. Robbins and Israel Rubinstein "Nitric Oxide synthase inhibitors attenuate human monocyte chemotaxis in vitro" Journal of Leukocyte biology, 1993, Vol. 53:498-503.

Singer I. I., Kawka D. W., Kazazis D. M. and Clark R. A. F. "In vivo co-distribution of fibronectin and actin fibers in granulation tissue: Immunofluorescence and electron microscope studies of the fibronexus at the myofibroblast surface". Journal Cell Biology, 1984, 98:2091-2106.

Singer I. I., Kazazis D. M and Kawka D. W. "Localization of the fibronexus at the surface of granulation tissue myofibroblasts using double-label immunogold electron microscopy on ultrathin frozen sections". Eur. Journal Cell Biology, 1985, 38:94-101.

Shanthi Wasser, Ganymede Y Lim, Choon-Nam Ong and Carolyn E L Tan. "Anti-oxidant ebselen causes the resolution of experimentally induced hepatic fibrosis in rats". Journal of Gastroenterology and Hepatology, 2001, Vol 16, Issue 11:1244.

Sheree E. Cross, Melanie J. Thompson and Michael S. Roberts. "Distribution of Systemically Administered Ampicillin, Benzylpenicillin, and Flucloxacillin in Excisional Wounds in Diabetic and Normal Rats and Effects of Local Topical Vasodilator Treatment". Antimicrobial agents and chemotherapy, 1996, Vol. 40, No. 7:1703–1710.

Shi H. P., Fishel R. S., Efron D. T., Williams J. Z., Fishel M. H., Barbul A. "Effect of supplemental ornithine on wound healing". J Surg Res, 2002, 106(2):299-302.

Shima D. T., Adanfis A. P., Yeo K. T., et al. Hypoxic induction of endothelial cell growth factors in retinal cells: identification and characterization of vascular endothelial growth factor (VEGF) as the mitogen. Mol Med 1995, 1:182-193.

Shimokado K., Raines E. W., Madtes D. K., Barrett T. B., Benditt E. P., Ross R. A. "Significant part of macrophage-derived growth factor consists of at least two forms of PDOF". Cell, 1985,43:277-286.

Shing Y., Folkman J., Haudenschild C., Lund D., Crum R., Klagsbrun M. "Angiogenesis is stimulated by a tumor-derived endothelial growth factor". Journal Cell Biochem., 1985,29:275-287.

Shiro Uemura, Hidetsugu Matsushita, Wei Li, Alexander J. Glassford, Tomoko Asagami, Keun-Ho Lee, David G. Harrison and Philip S. Tsao. "Diabetes Mellitus Enhances Vascular Matrix Metalloproteinase Activity Role of Oxidative Stress". Circulation Research, 2001, 88:1291.

Sholley M. M., Ferguson G. P., Seibel H. R., Montour J. L. and Wilson J. D. "Mechanisms of neovascularization. Vascular sprouting can occur without proliferation of endothelial cells". Lab. Invest., 1984, 51:624-634.

Shoshan S. "Wound Healing". Int Rev Connect Tissue Res, 1981, Vol. 9:1-26.

Shull M. M., Ormsby 1, Kier A. B., et al. "Targeted disruption of the mouse transforming growth factor-@ I gene results in multifocal inflammatory disease". Nature 1992, 359:693-699.

Shweiki D, ltin A, Soffer D, Keshet E. "Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis". Nature 1992, 359:843-845.

Silverstein RJ, Landsman AS, "The effects of a moderate and high dose of vitamin C on wound healing in a controlled guinea pig model". J Foot Ankle Surg., 1999, 38(5):333-338.

Singh J. P., Chaikin M. A., Stiles C. D.. "Phylogenetic analysis of platelet-derived growth factor by radio-receptor assay". Journal Cell Biol., 1982, 95:667-671.

Skidgel R. A., Gao X. P., Brovkovych V, Rahman A, Jho D, Predescu S, Standiford T. J., Malik A. B. "Nitric oxide stimulates macrophage inflammatory protein-2 expression in sepsis". J Immunol 2002,169(4):2093-2101.

Smith D. J., Dunphy M. J., Strang L. N., Marletta M. A. "The influence of wound healing on urinary nitrate levels in rats". Wounds 1991, 3: 50-58.

Sporn M. B., Roberts A. B. "Transforming growth factor-β: recent progress and new challenges". J Cell Biol 1992, 119:1017-1021.

Steed D. L. Group DUS. "Clinical evaluation of recombinant human platelet-derived growth factor for the treatment of lower extremity diabetic ulcers". Vasc Surg 1995, 21:71-81.

Stefan Frank, Birgit Stallmeyer, Heiko Kämpfer, Nicole Kolb and Josef Pfeilschifter. "Nitric oxide triggers enhanced induction of vascular endothelial growth factor expression in cultured keratinocytes (HaCaT) and during cutaneous wound repair". The FASEB Journal, 1999, 13:2002-2014.

Stefan Frank, Heiko Kampeer, Christian Wetzler and Josef Pfeilschifter. "Nitric Oxide drives skin repair: Novel functions of an established mediator." Kidney International, 2002, Vol. 61:882-888.

Stefania Picunio, Monica Simioni and Maria Gabriella Doni "Platelet Activation and Modulation of the induction if Nitric Oxide Synthase in the conscious rat". Life Sciences, 1999, Vol. 65, No. 14:1463-1475.

Stenn K. S., Madri J. A. and Roll F. J. "Migrating epidermis produces AB_2 collagen and requires continual collagen synthesis for movement". Nature(Lond.), 1979, 277:229-232.

Stenn K. S., Madri J. A., Tinghitella T., Terranova V. P. "Multiple mechanisms of dissociated epidermal cell spreading". Journal Cell Biol., 1983, 96(1):63-67.

Stetler-Stevenson W. G., Krutzsch H. C. and Liotta L. A. "Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase inhibitor family". Journal Biol. Chem., 1989, 264, 17374-17378.

Stetler-Stevenson W. G., Krutzsch H. C., Wacher M. P., Margulies I. M. and Liotta L. A. "The activation of human type IV collagenase proenzyme. Sequence identification of the major conversion product following organomercurial activation". Journal Biol. Chem., 1989, 264:1353-1356.

Steven T. Boyce, Andrew P. Supp, Viki B. Swope, and Glenn D. Warden. "Vitamin C Regulates Keratinocyte Viability, Epidermal Barrier, and Basement Membrane *In Vitro*, and Reduces Wound Contraction After Grafting of Cultured Skin Substitutes". Journal of Investigative Dermatology, 2002, Volume 118 Issue 4: Page 565.

Studer R. K., De Rubertis F. R., Craven P. A. "Nitric oxide suppresses increases in mesangial cell protein kinase C, transforming growth factor β and fibronectin synthesis induced by thromboxane". Journal Am. Soc. Nephrol, 1996, 7: 999-1005.

Subbu Arumugam, Young-Chul Jang, Christine Chen-Jensen, Nicole S. Gibran and F. Frank Isik. "Temporal activity of plasminogen activators and matrix metalloproteinases during cutaneous wound repair." Surgery, 1999, 125:587-593.

Suresh C. Tyagi, Anna Ratajska and Karl T. Weber "Myocardial matrix metalloproteinase(s): Localization and activation" Molecular and Cellular Biochemistry, 1993, 126:49-59.

Suresh C. Tyagi, Suresh Kumar G and Susan Borders. "Reduction-Oxidation (Redox) state regulation of extracellular matrix metalloproteinases and tissue inhibitors in cardiac normal and transformed fibroblast cells". Journal of cellular biochemistry, 1996, 61:139-151.

Suzuki N., Okiji T., Suda H. "Enhanced expression of activation-associated molecules on macrophages of heterogeneous populations in expanding periapical lesions in rat molars". Archives of Oral Biology, 1999, 44:67-79.

Szabo S., Folkman J., Vattay P., Morales R. E., Pinkus G. S., Kato K. "Accelerated healing of duodenal ulcers by oral administration of a mutein of basic fibroblast growth factor in rats". Gastroenterology, 1994, 106:1106-111 1.

Szymanowicz G and Laurain G. "An automatic method of 4-Hydroxyproline determination convenient for large series". Analytical Biochemistry, 1981, 113:58-61.

Takahashi S, Fujita T, Yamamoto A. "Role of nuclear factor-kappaB in gastric ulcer healing in rats". Am J Physiol Gastrointest Liver Physiol., 2001, 280(6):G1296-G1304.

Takashi Oono, Ulrich Specks, Beate Eckes, Slawomir Majewski, Nicolas Hunzelmann, Rupert Timpl and Thomas Krieg. "Expression of Type VI collagen mRNA during wound healing". J Invest Dermatol., 1993, 100:329-334.

Tarlton J. F., Vickery C.J., Leaper D.J. and Bailey A.J. "Postsurgical wound progression monitored by temporal changes in the expression of matrix metalloproteinase-9" British Journal of Dermatology, 1997, 137:506-516.

Tatsuya Okamoto, Giuseppe Valacchi, Kishorchandra Gohil, Takaaki Akaike and Albert van der Vliet. "S-Nitrosothiols Inhibit Cytokine-Mediated Induction of Matrix Metalloproteinase-9 in Airway Epithelial Cells". American Journal of Respiratory Cell and Molecular Biology, 2002, Vol. 27: 463-473.

Tatsuya Okamoto, Takaaki Akaike, Tomohiro Sawa, Yoichi Miyamoto, etal. "Activation of Matrix Metalloproteinases by Peroxynitrite-induced Protein S-Glutathiolation via Disulfide S-Oxide Formation" JBC, 2001, Vol. 276(31):29596-29602.

Teresa Di Colandrea, Lisy Wang, John Wille, Jeanine D. Armiento and Kiran K.Chada. "Epidermal expression of collagenase delays wound-healing in transgenic mice." J. Invest Dermatol., 1998, 111: 1029-1033.

Tessler S, RockweU P, Hicklin D, et al. "Heparin modulates the interaction of VEGF,,, with soluble and cell associate flk-1 receptors". J Biol Chem 1994, 269:12456-12461.

Thang P. T., Patrick S, Teik L. S, Yung C. S. "Anti-oxidant effects of the extracts from the leaves of Chromolaena odorata on human dermal fibroblasts and epidermal keratinocytes against hydrogen peroxide and hypoxanthine-xanthine oxidase induced damage". Burns, 2001, 27(4):319-327.

Themis R. Kyriakides, Jessica W. Y. Tam and Paul Bornstein. "Accelerated wound healing in mice with a disruption of the thrombospondin 2 gene". J. Invest Dermatol 1999, 113:782-787.

Thomas A. Mustoe, Glenn F. Pierce, Arlen Thomason, Peggy Gramates, Michael B. Sporn, Thomas F. Deuel. "Accelerated healing of incisional wounds in rats induced by transforming growth factor-β". Science, 1987, Vol. 237: 1333-1336.

Tirmenstein M. A, Nicholls-Grzemski F. A, Schmittgen T. D, Zakrajsek B. A and Fariss M. W. "Glutathione-dependent regulation of nitric oxide production in isolated rat hepatocyte suspensions". Antioxid Redox Signal 2000 Winter, 2(4):767-777.

Tomoo Tamura, Tohru Nakanishi, YusukeKimura, Takako Hattori, Kazhuhiro Sasaki, Hiromichi Norimatsu, Kojiro Takahashi and Masaharu Takigawa. "Nitric Oxide Mediates Interleukin-1-Induced Matrix Degradation and Basic Fibroblast Growth Factor Release in Cultured Rabbit Articular Chondrocytes: A possible Mechanism of Pathological Neovascularisation in Arthritis". Endocrinology 1996, 137: 3729-3737.

Trenam C. W., Blake D. R., Morris C. "Skin inflammation: reactive oxygen species and the role of iron". Journal Invest Dermatol, 1992, 99:675.

Tsuneko Fujii, Rieko Hamaoka, Junichi Fujii and Naoyuki Taniguchi "Redox capacity of cells affects inactivation of glutathione reductase by nitrosative stress" Archives of Biochemistry and Biophysics, 2000, Vol.378, No. 1:123-130.

Turck J., Pollock A. S., Lee L. K., Marti H. P. and Lovett D. H. "Matrix metalloproteinase 2(gelatinase A) regulates glomerular mesangial cell proliferation and differentiation". Journal Biol. Chem., 1996, 271:15074-15083.

Turk J. L., Heather C. J. and Diengdoh J. V. "A histochemical analysis of mononuclear cell infiltrates of the skin with particular reference to delayed hypersensitivity in the guinea pig". Int. Arch. Allergy Appl. Immunol., 1976, 29:278-289.

Tuula Salo, Marja Makela, Meeri Kylmaniemi, Helena Autio-Harmainen and Hannu Larjava. "Expression of matrix metalloproteinase-2 and –9 during early human wound healing" Laboratory Investigation, 1994, Vol. 70, No.2: 176-182.

Tzyh-Chyuan Hour, Shoei-Yn Lin Shiau and Jen-Kun Lin. "Suppression of *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine- and *S*-nitrosoglutathione-induced apoptosis by Bcl-2 through inhibiting glutathione-*S*-transferase in NIH3T3 cells". Toxicology Letters, 1999, Volume 110, Issue 3: 191-202.

Unemori E. N., Ferrara N, Bauer E. A., Amento E. P. "Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells". J Cell Physiol 1992, 153:557-562.

Valeri Kolpokov, David Gordon and Thomas J Kulik. "Nitric Oxide Generating Compounds Inhibit total Protein and Collagen Synthesis in Cultured Vascular Smooth Muscle Cells". Circ Res 1995, 76:305-309.

Van Wart H. E. and Birkedal-Hansen H. "The cysteine switch: A principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family". Proc. Natl. Acad. Sci. USA, 1990, 87, 14:5578-5582.

Vaxman F, Olender S, Lambert A, Nisand G, Grenier JF. "Can the wound healing process be improved by vitamin supplementation? Experimental study on humans". Eur Surg Res., 1996, 28(4):306-314.

Veli-Matti Kahari and Ulpu Saarialho-Kere. "Matrix metalloproteinases in skin" Exp. Dermatol., 1997, 6:199-213.

Victor H. Cohn and Jerry Lyle "A Fluorometric assay for glutathione" Analytical Biochemistry, 1966, 14:434-440.

Villaschi S., Nicosia R.F. "Angiogenic role of endogenous basic fibroblast growth factor released by rat aorta after injury". Am J Pathol., 1993, 143:181-190.

Vinita Ahuja, Sara E. Miller and David N. Howell. "Identification of two subpopulation of rat monocytes expressing disparate molecular forms and quantities of CD43". Cellular Immunology, 1995, 163: 59-69.

Vira´g L, Szabo´ E´ , Bakondi E, Bai P, Gergely P, Hunyadi J, Szabo´ C. "Nitric oxide-peroxynitrite-poly(ADP-ribose) polymerase pathway in the Skin". Exp Dermatol, 2002, 11:189–202.

Vlodavsky 1, Folkman J., Sullivan R., Fridman R., Ishai-Michaeli R., Sasse J., Klagsbrun M. "Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix". Proc Natl Acad Sci USA, 1987,84:2292-2296.

Vodovotz Y, Bogdan C. "Control of nitric oxide production by transforming growth factor-β: implications for homeostasis". Prog. Growth Factor Res, 1994, 5: 341-351.

Wahl S. M., Hunt D. A., Wakefield I. M., et al. "Transforming growth factor-beta (TGF-β) induces monocyte chemotaxis and growth factor production". Proc Natl Acad Sci USA 1987, 84:5788-5792.

Wang Y, Huang D. S., Wood S and Watson R. R. "Modulation of immune function and cytokine production by various levels of vitamin E supplementation during murine AIDS". Immunopharmacology, 1995, 29:225.

Wassima A. El-Harake, Mikhail A. Furman, Brian Cook, K. Sreekumaran Nair, Jayme Kukowski and Irwin G. Brodsky. "Measurement of dermal collagen synthesis rate in vivo in humans". Am. J. Physiol., 1998, 274:E586-E591.

Wei X. Q., Charles I. G., Smith A, et al. Altered Immune responses in mice lacking inducible nitric oxide Synthase". Nature, 1995, 375:408-411.

Weller R, Patullo S Smith et al. "Nitric Oxide is generated on the skin surface by the reduction of Sweat nitrate". J Invest Dermat, 1995, 107: 327-331.

Weller R. "Nitric Oxide – a newly Discovered Chemical Transmitter In Human Skin". Bri J. Dermat., 1997, 137: 665-672.

Werb Z., Tremble P. M., Behrendtsen O., Crowley E. and Damsky C. H. "Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression". Journal Cell. Biol., 1989, 109:877-889.

Werner S and Smola H. "Paracrine regulation of keratinocyte proliferation and differentiation Trends". Cell Biol., 2001, 11(4):143-146.

Willenbrock F., Crabbe T., Slocombe P. M., Sutton C. W., Docherty A. J., Cockett M. I., O'Shea M., Brocklehurst K., Phillips I. R. and Murphy G. "The activity of the tissue inhibitors of metalloproteinases is regulated by C-terminal domain interactions: A kinetic analysis of the inhibition of gelatinase". A. Biochemistry, 1993, 32:4330-4337.

Winter G. D. "Formation of the scab and the rate of epithelialization of superficial wounds in the skin of the young domestic pig". Nature (Lond.), 1962, 193:293:294.

Winter G. D. "Oxygen and epidermal wound healing: Oxygen transport to tissue III". Eds. Advances in experimental medicine and biology, Plenum, New York, 1978, 94:673-678.

Witte M. B, Schaffer M. R, Barbul A. "Phenotypic induction of nitric oxide is critical for synthetic function in wound fibroblasts". Surg Forum, 1996, 47:703-705.

Witte M. B. and Barbul A. "Role of nitric oxide in wound repair". Am J Surg 2002, 183(4):406-412.

Witte M. B., Kiyama T, Barbul A. Nitric oxide enhances experimental wound healing in diabetes. Br J Surg 2002, 89(12):1594-1601.

Witte M. B, Thronton F. J., Tantri U and Barbul A. "L-arginine supplementation enhances diabetic wound healing:involvement of nitric oxide synthase and arginase pathway". Metabolism, 2002, 51(10): 1269-1273.

Woessner JF Jr. "The determination of hydroxyproline in tissues and protein samples containing small proportions of this imino acid". Arch Biochem Biophys, 1961, 93:440-447.

Woodley D. T., Liotta L. A. and Brundage R. "Adult human epidermal cells migrating on nonviable matrix produce a Type IV collagenase". Clin. Res., 1982, 30:266(abst).

Woodley D. T., O'Keefe E. J. and Prunieras M. "Cutaneous wound healing: A model for cell-matrix interactions". Journal Am. Acad. Dermatol., 1985, 12:420-433.

Wright C. D., Mulsch A, Busse R, Oswald H. "Generation of Nitric oxide by human neutrophils". Biochem Biophys Res Commun., 1989, 160: 813-819.

Yamasaki K, Edington H. D., McClosky C, Tzeng E, Lizonova A, Kovesdi I, Steed D. L., Billiar T. R. "Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer". J Clin Invest 1998, 101(5):967-971.

Yared Herouy, Andreas E. May, Gudula Pornschlegel, Christoph Stetter, Harald Grenz, Klaus T. Preissner, Erwin Schopf, Johannes Norgauer and Wolfgang Vanscheidt. "Lipodermatosclerosis is characterized by elevated expression and activation of matrix metalloproteinases: Implications for venous ulcer formation" J. Invest Dermatol., 1998, 111:822-827.

Yi-Hui Hong, Hai-Bing Peng, Vito La Fata and James K. Liao "Hydrogen Peroxide-Mediated Transcriptional Induction of Macrophage Colony-Stimulating Factor by TGF-$\beta 1^{1}$" The Journal of Immunology, 1997, 159:2418-2423.

Yoshitaka Hayashi, Yoshiki Sawa, Naoto Fukuyama, Hiroe Nakazawa and Hikaru Matsuda. "Inducible nitric oxide production is an adaptation to cardiopulmonary bypass-induced inflammatory response". The Annals of Thoracic Surgery, 2001, Volume 72, Issue 1: 149-155.

Young P. K. and Grinell F. "Metalloproteinase activation cascade after burn injury: A longitudinal analysis of the human wound environment". Journal Invest. Dermatol., 1994, 103, 5, 660-664.

Yuichiro Justin Suzuki, Henry Jay Forman and Alex Sevanian "Oxidants as stimulators of signal transduction" Free Radical Biology & Medicine, 1997, Vol.22 Nos. 1 / 2:269-285.

Zeitz M., Ruiz-Torres A., Merker H. J. "Collagen metabolism in granulating wounds of rat skin". Arch Dermatol Res., 1978, 263(2):207-214.

Appendices

Appendix A

Founded 1905

THE NATIONAL UNIVERSITY *of* SINGAPORE

Ref: 11th April 1998 **Animal Holding Unit**

A/P Walter Tan
Dept of Surgery

Fax: 773-5340

Dear Prof Tan,

APPLICATION FOR USE OF EXPERIMENTAL ANIMALS

I refer to your application dated 9-4-98 which we received on 9-4-98.

Title of Project : "Cellular Mechanisms in Wound Healing ...
Animal Species : Rats.

*Project Number Assigned by AHU : **113/98.***
(Please quote this number when placing an order for animals).

Please note the following recommendations:

Access to AHU will be allowed using the NUS Staff/student card:

Mon. - Fri.	0830 - 1700hrs
Sat.	0830 - 1300hrs.

Thank you.

Yours sincerely,

Dr Leslie Retnam
Veterinary Surgeon

Copy to: A/P Yap Eu Hian
Director

A4:project/113

Appendix B

Items Covered by Grant of Permission

The Publishing Division
of the Massachusetts Medical Society

Department of Permissions & Licensing
860 Winter Street, Waltham, Massachusetts 02451-1413 USA
Telephone: (781) 434-7382 fax: (781) 434-7633

MMS Reference Number: PS - 2003 - 1453
MMS Invoice Number: RY - 2003 - 1453

Source Information							Further Conditions				
Source: The New England Journal of Medicine											
Volume	Pages	Pub. Date	Author(s)	Article Title	Type	Item	Format	Language	Adapted	Amount	Customer Reference
341	738- 746	09/02/1999	Singer, Clark	Mechanisms of Disease: Cutaneous Wound Healing.	F	F1	Print	English*	N	$0.00	
341	738- 746	09/02/1999	Singer, Clark	Mechanisms of Disease: Cutaneous Wound Healing.	F	F2	Print	English*	N	$0.00	

The following information has been provided for us in your letter of request.

End Use: Doctoral thesis - "Role of Nitric Oxide in Wound Healing"
User: Achuth HN
Sponsor: National University of Singapore
Date of Use:
Number of Copies:

*Figure may be translated for use in thesis

A - Abstract, F - Figure, FT-Full Text, I - Images in Clinical Medicine, T - Table, Q - Excerpt/Quote

Appendix C

Introduction:

Type IV Collagenases or Matrix Metalloproteinases [MMP-2(Gelatinase A) and MMP-9 (Gelatinase B)] are collagen decomposing enzymes which are involved in normal and pathophysiological processes such as metastasis and tumor growth. A clear understanding of the cellular stimuli and control mechanisms of these enzymes may provide a key to the cause and treatment of matrix metalloproteinase-associated pathologies. The CHEMICON Type IV Collagenase / MMP-2,9 Activity Assay Kit offers a versatile design, which can be customized to accommodate the needs of individual investigators. The primary advantages of this kit include: 1) the absence of radioisotopes, 2) simplicity of sample measurement, and 3) the inclusion of human collagenase for positive control purposes.

The principle of the CHEMICON Type IV Collagenase / MMP-2,9 Activity Assay Kit is based upon fluorescent measurement of collagen fragments upon cleavage by Type IV Collagenases. It is known that collagen decomposition fragments differ from whole collagen in their temperature of denaturation and ethanol solubility. Upon Type IV Collagenase cleavage of fluorescently-labeled Collagen Type IV, decomposition fragments are produced. These fragments are selectively denatured and extracted with ethanol. Fluorescence intensity of the extracted product is measured and correlates with Type IV Collagenase activity.

Application:

The CHEMICON Type IV Collagenase / MMP-2,9 Activity Assay Kit is intended for research use only; not for diagnostic or therapeutic applications.

It should be noted that many proteinases exhibit collagen-decomposing activity, including members of the serine and metalloproteinase families. Individual investigators should include adequate experimental controls, as well as appropriate proteinase inhibitors to differentiate MMP activity from other proteinase activities. Common proteinase inhibitors include: PMSF (serine proteinases), *o*-Phenanthroline (metalloproteinases), and EGTA (metalloproteinases). Investigators should consult published literature for specific examples of common inhibitor cocktails.

Samples containing serum or synovial fluid should first be pre-treated with reagents contained in the Sample Preatment Kit for Type IV Collagenase Activity Assay (Catalog Number ECM484), as these fluids contain endogenous inhibitors of MMP activity which may give artificially low activity readings.

Kit Components:

1. **FITC Labeled Type IV Collagen Solution (1 mg/mL.):** 7.5 mL.
2. **Buffer Solution (0.1M Tris-HCl pH 7.5, containing NaCl, $CaCl_2$, NaN_3):** 15 mL.
3. **Enzyme Stop Reagent/Extraction Solution (0.05M Tris-HCl, pH 9.5/ ethanol containing NaCl, *o*-phenanthroline):** 1 x 30 mL, 1 x 60 mL
4. **MMP-9 Standard, human (0.5 unit*/mL):** 0.5 mL.

* One unit of MMP activity is defined as the amount of enzyme degrading 1 µg of collagen per minute under the experimental conditions described for this assay.

Materials Required But Not Provided:

1. 30-50°C water bath or incubator
2. Centrifuge: more than 8,000 X g
3. Vortex mixer
4. Fluorometer
5. 0.5 mL microcell for fluorometer
6. Microcentrifuge tubes, 1.5 mL size.
7. Trypsin / Soy Bean Trypsin Inhibitor or 4-Aminophenylmercuric Acetate (AMPA) for the activation of latent (proenzyme form) of Collagenase Type-IV.

Analytical Sensitivity and Detection Limits:

The CHEMICON Type IV Collagenase / MMP-2,9 Activity Assay can detect enzyme activity of 0.05 unit / mL (42°C, 2h) under standard assay conditions (42°C, 2h).

Storage and Stability:

Store kit at -20°C for up to 6 months.

Important Note: *Due to the small volumes contained within the kit, we recommend gently tapping the vials on a hard surface or briefly centrifuging the vials in a tabletop centrifuge* ***before opening*** *to dislodge any liquid entrapped in the containers' caps during shipment.*

Preparation of Samples Containing Latent Enzyme:

To activate Type IV Collagenase which is present in its precursor form or in a complex with its inhibitor, the sample should be pre-treated with trypsin or APMA (4-aminophenylmercuric acetate).

1. Trypsin pre-treatment: To activate latent Type IV Collagenase, 100 µL of the sample solution is incubated with 1 µg of trypsin at 37°C for 5 min. Residual trypsin is inactivated by adding a 5-fold molar excess of soybean trypsin inhibitor (SBTI).
2. APMA (4-aminophenylmercuric Acetate) pre-treatment: Add to sample from 100 mM stock to obtain 1 mM final concentration. Incubate for 30 minutes at room temperature.

Note: *Conditions for enzyme activations are suggested, and must be optimized by investigator to satisfy individual experimental conditions.*

Samples containing serum or synovial fluid should first be pre-treated with reagents contained in the Sample Preatment Kit for Type IV Collagenase Activity Assay (Catalog Number ECM484), as these fluids contain endogenous inhibitors of MMP activity which may give artificially low activity readings.

Preparation of Standard Curve:

Develop a standard curve by preparing the following solutions:

Final MMP-9 Conc. (U/mL)	MMP-9 Standard Solution (µL)	Buffer solution (µL)
0.1 U/mL.	30 µL	120 µL
0.2 U/mL.	60 µL	90 µL
0.3 U/mL.	90 µL	60 µL
0.4 U/mL.	120 µL	30 µL

The results of the standard curve may be displayed by graphing MMP-9 concentration (abscissa) and Fluorescence Intensity (ordinate).

Method of Type IV Collagenase Activity Assay

1. Add 25 µL of Buffer Solution to all microcentrifuge tubes at 4°C.
2. Add 25 µL of FITC-labeled Type IV Collagen solution at 4°C and mix well.
3. Add 50 µL of sample to all microcentrifuge tubes, except Blank and Total tubes. Add 50 µL Buffer Solution which has been diluted 2-fold to Blank and Total tubes. Vortex gently for 3-5 seconds.
4. Store all tubes at room temperature for 20 minutes.
5. Incubate all tubes except for Total tube for desired amount of time (typically 1-6 hours) at 42°C in a water bath. Boil Total tube for 5 minutes.
6. Next, place in ice bath for a period of 5 minutes to stop enzyme activation.
7. Add 300 µL of cold enzyme stop reagent/extraction solution to all tubes. Vortex for 3-5 seconds to mix thoroughly, then place in ice bath for 5 minutes.
8. Store tubes for 30 min. in an ice bath.
9. At 4°C, centrifuge all tubes, except for Total tube, for 10 minutes at greater than 8,000 x g to precipitate residual undigested collagen.
10. Within 10 minutes, remove 300 µL of supernatant and place into microcell fluorescence cuvette. Measure fluorescence intensity of supernatant at 520 nm (Em) / 495nm (Ex).

Calculation of Results:

Type IV Collagenase Activity (units / mL) may be calculated as follows:

Type IV Collagenase (units / mL) is equal to:

$$\frac{FI^{Sample}-FI^{Blank}}{FI^{Total}-FI^{Blank}} \times \frac{1}{\text{Reaction Time (min.)}} \times \frac{Y\ \mu g/tube\ (substrate\ amount)}{X\ mL/tube\ (sample\ volume)}$$

Note: One unit of collagenase activity is defined as the amount of enzyme degrading 1 µg of collagen per. min. under the conditions employed. 1 unit Collagenase = 1 µg collagen degraded/min.

Collagenase Activity may also be determined by comparison versus a standard curve of control MMP-9.

References:

1. Tarin, D., (1967) Int. J. Cancer **2**: 195.
2. Liotta, L.A., et al. (1980) Nature, **284**: 67.
3. Biswaz, C. (1984) J. Cell. Biochem. **28**: 39.
4. Erkell, L.J. and V. Schirramacher (1988) Cancer Res. **48**: 6633.
5. Otani, Y. and J. Keio. (1990) J. Med. **39**: 159.

Appendix D

Glutathione Assay Kit

Catalog No. 703002

TABLE OF CONTENTS

Contents of the Kit 1

Precautions 2

Warranty and Limitations of Remedy 2

If You Have Problems 2

Storage and Stability 2

Additional Items Required 2

About this Assay 2

Sensitivity 3

Pre-Assay Preparation 3

Sample Preparation 4

Performing the Assay 6

Calculating the Results 7

Interferences 9

Troubleshooting 9

References 10

Related Products 10

Plate Template 11

Notes 11

CONTENTS OF THE KIT

Number	Item	Quantity
1	MES Buffer (2X)	1 vial
2	GSSG Standard	1 vial
3	Cofactor Mixture	1 vial
4	Enzyme Mixture	1 vial
5	DTNB	4 vials
6	96 Well Plate	1 plate
7	Plate Cover	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

PRECAUTIONS

- Please read these instructions carefully before beginning this assay.
- For research use only. Not for human or diagnostic use.

WARRANTY AND LIMITATION OF REMEDY

Cayman Chemical Company makes no warranty of any kind, expressed or implied, including, but not limited to, the warranties of fitness for a particular purpose and merchantability, which extends beyond the description of the chemicals on the face hereof, except that the material will meet our specifications at the time of delivery. Buyer's exclusive remedy and Cayman Chemical Company's sole liability hereunder shall be limited to refund of the purchase price of, or at Cayman Chemical Company's option, the replacement of, all material that does not meet our specifications. Cayman Chemical Company shall not be liable otherwise or for incidental or consequential damages, including, but not limited to, the costs of handling. Said refund or replacement is conditioned on Buyer giving written notice to Cayman Chemical Company within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within said thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

IF YOU HAVE PROBLEMS

Our technical support staff may be reached by phone (800-364-9897, 734-971-3335), fax (734-971-3640), or E-Mail (techserv@caymanchem.com) Monday through Friday 8:00 AM to 6:00 PM EST. In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

STORAGE AND STABILITY

This kit will perform as specified if stored at 4°C and used before the expiration date indicated on the outside of the box.

ADDITIONAL ITEMS REQUIRED

1. A plate reader with a 414 or 405 nm filter.
2. An adjustable pipettor, repeat pipettor, and an eight channel pipettor (optional).
3. A source of pure water. Glass distilled water or HPLC-grade water is acceptable.
4. Metaphosphoric acid and triethanolamine (described on page 6).
5. 2-vinylpyridine (optional, described on page 6).

ABOUT THIS ASSAY

Glutathione (GSH) is a tripeptide (γ-glutamylcysteinylglycine) widely distributed in both plants and animals.[1,2] GSH serves as a nucleophilic co-substrate to glutathione transferases in the detoxification of xenobiotics and is an essential electron donor to glutathione peroxidases in the reduction of hydroperoxides.[2,3] GSH is also involved in amino acid transport and maintenance of protein sulfhydryl reduction status.[4,5] Concentration of GSH ranges from a few micromolar in plasma to several millimolar in tissues such as liver.[6,7]

Cayman's GSH assay kit utilizes a carefully optimized enzymatic recycling method, using glutathione reductase, for the quantification of GSH (see Figure 1, page 3).[8-10] The sulfhydryl group of GSH reacts with DTNB (5,5'-dithiobis-2-nitrobenzoic acid, Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. Measurement of the absorbance of TNB at 405 or 414 nm provides an accurate estimation of GSH in the sample.

GSH is easily oxidized to the disulfide dimer GSSG. GSSG is produced during the reduction of hydroperoxides by glutathione peroxidase. GSSG is reduced to GSH by glutathione reductase and it is the reduced form that exists mainly in biological systems. Because of the use of glutathione reductase in the Cayman GSH assay kit, both GSH and GSSG are measured and the assay reflects total glutathione. The kit can also be used to measure only GSSG by following the protocol given on page 6. GSH measurement can be done in plasma, serum, erythrocyte lysates, tissue samples, and cultured cells using this kit. However, plasma and serum samples will have to be concentrated before assaying, and nearly all samples will require deproteination (see page 6 for more details).

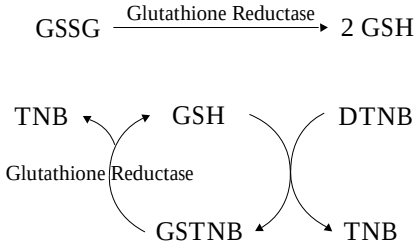


Figure 1. GSH Recycling

SENSITIVITY

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0 - 16 µM GSH (or 0 - 8 µM GSSG).

PRE-ASSAY PREPARATION

Reconstitution of the Reagents

Some of the kit components are in lyophilized form and need to be reconstituted prior to use. Follow the directions carefully to ensure proper volumes of water or Assay Buffer are used to reconstitute the vial components.

1. MES Buffer (2X) - (vial #1)

   The buffer consists of 0.4 M 2-(N-morpholino)ethanesulphonic acid, 0.1 M phosphate, and 2 mM EDTA, pH 6.0. Dilute the buffer with equal volume of water before use. Hereafter, MES Buffer refers to this diluted buffer.

2. GSSG Standard - (vial #2)

   The vial contains 25 µM GSSG in MES buffer. This standard is ready to use as supplied. NOTE: GSSG is provided as a standard instead of GSH. Under the assay conditions, GSSG is immediately reduced to GSH thereby providing the necessary standard. The standard is stable for at least 6 months if stored as supplied at 0 - 4°C.

3. Cofactor Mixture - (vial #3)

   The vial contains a lyophilized powder of $NADP^+$ and glucose-6-phosphate. Reconstitute the contents of the vial with 0.5 ml of water and mix well. The reconstituted reagent will be stable for 2 weeks if stored at 0 - 4 °C.

4. Enzyme Mixture - (vial #4)

   The vial contains glutathione reductase and glucose-6-phosphate dehydrogenase in 0.2 ml buffer. Carefully open the vial without spilling any liquid from the cap. Add 2 ml of diluted MES Buffer to the vial, replace the cap, and mix well. The reconstituted Enzyme Mixture will be stable for 2 weeks if stored at 0 - 4 °C.

5. DTNB - (vial #5)*

   Each vial contains a lyophilized powder of DTNB (5,5'-dithio*bis*-2-nitrobenzoic acid, Ellman's reagent). Reconstitute the contents of the vial with 0.5 ml of water and mix well. The reconstituted reagent must be used within 10 minutes.

*Reconstitution of this reagent should be done just prior to its addition to the Assay Cocktail described on page 7. Four vials of this reagent are provided to reconstitute each time the Assay Cocktail is prepared.

Plate configuration

There is no specific pattern for using the wells on the plate. A typical layout of standards and samples to be measured in triplicate is given below (see Figure 2). We suggest you record the contents of each well on the template sheet provided (see page 11).

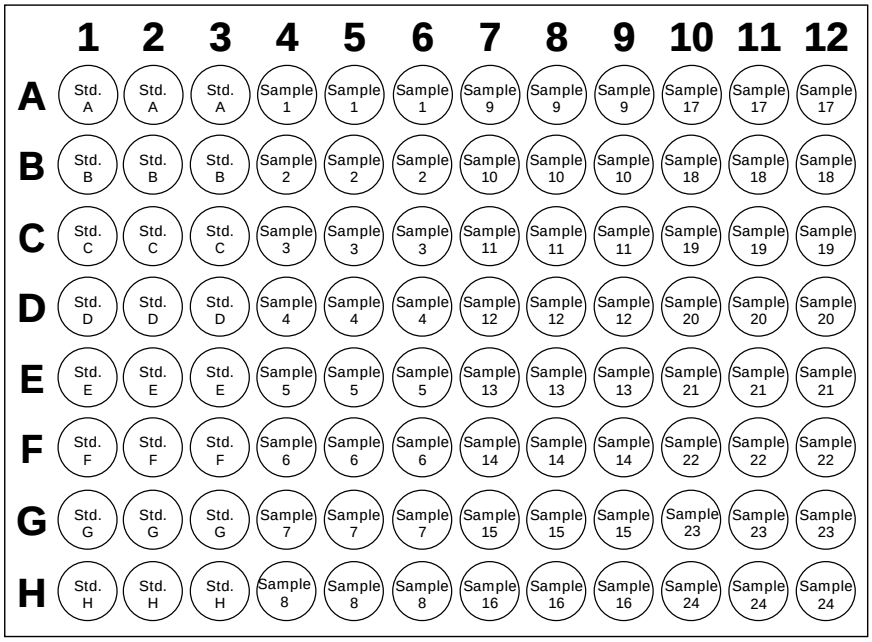


Figure 2. Sample Plate Format

PIPETTING HINTS

- **When pipetting the Assay Cocktail (see below), we recommend an eight channel pipet be used to save time and maintain more precise times of incubation.**
- **Before pipetting each reagent, equilibrate the pipet tip (i.e., fill the tip and expel the contents several times)**
- **Do not expose the pipet tip to the reagent(s) already in the well.**

SAMPLE PREPARATION

CAUTION: Thiol compounds such as mercaptoethanol, dithiothreitol, etc., or thiol alkylating agents such as N-ethylmaleimide should not be added to the samples at any stage of sample collection or preparation. If the samples contain any of these compounds they are unsuitable for GSH quantification.

Tissue Homogenate

1. Prior to dissection, perfuse tissue with a PBS (phosphate buffered saline) solution, pH 7.4, containing 0.16 mg/ml heparin to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold buffer (i.e., 50 mM MES or phosphate, pH 6-7, containing 1 mM EDTA) per gram tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice.
5. The supernatant will have to be deproteinated before assaying (see page 6). If not assaying on the same day, the sample will still have to be deproteinated, and then stored at -20°C. The sample will be stable for at least six months.

Cell Lysate

1. Collect cells by centrifugation (i.e., 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. The cell pellet can be homogenized or sonicated in 1-2 ml of cold buffer (i.e., 50 mM MES or phosphate, pH 6-7, containing 1 mM EDTA).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice.
5. The supernatant will have to be deproteinated before assaying (see page 6). If not assaying on the same day, the sample will still have to be deproteinated, and then stored at -20°C. The sample will be stable for at least six months.

Plasma and Erythrocyte lysate

1. Collect blood using an anticoagulant such as heparin, citrate, or EDTA.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipet off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice.
3. Remove the white buffy layer (leukocytes) and discard.
4. Lyse the erythrocytes (red blood cells) in 4 times its volume of ice-cold HPLC-grade water.
5. Centrifuge at 10,000 x g for 15 minutes at 4°C.
6. Collect the supernatant (erythrocyte lysate) and store on ice.
7. The plasma and erythrocyte lysate will have to be deproteinated before assaying (see page 6). If not assaying on the same day, the samples will still have to be deproteinated, and then stored at -20°C. The samples will be stable for at least six months. NOTE: Plasma samples contain glutathione levels below the detection limit of the assay and thus can not be measured directly. Before assaying, add TEAM reagent to the deproteinated plasma sample (see page 6), concentrate by lyophilization, and then reconstitute the sample with MES Buffer to one third of its original volume. You will only be able to determine the total GSH content. We do not guarantee the accuracy of the GSSG content due to the many manipulations that the plasma sample has endured.

Serum

1. Collect blood without using an anticoagulant such as heparin, citrate, or EDTA. Allow blood to clot for 30 minutes at 25°C.
2. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipet off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice.
3. The serum will have to be deproteinated before assaying (see page 6). If not assaying on the same day, the sample will still have to be deproteinated, and then stored at -20°C. The sample will be stable for at least six months. NOTE: Serum samples contain glutathione levels below the detection limit of the assay and thus can not be measured directly. Before assaying, add TEAM reagent to the deproteinated serum sample (see page 6), concentrate by lyophilization, and then reconstitute the sample with MES Buffer to one third of its original volume. You will only be able to determine the total GSH content. We do not guarantee the accuracy of the GSSG content due to the many manipulations that the serum sample has endured.

Recommended procedure for deproteination of samples:

Almost all biological samples used for GSH measurement contain large amounts of proteins, e.g., erythrocyte lysate, tissue homogenates, etc. It is necessary to remove as much protein as possible from the sample to avoid interferences due to particulates and sulfhydryl groups on proteins in the assay. Samples that are low in protein (<1 mg/ml) and are devoid of particulates can be assayed directly.

1. MPA reagent: Dissolve 5 g of metaphosphoric acid (Aldrich, Cat# 43157-5 or 23927-5) in 50 ml water. The MPA solution is stable for 4 hours at 25°C.
2. Add an equal volume of the MPA reagent to the sample and mix it on a vortex mixture. Allow the mixture to stand at room temperature for 5 minutes and centrifuge at >2000 g for at least 2 minutes (a microfuge will be sufficient for the centrifugation). Carefully collect the supernatant without disturbing the precipitate. The supernatant can be stored at this stage for long periods of time (up to 6 months) at -20°C without any degradation of GSH or GSSG. Do not add TEAM reagent until you are ready to assay the sample.
3. TEAM reagent: Prepare a 4 M solution of triethanolamine (Aldrich, Cat# T5830-0) in water by mixing 531 µl of triethanolamine with 469 µl of water. The TEAM solution is stable for 4 hours at 25°C.
4. Add 50 µl of TEAM reagent per ml of the supernatant and vortex immediately. The TEAM reagent will increase the pH of the sample. The sample is ready for assay of total GSH (i.e., both oxidized and reduced). Any necessary dilutions of the sample should be done at this stage with MES Buffer.

Sample preparation for exclusive measurement of GSSG:

Quantification of GSSG, exclusive of GSH, is accomplished by first derivatizing GSH with 2-vinylpyridine.[11] This can be achieved as follows:

1. Prepare a 1 M solution of 2-vinylpyridine (Aldrich, Cat# 13229-2) in ethanol by mixing 108 µl of 2-vinylpyridine and 892 µl of ethanol.
2. Add 10 µl of the 2-vinylpyridine solution per ml of sample from step 4 above. Mix well on a vortex mixer and incubate at room temperature for about 60 minutes and assay the sample.* This procedure can derivatize up to 1 mM GSH. More concentrated samples should be diluted with MES Buffer before derivatization.

*2-Vinylpyridine inhibits color development in the assay to some extent. Hence, it is essential to prepare the standards also the same way by adding 2-vinylpyridine (i.e., add 5 µl of 2-vinylpyridine solution per tube described below) and incubating to the same length of time as the sample.

PERFORMING THE ASSAY

- *All reagents must be equilibrated to room temperature before beginning the assay.*
- *The volume of sample and standards added to the wells is 50 µl and the final volume of the assay is 200 µl in all the wells.*
- *It is not necessary to use all the wells on the plate at one time. However, a standard curve must be run simultaneously with each set of samples.*
- *Add TEAM reagent to the deproteinated samples (see deproteination procedure, page 6).*
- *Use diluted MES Buffer in the assay.*
- *If the expected concentration of GSH in the sample is not known or if it is expected to be beyond the range of the standard curve, it is prudent to assay the sample at several dilutions.*
- *It is recommended that the samples and standards be assayed at least in duplicate.*
- *Prepare the Assay Cocktail (see below) just before its addition to the wells.*
- *Addition of the Assay Cocktail (see below) to the wells must be done as quickly as possible. The time difference in addition between the first well to the last should not be more than 2 minutes.*

1. Preparation of the standards: Take eight clean test tubes and mark them A-H. Aliquot the GSSG standard (vial #2) and MES Buffer to each tube as described in Table 1.

Tube	GSSG Standard (µl)	MES Buffer (µl)	Final Concentration (µM GSSG)	Equivalent Total GSH (µM)*
A	0	500	0	0
B	5	495	0.25	0.5
C	10	490	0.5	1.0
D	20	480	1.0	2.0
E	40	460	2.0	4.0
F	80	420	4.0	8.0
G	120	380	6.0	12.0
H	160	340	8.0	16.0

*Under the assay conditions GSSG is reduced to produce 2 mole equivalents of GSH.

Table 1

2. Add 50 µl of standard (tubes A - H) per well in the designated wells on the plate (see suggested plate configuration, Figure 2, page 4).
3. Add 50 µl of sample to each of the sample wells.
4. Cover the plate with the plate cover provided.
5. Prepare the Assay Cocktail by mixing the following reagents in a 20 ml vial: MES Buffer (11.25 ml), reconstituted Cofactor Mixture (0.45 ml), reconstituted Enzyme Mixture (2.1 ml), water (2.3 ml), and reconstituted DTNB (0.45 ml).

   NOTE: *The volumes of reagents given are for the use of the entire plate. Adjust the volumes of the reagents accordingly if only a part of the plate is used.*

   Prepare fresh Assay Cocktail and run a standard curve each time the assay is performed.

   Use the Assay Cocktail within 10 minutes of preparation.
6. Remove the plate cover and add 150 µl of the freshly prepared Assay Cocktail to each of the wells containing standards and samples using a multichannel pipet. Replace the plate cover and incubate the plate in the dark on an orbital shaker.
7. Measure the absorbance in the wells at 405 or 414 nm using a plate reader at 5 minutes intervals for 30 minutes (a total of 6 measurements). (NOTE: If only the end point method of calculation (see below) is used, one measurement at 25 minutes is enough.) Expected absorbance of the lowest standard (standard A) at 405 nm is 0.15 - 0.25 AU and that of the highest standard (standard H) is 0.6 - 0.8 AU in 30 minutes.

CALCULATING THE RESULTS

GSH concentration of the samples can be determined either by the End Point Method or the Kinetic Method. The End Point Method is adequate for most purposes. However, if the levels of cysteine or other thiols in the samples are expected to be significant compared to GSH, the Kinetic Method should be used.

End Point Method

1. Calculate the average absorbance from the 25 minutes measurement for each standard and sample.
2. Subtract the absorbance value of the standard A from itself and all other values (both standards and samples). This is the corrected absorbance.
3. Plot the corrected absorbance values (from step 2 above) of each standard as a function of the concentration of GSSG or Total GSH of Table 1 (see Figure 3, page 8).

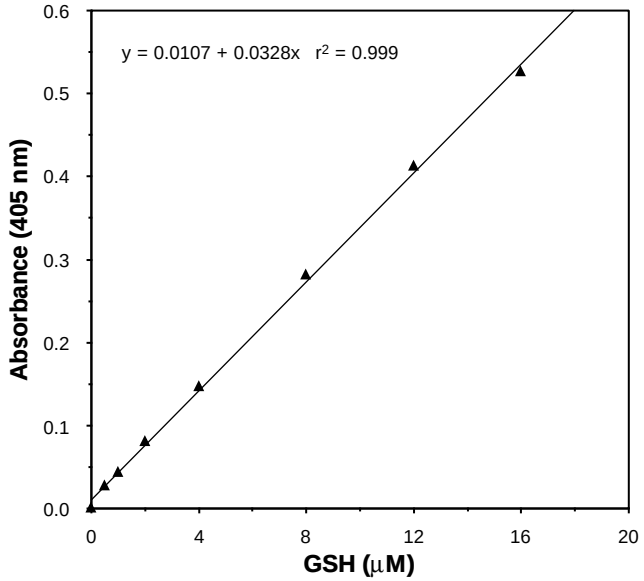


Figure 3. Plot of corrected Absorbance at 25 minutes vs. GSH concentration (µM)

4. Calculate the values of GSSG or Total GSH for each sample from the standard curve.

[Total GSH] or [GSSG] = {(absorbance at 405 or 414 nm) - (y-intercept)}/slope x 2* x sample dilution

*NOTE: If your sample required deproteination, multiply by "2" to account for the addition of MPA Reagent.

Kinetic Method

1. Plot the average absorbance values of each standard and sample as a function of time and determine the slope for each curve (see Figure 4). This is called i-slope.

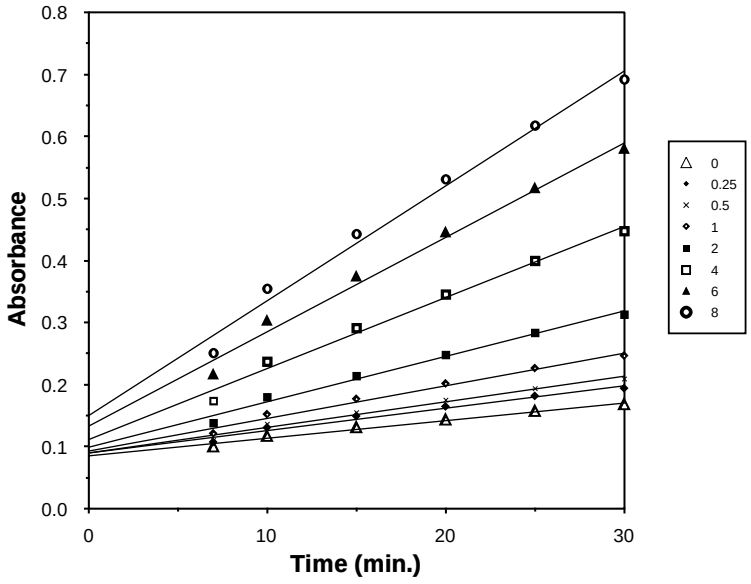


Figure 4. Plot of Absorbance vs. Time for each standard

2. Plot the i-slopes of each standard as a function of the concentration of GSSG or total GSH of Table 1 (see Figure 5, page 9). The slope of this curve is called f-slope.

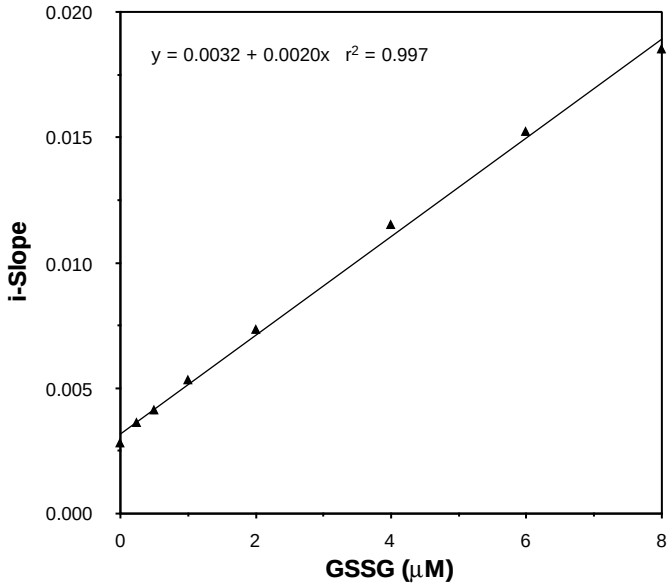


Figure 5. Plot of Slope vs. GSSG concentration

3. Calculate the values of GSSG or total GSH for each sample from their respective slopes using the slope *versus* GSSG or GSH standard curve.

[Total GSH] or [GSSG] = {(i-slope for the sample) - (y-intercept)}/f-slope x 2* x sample dilution

*NOTE: If your sample required deproteination, multiply by "2" to account for the addition of MPA Reagent.

INTERFERENCES

Added thiols such as mercaptoethanol, dithiothreitol, etc., and high levels of cysteine will consume all the DTNB and cause severe interference in the estimation of GSH. Thiol alkylating agents such as N-ethylmaleimide will inhibit glutathione reductase thereby rendering the assay ineffective.

TROUBLESHOOTING

Problem: Erratic values; dispersion of duplicates.
Cause: Poor pipetting/technique. -*or*- Bubble(s) in the well.

Problem: No color development.
Cause: One or more of the constituents of Assay Cocktail missing -*or*- Standards not added to the wells.
Solution: Make sure to add all components to the Assay Cocktail and the wells.

Problem: Non-linear standard curve.
Cause: Absorbance values too high (>1.2) at high GSH concentrations.
Solution: Use the absorbance measured at lower time points. Ideally, the highest point on the standard curve should have an absorbance value less than 1.2 AU.

Problem: No color in the sample above the background.
Cause: Concentration of GSH in the sample is too low (<0.25 µM).
Solution: Concentrate the sample by lyophilization and reconstitute in a smaller volume of MES Buffer than the original volume of the sample. Concentration should be performed after deproteination of the sample.

Problem: Absorbance value of the sample higher than the highest point of the standard curve.
Cause: Sample is too concentrated and/or high concentration of other thiols.
Solution: Dilute the sample and reassay. Analyze the data using Kinetic Method. If the absorbance value falls within the range of any of the standards but the i-slope does not, thiols other than GSH are interfering with the assay.

REFERENCES

1. Foyer, C.H., Lelandais, M., Kunert, K.J. Photooxidative stress in plants. *Physiol. Plant.* 92, 696-717 (1994).
2. Glutathione: Metabolism and function, Arias, I.M. and Jakoby, W.B. editors. Raven Press, New York (1976).
3. Baillie, T.A. and Slatter, J.G. Glutathione: A vehicle for the transport of chemically reactive metabolites *in vivo*. *Acc. Chem. Res.* 24, 264-270 (1991).
4. Inoue, M., Saito, Y., Hirata, E., *et al.* Regulation of redox states of plasma proteins by metabolism and transport of glutathione and related compounds. *Journal of Protein Chemistry* 6, 207-225 (1987).
5. Inoue, M. Interorgan metabolism and membrane transport of glutathione and related compounds, Chapter 6, *in* Renal Biochemistry. Kinne, R.K.H. editor. Elsevier Science Publishers B.V. London, 225-269 (1985).
6. Lash, L.H. and Jones, D.P. Distribution of oxidized and reduced forms of glutathione and cysteine in rat plasma. *Arch. Biochem. Biophys.* 240, 583-592 (1985).
7. Wendel, A. and Cikryt, P. The level and half-life of glutathione in human plasma. *FEBS Lett.* 120, 209-211 (1980).
8. Tietze, F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. *Anal. Biochem.* 27, 502-522 (1969).
9. Eyer, P. and Podhradsky, D. Evaluation of the micromethod for determination of glutathione using enzymatic cycling and Ellman's reagent. *Anal. Biochem.* 153, 57-66 (1986).
10. Baker, M.A., Cerniglia, G.J., Zaman, A. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Anal. Biochem.* 190, 360-365 (1990).
11. Griffith, O.W. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* 106, 207-212 (1980).

RELATED PRODUCTS

4-hydroxy Hexenal - Cat. No. 32060 • 4-hydroxy Nonenal - Cat. No. 32100 • 8-Isoprostane EIA Kit - Cat. No. 516351 • Glutathione Peroxidase Assay Kit - Cat. No. 703102 • Glutathione Reductase Assay Kit - Cat. No. 703202 • Glutathione S-Transferase Assay Kit - Cat. No. 703302 • Lipid Hydroperoxide Assay Kit - Cat. No. 705002

PLATE TEMPLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

NOTES