EFFECT OF ANTIHYPERTENSIVE DRUGS TREATMENT ON OXIDATIVE STRESS IN HEART OF N-nitro-L-arginine methyl ester (L-NAME) ADMINISTERED SPONTANEOUSLY HYPERTENSIVE RAT

By

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KESAN UBATAN ANTIHIPERTENSI KE ATAS TEKANAN OKSIDATIF JANTUNG TIKUS HIPERTENSI SPONTAN ARUHAN

N-nitro-L-arginina metil ester (L-NAME)

Oleh

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Tesis yang diserahkan untuk

memenuhi keperluan bagi

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS ……ii

TABLE OF CONTENTS ……...…….iii

LIST OF TABLES ……..xi

LIST OF FIGURES ……..……xvii

LIST OF ABBREVIATIONS, SYMBOLS, ACRONYMS USED ……...…….xxiii

ABSTRAK ……..……xxvi

ABSTRACT ……xxix

CHAPTER 1

GENERAL INTRODUCTION ……1

1.1 BACKGROUND OF THE STUDY ……..1

1.2 LITERATURE REVIEW ……..5

1.2.1 Free radicals and its formation ...……....5

1.2.1.1 Superoxide anion ($O_2^{\cdot -}$) ……...……7

1.2.1.2 Hydrogen peroxide (H_2O_2) ……...……8

1.2.1.3 Hydroxyl radical ($^{\cdot}OH$) …….....9

1.2.1.4 Nitric Oxide ($NO^{\cdot}$) …….....……10

1.2.1.5 Peroxynitrite anion ($ONOO^-$) …….……12

1.2.2 Oxidative damage to lipid, protein and DNA ……..…….15

1.2.3 Antioxidant defence mechanism ……..17

1.2.3.1 Enzymatic antioxidants……18

1.2.3.1.1 Superoxide dismutase (SOD...…….……18

1.2.3.1.2 Catalase (CAT) ……..…20

1.2.3.1.3 Glutathione peroxidase (GPx) ..……..…..21

1.2.3.1.4 Glutathione reductase (GR) ……..….22

1.2.3.1.5 Glutathione S- transferase (GST) …….23

1.2.3.2 Non-enzymatic antioxidants ……..…24

1.2.3.3 Total antioxidant status (TAS) …….26

1.2.4 Oxidative stress……...27

1.2.5 Hypertension ……..…..27

1.2.6 Role of oxidative stress in hypertension and cardiovascular diseases……29

1.2.7 Antihypertensive drugs ……...33

1.2.8 Animal models of hypertension ……..37

1.3 RESEARCH QUESTIONS, HYPOTHESIS AND GENERAL OBJECTIVES OF THE STUDY ……40

1.3.1 Hypothesis ……..……..40

1.3.2 Objectives ……...……41

CHAPTER 2

GENERAL MATERIAL AND METHOD ……..……..43

2.1 Materials and Chemicals ……43

2.1.1 Chemicals and reagents……43

2.1.2 Consumables ……46

2.1.3 Kits ……..47

2.1.4 Laboratory equipment ……..……..48

2.2 Experimental design ……...……....49

2.2.1 Animals ……..……...……...49

2.2.2 Blood pressure measurement ……...……....50

2.2.3 Preparation of homogenate ...……...……51

2,3 Estimation of protein……...……54

2.3.1 Calculation of protein concentration ……..…….55

2.4 Estimation of TAS ……...……...55

2.4.1 Preparation of reagents……..……..…..……55

2.4.2 Procedure ……..…...57

2.4.3 Calculation of TAS ……...……59

2.5 Estimation of TBARS ……...…..59

2.5.1 Preparation of reagents ……..……..…60

2.5.2 Procedure ……..……...61

2.5.3 Calculation of TAS ……...……61

2.6 Estimation of PCO ……...……...62

2.6.1 Preparation of reagents …….……..…62

2.6.2 Procedure ……..…...64

2.6.3 Calculation of TAS ……..…65

2.7 Estimation of SOD activity ...…….....65

2.7.1 Preparation of reagents …….……..…66

2.7.2 Procedure …….....66

2.7.3 Calculation of TAS ……..…67

2.8 Estimation of CAT activity ...……..…...68

2.8.1 Preparation of reagents ……..……68

2.8.2 Procedure ……..…...69

2.8.3 Calculation of TAS ……..……69

2.9 Estimation of GPx activity ...…….……..…...70

2.9.1 Preparation of reagents ……..…70

2.9.2 Procedure ……....71

2.9.3 Calculation of GPx activity……..…72

2.10 Estimation of GR activity ...…….…….…….....72

2.10.1 Preparation of reagents ……..…73

2.10.2 Procedure ……...…….…...73

2.10.3 Calculation of GR activity……...……..……74

2.11 Estimation of GST activity ...…….....…….…...75

2.11.1 Preparation of reagents ……..…75

2.11.2 Procedure ……...……....75

2.11.3 Calculation of GST activity……...……..……...76

2.12 Estimation of GSH and GSSG levels ...…….....77

2.12.1 Preparation of reagents ……..…77

2.12.2 Procedure ……...…….…...79

2.12.3 Calculations ……...……..……..81

2.13 Estimation of Nitric oxide (NO) ……..…….……...82

2.13.1 Preparation of reagents …….……83

2.13.2 Procedure ……...…….……...84

2.13.3 Calculations ……...……..……..86

2.14 Statistical analysis …….……...…..……...87

CHAPTER 3

COMPARISON OF OXIDATIVE STRESS MARKERS AND ANTIOXIDANT DEFENCE SYSTEM IN SPONTANEOUSLY HYPERTENSIVE AND NORMOTENSIVE WISTAR-KYOTO RAT ADMINISTERED WITH L-NAME.....88

3.1 INTRODUCTION ……..…88

3.2 RESEARCH OBJECTIVES ……...……..……..92

3.3 METHODS AND MATERIALS ……..…...……..94

3.4 RESULTS ……...……....96

3.4.1 SBP in SHR and age-matched WKY rats administered with L-NAME..99

3.4.2 Changes in TAS level with age in heart of WKY and SHR ……..…102

3.4.3 Changes in TBARS level with age in heart of WKY and SHR ……105

3.4.4 Changes in PCO level with age in heart of WKY and SHR ……..……110

3.4.5 Changes in SOD activity with age in heart of WKY and SHR ……115

3.4.6 Changes in CAT activity with age in heart of WKY and SHR ……119

3.4.7 Changes in GPx activity with age in heart of WKY and SHR ……..…125

3.4.8 Changes in GR activity with age in heart of WKY and SHR …...……130

3.4.9 Changes in GST activity with age in heart of WKY and SHR ….……136

3.4.10 Changes in GSH level with age in heart of WKY and SHR ……..…142

3.4.11 Changes in GSSG level with age in heart of WKY and SHR ……..…147

3.4.12 Changes in GSH : GSSG ratio with age in heart of WKY and SHR...152

3.4.13 Changes in NO level with age in heart of WKY and SHR …….157

3.5 DISCUSSION ……..……..162

3.6 SUMMARY AND CONCLUSION……..169

CHAPTER 4

EFFECTS OF CLONIDINE TREATMENT ON OXIDATIVE STRESS IN HEART OF SHR AND SHR+L-NAME ……..….171

4.1 INTRODUCTION ……171

4.2 RESEARCH OBJECTIVES ……174

4.3 METHODS AND MATERIALS ……..……175

4.4 RESULTS ……177

4.4.1 SBP in Clonidine treated SHR and age-matched untreated SHR and SHR administered with L-NAME ……..…..…179

4.4.2 TAS in heart of SHR and Clonidine treated SHR ……182

4.4.3 TBARS in heart of SHR and Clonidine treated SHR ……....186

4.4.4 PCO in heart of SHR and Clonidine treated SHR ……190

4.4.5 SOD activity in heart of SHR and Clonidine treated SHR ……194

4.4.6 CAT activity in heart of SHR and Clonidine treated SHR ……..….197

4.4.7 GPx activity in heart of SHR and Clonidine treated SHR ……201

4.4.8 GR activity in heart of SHR and Clonidine treated SHR ……..205

4.4.9 GST activity in heart of SHR and Clonidine treated SHR ……209

4.4.10 GSH level in heart of SHR and Clonidine treated SHR ……..213

4.4.11 GSSG level in heart of SHR and Clonidine treated SHR ……...….217

4.4.12 GSH : GSSG ratio in heart of SHR and Clonidine treated SHR …….221

4.4.12 NO level in heart of SHR and Clonidine treated SHR ……...….225

4.5 DISCUSSION ……..……..……..229

4.6 SUMMARY AND CONCLUSION……..……..234

CHAPTER 5

EFFECTS OF ENALAPRIL TREATMENT ON OXIDATIVE STRESS IN HEART OF SHR AND SHR+L-NAME ……....236

5.1 INTRODUCTION ……...….236

5.2 RESEARCH OBJECTIVES …….238

5.3 METHODS AND MATERIALS …….239

5.4 RESULTS ……..……241

5.4.1 SBP in Enalapril treated SHR and age-matched untreated SHR and SHR administered with L-NAME ……..…243

5.4.2 TAS in heart of SHR and Enalapril treated SHR ……..246

5.4.3 TBARS in heart of SHR and Enalapril treated SHR ……....250

5.4.4 PCO in heart of SHR and Enalapril treated SHR ……..254

5.4.5 SOD activity in heart of SHR and Enalapril treated SHR …….258

5.4.6 CAT activity in heart of SHR and Enalapril treated SHR …….261

5.4.7 GPx activity in heart of SHR and Enalapril treated SHR ……..265

5.4.8 GR activity in heart of SHR and Enalapril treated SHR ……...……269

5.4.9 GST activity in heart of SHR and Enalapril treated SHR …… ……...273

5.4.10 GSH level in heart of SHR and Enalapril treated SHR …… …..277

5.4.11 GSSG level in heart of SHR and Enalapril treated SHR …… …281

5.4.12 GSH : GSSG ratio in heart of SHR and Enalapril treated SHR ...…...285

5.4.12 NO level in heart of SHR and Enalapril treated SHR …….289

5.5 DISCUSSION ……..……..293

5.6 SUMMARY AND CONCLUSION……..299

CHAPTER 6

SUMMARY AND CONCLUSION …….300

REFERENCES ……...……..308

APPENDICES ……..A1

LIST OF TABLES

Table 1.1	Classification of blood pressure for adults aged 18 and above	3
Table 1.2	Oral antihypertensive drugs	35
Table 2.1	List of chemicals and reagents	43
Table 2.2	List if consumables	46
Table 2.3	List of commercials kits	47
Table 2.4	List of laboratory equipment used	48
Table 2.5	Procedure for determination of total antioxidant status	58
Table 2.6	GSSG standard preparation	80
Table 2.7	Nitrate standard preparation	85
Table 3.4.1	SBP in SHR and age-matched WKY rats administered with L-NAME	99
Table 3.4.2.1	Changes in TAS level in heart with age of WKY and SHR	102
Table 3.4.2.2	TAS level in heart of SHR and age-matched WKY rats	103
Table 3.4.2.3	TAS level in heart of WKY and SHR administered with L-NAME	104
Table 3.4.3.1	Changes in TBARS level in heart with age of WKY and SHR	105
Table 3.4.3.2	TBARS level in heart of SHR and age-matched WKY rats	107
Table 3.4.3.3	TBARS level in heart of WKY and SHR administered with L-NAME	108
Table 3.4.4.1	Changes in PCO level in heart with age of WKY and SHR	110
Table 3.4.4.2	PCO level in heart of SHR and age-matched WKY rats	112
Table 3.4.4.3	PCO level in heart of WKY and SHR administered with L-NAME	113
Table 3.4.5.1	Changes in SOD activity in heart with age of WKY and SHR	115

Table 3.4.5.2	SOD activity in heart of SHR and age-matched WKY rats	117
Table 3.4.5.3	SOD activity in heart of WKY and SHR administered with L-NAME	118
Table 3.4.6.1	Changes in CAT activity in heart with age of WKY and SHR	119
Table 3.4.6.2	CAT activity in heart of SHR and age-matched WKY rats	121
Table 3.4.6.3	CAT activity in heart of WKY and SHR administered with L-NAME	123
Table 3.4.7.1	Changes in GPx activity in heart with age of WKY and SHR	125
Table 3.4.7.2	GPx activity in heart of SHR and age-matched WKY rats	127
Table 3.4.7.3	GPx activity in heart of WKY and SHR administered with L-NAME	128
Table 3.4.8.1	Changes in GR activity in heart with age of WKY and SHR	130
Table 3.4.8.2	GR activity in heart of SHR and age-matched WKY rats	132
Table 3.4.8.3	GR activity in heart of WKY and SHR administered with L-NAME	134
Table 3.4.9.1	Changes in GST activity in heart with age of WKY and SHR	136
Table 3.4.9.2	GST activity in heart of SHR and age-matched WKY rats	138
Table 3.4.9.3	GST activity in heart of WKY and SHR administered with L-NAME	140
Table 3.4.10.1	Changes in GSH level in heart with age of WKY and SHR	142
Table 3.4.10.2	GSH level in heart of SHR and age-matched WKY rats	144
Table 3.4.10.3	GSH level in heart of WKY and SHR administered with L-NAME	146
Table 3.4.11.1	Changes in GSSG level in heart with age of WKY and SHR	147
Table 3.4.11.2	GSSG level in heart of SHR and age-matched WKY rats	149
Table 3.4.11.3	GSSG level in heart of WKY and SHR administered with L-NAME	150

Table 3.4.12.1	Changes in GSH : GSSG ratio in heart with age of WKY and SHR	152
Table 3.4.12.2	GSH : GSSG ratio in heart of SHR and age-matched WKY rats	154
Table 3.4.12.3	GSH : GSSG ratio in heart of WKY and SHR administered with L-NAME	155
Table 3.4.13.1	Changes in NO level in heart with age of WKY and SHR	157
Table 3.4.13.2	NO level in heart of SHR and age-matched WKY rats	159
Table 3.4.13.3	NO level in heart of WKY and SHR administered with L-NAME	160
Table 4.4.1	SBP in Clonidine treated SHR and age-matched untreated SHR and SHR administered with L-NAME	179
Table 4.4.2.1	TAS level in heart of untreated SHR and Clonidine treated SHR in different age groups	182
Table 4.4.2.2	TAS level in Clonidine treated and untreated SHR and SHR administered with L-NAME	184
Table 4.4.3.1	TBARS level in heart of untreated SHR and Clonidine treated SHR in different age groups	186
Table 4.4.3.2	TBARS level in Clonidine treated and untreated SHR and SHR administered with L-NAME	188
Table 4.4.4.1	PCO level in heart of untreated SHR and Clonidine treated SHR in different age groups	190
Table 4.4.4.2	PCO level in Clonidine treated and untreated SHR and SHR administered with L-NAME	192
Table 4.4.5.1	SOD activity in heart of untreated SHR and Clonidine treated SHR in different age groups	194
Table 4.4.5.2	SOD activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	196
Table 4.4.6.1	CAT activity in heart of untreated SHR and Clonidine treated SHR in different age groups	197
Table 4.4.6.2	CAT activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	199

Table 4.4.7.1	GPx activity in heart of untreated SHR and Clonidine treated SHR in different age groups	201
Table 4.4.7.2	GPx activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	203
Table 4.4.8.1	GR activity in heart of untreated SHR and Clonidine treated SHR in different age groups	205
Table 4.4.8.2	GR activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	207
Table 4.4.9.1	GST activity in heart of untreated SHR and Clonidine treated SHR in different age groups	209
Table 4.4.9.2	GST activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	211
Table 4.4.10.1	GSH level in heart of untreated SHR and Clonidine treated SHR in different age groups	213
Table 4.4.10.2	GSH level activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	215
Table 4.4.11.1	GSSG level in heart of untreated SHR and Clonidine treated SHR in different age groups	217
Table 4.4.11.2	GSSG level activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	219
Table 4.4.12.1	GSH : GSSG ratio in heart of untreated SHR and Clonidine treated SHR in different age groups	221
Table 4.4.12.2	GSH : GSSG ratio in Clonidine treated and untreated SHR and SHR administered with L-NAME	223
Table 4.4.13.1	NO level in heart of untreated SHR and Clonidine treated SHR in different age groups	225
Table 4.4.13.2	NO level activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	227
Table 5.4.1	SBP in Enalapril treated SHR and age-matched untreated SHR and SHR administered with L-NAME	243
Table 5.4.2.1	TAS level in heart of untreated SHR and Enalapril treated SHR in different age groups	246

Table 5.4.2.2	TAS level in Enalapril treated and untreated SHR and SHR administered with L-NAME	248
Table 5.4.3.1	TBARS level in heart of untreated SHR and Enalapril treated SHR in different age groups	250
Table 5.4.3.2	TBARS level in Enalapril treated and untreated SHR and SHR administered with L-NAME	252
Table 5.4.4.1	PCO level in heart of untreated SHR and Enalapril treated SHR in different age groups	254
Table 5.4.4.2	PCO level in Enalapril treated and untreated SHR and SHR administered with L-NAME	256
Table 5.4.5.1	SOD activity in heart of untreated SHR and Enalapril treated SHR in different age groups	258
Table 5.4.5.2	SOD activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	260
Table 5.4.6.1	CAT activity in heart of untreated SHR and Enalapril treated SHR in different age groups	261
Table 5.4.6.2	CAT activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	263
Table 5.4.7.1	GPx activity in heart of untreated SHR and Enalapril treated SHR in different age groups	265
Table 5.4.7.2	GPx activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	267
Table 5.4.8.1	GR activity in heart of untreated SHR and Enalapril treated SHR in different age groups	269
Table 5.4.8.2	GR activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	271
Table 5.4.9.1	GST activity in heart of untreated SHR and Enalapril treated SHR in different age groups	273
Table 5.4.9.2	GST activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	275
Table 5.4.10.1	GSH level in heart of untreated SHR and Enalapril treated SHR in different age groups	277

Table 5.4.10.2	GSH level activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	279
Table 5.4.11.1	GSSG level in heart of untreated SHR and Enalapril treated SHR in different age groups	281
Table 5.4.11.2	GSSG level activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	283
Table 5.4.12.1	GSH : GSSG ratio in heart of untreated SHR and Enalapril treated SHR in different age groups	285
Table 5.4.12.2	GSH : GSSG ratio in Enalapril treated and untreated SHR and SHR administered with L-NAME	287
Table 5.4.13.1	NO level in heart of untreated SHR and Enalapril treated SHR in different age groups	289
Table 5.4.13.2	NO level in Enalapril treated and untreated SHR and SHR administered with L-NAME	291
Table 6.1	Summary of findings on comparison of oxidative stress in WKY, SHR, WKY+L-NAME and SHR+L-NAME	301
Table 6.2	Summary of the finding on the effect of antihypertensive drugs treatment on SBP and oxidant/antioxidant status in heart of SHR and SHR+L-NAME	304

LIST OF FIGURES

Figure 1.1	Vascular ROS	14
Figure 1.2	Events linking oxidative stress and heart failure	32
Figure 2.1	Summary of experimental design	52
Figure 3.4.1	SBP in SHR and age-matched WKY rats administered with L-NAME	100
Figure 3.4.2.1	Changes in TAS level in heart with age of WKY and SHR	102
Figure 3.4.2.2	TAS level in heart of SHR and age-matched WKY rats	103
Figure 3.4.2.3	TAS level in heart of WKY and SHR administered with L-NAME	104
Figure 3.4.3.1	Changes in TBARS level in heart with age of WKY and SHR	105
Figure 3.4.3.2	TBARS level in heart of SHR and age-matched WKY rats	107
Figure 3.4.3.3	TBARS level in heart of WKY and SHR administered with L-NAME	108
Figure 3.4.4.1	Changes in PCO level in heart with age of WKY and SHR	110
Figure 3.4.4.2	PCO level in heart of SHR and age-matched WKY rats	112
Figure 3.4.4.3	PCO level in heart of WKY and SHR administered with L-NAME	113
Figure 3.4.5.1	Changes in SOD activity in heart with age of WKY and SHR	115
Figure 3.4.5.2	SOD activity in heart of SHR and age-matched WKY rats	117
Figure 3.4.5.3	SOD activity in heart of WKY and SHR administered with L-NAME	118
Figure 3.4.6.1	Changes in CAT activity in heart with age of WKY and SHR	119
Figure 3.4.6.2	CAT activity in heart of SHR and age-matched WKY rats	121
Figure 3.4.6.3	CAT activity in heart of WKY and SHR administered with L-NAME	123

Figure 3.4.7.1	Changes in GPx activity in heart with age of WKY and SHR	125
Figure 3.4.7.2	GPx activity in heart of SHR and age-matched WKY rats	127
Figure 3.4.7.3	GPx activity in heart of WKY and SHR administered with L-NAME	128
Figure 3.4.8.1	Changes in GR activity in heart with age of WKY and SHR	130
Figure 3.4.8.2	GR activity in heart of SHR and age-matched WKY rats	132
Figure 3.4.8.3	GR activity in heart of WKY and SHR administered with L-NAME	134
Figure 3.4.9.1	Changes in GST activity in heart with age of WKY and SHR	136
Figure 3.4.9.2	GST activity in heart of SHR and age-matched WKY rats	138
Figure 3.4.9.3	GST activity in heart of WKY and SHR administered with L-NAME	140
Figure 3.4.10.1	Changes in GSH level in heart with age of WKY and SHR	142
Figure 3.4.10.2	GSH level in heart of SHR and age-matched WKY rats	144
Figure 3.4.10.3	GSH level in heart of WKY and SHR administered with L-NAME	146
Figure 3.4.11.1	Changes in GSSG level in heart with age of WKY and SHR	147
Figure 3.4.11.2	GSSG level in heart of SHR and age-matched WKY rats	149
Figure 3.4.11.3	GSSG level in heart of WKY and SHR administered with L-NAME	150
Figure 3.4.12.1	Changes in GSH : GSSG ratio in heart with age of WKY and SHR	152
Figure 3.4.12.2	GSH : GSSG ratio in heart of SHR and age-matched WKY rats	154
Figure 3.4.12.3	GSH : GSSG ratio in heart of WKY and SHR administered with L-NAME	155
Figure 3.4.13.1	Changes in NO level in heart with age of WKY and SHR	157
Figure 3.4.13.2	NO level in heart of SHR and age-matched WKY rats	159

Figure 3.4.13.3	NO level in heart of WKY and SHR administered with L-NAME	160
Figure 4.3	Experimental Rat Groups	176
Figure 4.4.1	SBP in Clonidine treated SHR and age-matched untreated SHR and SHR administered with L-NAME	180
Figure 4.4.2.1	TAS level in heart of untreated SHR and Clonidine treated SHR in different age groups	182
Figure 4.4.2.2	TAS level in Clonidine treated and untreated SHR and SHR administered with L-NAME	184
Figure 4.4.3.1	TBARS level in heart of untreated SHR and Clonidine treated SHR in different age groups	186
Figure 4.4.3.2	TBARS level in Clonidine treated and untreated SHR and SHR administered with L-NAME	188
Figure 4.4.4.1	PCO level in heart of untreated SHR and Clonidine treated SHR in different age groups	190
Figure 4.4.4.2	PCO level in Clonidine treated and untreated SHR and SHR administered with L-NAME	192
Figure 4.4.5.1	SOD activity in heart of untreated SHR and Clonidine treated SHR in different age groups	194
Figure 4.4.5.2	SOD activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	196
Figure 4.4.6.1	CAT activity in heart of untreated SHR and Clonidine treated SHR in different age groups	197
Figure 4.4.6.2	CAT activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	199
Figure 4.4.7.1	GPx activity in heart of untreated SHR and Clonidine treated SHR in different age groups	201
Figure 4.4.7.2	GPx activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	203
Figure 4.4.8.1	GR activity in heart of untreated SHR and Clonidine treated SHR in different age groups	205

Figure 4.4.8.2	GR activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	207
Figure 4.4.9.1	GST activity in heart of untreated SHR and Clonidine treated SHR in different age groups	209
Figure 4.4.9.2	GST activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	211
Figure 4.4.10.1	GSH level in heart of untreated SHR and Clonidine treated SHR in different age groups	213
Figure 4.4.10.2	GSH level activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	215
Figure 4.4.11.1	GSSG level in heart of untreated SHR and Clonidine treated SHR in different age groups	217
Figure 4.4.11.2	GSSG level activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	219
Figure 4.4.12.1	GSH : GSSG ratio in heart of untreated SHR and Clonidine treated SHR in different age groups	221
Figure 4.4.12.2	GSH : GSSG ratio in Clonidine treated and untreated SHR and SHR administered with L-NAME	223
Figure 4.4.13.1	NO level in heart of untreated SHR and Clonidine treated SHR in different age groups	225
Figure 4.4.13.2	NO level activity in Clonidine treated and untreated SHR and SHR administered with L-NAME	227
Figure 5.3	Experimental Rat Groupd	240
Figure 5.4.1	SBP in Enalapril treated SHR and age-matched untreated SHR and SHR administered with L-NAME	243
Figure 5.4.2.1	TAS level in heart of untreated SHR and Enalapril treated SHR in different age groups	246
Figure 5.4.2.2	TAS level in Enalapril treated and untreated SHR and SHR administered with L-NAME	248
Figure 5.4.3.1	TBARS level in heart of untreated SHR and Enalapril treated SHR in different age groups	250

Figure 5.4.3.2	TBARS level in Enalapril treated and untreated SHR and SHR administered with L-NAME	252
Figure 5.4.4.1	PCO level in heart of untreated SHR and Enalapril treated SHR in different age groups	254
Figure 5.4.4.2	PCO level in Enalapril treated and untreated SHR and SHR administered with L-NAME	256
Figure 5.4.5.1	SOD activity in heart of untreated SHR and Enalapril treated SHR in different age groups	258
Figure 5.4.5.2	SOD activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	260
Figure 5.4.6.1	CAT activity in heart of untreated SHR and Enalapril treated SHR in different age groups	261
Figure 5.4.6.2	CAT activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	263
Figure 5.4.7.1	GPx activity in heart of untreated SHR and Enalapril treated SHR in different age groups	265
Figure 5.4.7.2	GPx activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	267
Figure 5.4.8.1	GR activity in heart of untreated SHR and Enalapril treated SHR in different age groups	269
Figure 5.4.8.2	GR activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	271
Figure 5.4.9.1	GST activity in heart of untreated SHR and Enalapril treated SHR in different age groups	273
Figure 5.4.9.2	GST activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	275
Figure 5.4.10.1	GSH level in heart of untreated SHR and Enalapril treated SHR in different age groups	277
Figure 5.4.10.2	GSH level activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	279
Figure 5.4.11.1	GSSG level in heart of untreated SHR and Enalapril treated SHR in different age groups	281

Figure 5.4.11.2	GSSG level activity in Enalapril treated and untreated SHR and SHR administered with L-NAME	283
Figure 5.4.12.1	GSH : GSSG ratio in heart of untreated SHR and Enalapril treated SHR in different age groups	285
Figure 5.4.12.2	GSH : GSSG ratio in Enalapril treated and untreated SHR and SHR administered with L-NAME	287
Figure 5.4.13.1	NO level in heart of untreated SHR and Enalapril treated SHR in different age groups	289
Figure 5.4.13.2	NO level in Enalapril treated and untreated SHR and SHR administered with L-NAME	291

LIST OF ABBREVIATIONS, SYMBOLS, ACRONYMS USED

Abbreviation	Definition
$^{\cdot}OH$	hydroxyl radical
$^{1}O_2$	singlet oxygen
ACE	angiotensin converting enzyme
ANOVA	analysis of variance
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CAT	catalase
CDNB	1-chloro-2,4-dinitrobenzene
Cu/Zn-SOD	copper/zinc superoxide dismutase
DNA	deoxyribonucleic acid
DOCA	deoxycorticosterone acetate
EC-SOD	extracellular superoxide dismutase
EDTA	Ethylenediaminetetraacetic acid
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione / glutathione
GSSG	oxidized glutathione / disulfide glutathione
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
kDa	kilo Dalton

L-NAME	N-nitro-L-arginine methyl ester
MDA	malondialdehyde
Mn-SOD	manganese superoxide dismutase
n	sample size
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
$O_2^{\bullet -}$	superoxide anion
$ONOO^-$	peroxynitrite
PCO	protein carbonyl
RAAS	renin-angiotensin-aldosterone system
RNS	reactive nitrogen species
ROS	reactive oxygen species
S.E.M	standard error of the mean
SBP	systolic blood pressure
SDS	Sodium dodecyl sulphate
SHR	spontaneously hypertensive rat
SOD	superoxide dismutase
TAS	total antioxidant status
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
UV	Ultraviolet

WKY	Wistar Kyoto

KESAN UBATAN ANTIHIPERTENSI KE ATAS TEKANAN OKSIDATIF JANTUNG TIKUS HIPERTENSI SPONTAN ARUHAN N-nitro-L-arginina metil ester (L-NAME)

ABSTRAK

Hipertensi adalah satu faktor risiko utama yang menyumbang kepada kardiovaskular, serebrovaskular dan kerosakan ginjal. Patogenesis hipertensi adalah multifaktorial dan sangat kompleks. Ketidakseimbangan status oksidan / antioksidan telah dicadangkan sebagai mekanisme patogen penting dalam tekanan darah tinggi dan juga kerosakan organ aruhan-tekanan darah tinggi terutamanya pada jantung. Memandangkan tekanan darah tinggi menyumbang kepada kerosakan organ, rawatan ubat antihipertensi bertujuan untuk mengurangkan bukan sahaja tekanan darah tetapi juga kerosahan organ aruhan-tekanan darah tinggi. Walau bagaimanapun, kesan ubat antihipertensi α_2-adrenergic agonist (Clonidine) dan perencat Angiotensine-Converting Enzim (ACE) (Enalapril) ke atas tekanan oksidatif pada jantung belum dikaji secara terperinci. Oleh itu, kajian ini telah dijalankan untuk mengkaji kesan Clonidine dan Enalapril pada penanda tekanan oksidatif, enzim antioksidan, antioksidan bukan enzim dan nitrik oksida dalam jantung tikus hipertensi spontan (SHR) dan SHR diaruh perencat enzim sintesis nitrik oksida, N-nitro-L-arginina metil ester (SHR + L-NAME). Dalam fasa pertama kajian, perbandingan penanda tekanan oksidatif dan sistem pertahanan antioksidan dalam normotensif tikus Wistar Kyoto (WKY) dan SHR, serta WKY+L-NAME dan SHR+L-NAME dinilai. SHR mengalami hipertesi bermula

minggu kelapan diiringi dengan peningkatan dalam penanda tekanan oksidatif TBARS dan PCO, peningkatan aktiviti CAT, GR dan GST, penurunan aktiviti GPx, peningkatan aras GSH dan pengurangan aras GSSG, nisbah GSH: GSSG, dan aras NO dalam jantung. Dalam WKY + L-NAME, perencatan NO sintase oleh L-NAME berjaya meningkatkan tekanan darah berterusan bermula pada minggu ke-20. Dalam model ini, aras TBARS dan PCO meningkat, aktiviti GPx menurun, aktiviti GR dan GST meningkat, aras GSSG meningkat dan NO menurun. Bagi SHR+L-NAME, nilai tekanan darah melebihi > 200 mmHg, mempamerkan keadaan hipertensi kronik. SHR+L_NAME menunjukkan peningkatan aras TBARS dan PCO, peningkatan aktiviti GR dan GST, pengurangan aktiviti GPx, peningkatan aras GSSG dan pengurangan NO. Dalam fasa kedua kajian, kesan rawatan Clonidine ke atas status oksidan / antioksidan dalam jantung SHR dan SHR+L-NAME dinilai. Rawatan Clonidine mengurangkan SBP dan meningkatkan aras TAS pada SHR dan SHR+L-NAME, pengurangan TBARS pada SHR+L-NAME, pengurangan PCO, aktiviti GST dan meningkatkan aras GSH dan nisbah GSH : GSSG, dan aktiviti CAT pada SHR dan SHR+L-NAME, penurunan aras GSSG dalam SHR+L-NAME dan peningkatan aras NO dalam SHR. Dalam fasa ketiga kajian, kesan rawatan Enalapril ke atas status oksidan / antioksidan pada jantung SHR dan SHR+L-NAME dinilai. Rawatannya mengurangkan SBP, TBARS, PCO, aktiviti GR dan GST, dan meningkatkan aras TAS, GSH, nisbah GSH:GSSG dan aktiviti CAT pada SHR SHR+L-NAME, pengurangan aras TBARS dan PCO pada SHR dan SHR+L-NAME, peningkatan aktiviti SOD pada SHR, peningkatan aktiviti CAT pada SHR dan SHR+L-NAME, peningkatan aktiviti SOD pada SHR dan pengurangan aras GSSG pada SHR+L-NAME. Kesimpulannya,

kajian ini mencadangkan bahawa tekanan oksidatif mungkin memainkan peranan dalam perkembangan dan / atau pengekalan hipertensi dan ketidakseimbangan status oksidan / antioksidan pada jantung membawa kepada kerosakannya pada tikus hipertensi. Rawatan ubat antihipertensi seperti Clonidine dan Enalapril menambahbaik tekanan oksidatif pada jantung yang berkemungkinan mengelakkannya daripada kerosakan di samping peranannya untuk mengurangkan tekanan darah.

EFFECT OF ANTIHYPERTENSIVE DRUGS TREATMENT ON OXIDATIVE STRESS IN HEART OF N-nitro-L-arginine methyl ester (L-NAME) ADMINISTERED SPONTANEOUSLY HYPERTENSIVE RAT

ABSTRACT

Hypertension is a major risk factor contributing to cardiovascular, cerebrovascular and renal diseases. The pathogenesis of essential hypertension is multifactorial and highly complex. An imbalance in the oxidant/antioxidant status has been proposed as an important pathogenic mechanism in hypertension as well as hypertension induced organ damage including heart. As hypertension contributes to organ damage, antihypertensive drug treatment aims to reduce not only the blood pressure but also hypertension induced organ damage. However, the effect of antihypertensive drugs α_2-adrenergic agonist (Clonidine) and Angiotensin-Converting Enzyme (ACE) inhibitor (Enalapril) on oxidative stress in heart has not been well studied. Therefore, this study investigate the effect of Clonidine and Enalapril on oxidative stress markers, enzymatic /non-enzymatic antioxidants and nitric oxide (NO) in heart of spontaneously hypertensive rat (SHR) and SHR administered NO synthase inhibitor, N-nitro-L-arginine methyl ester (SHR+L-NAME). In the first phase of study, the comparison of levels of oxidative stress markers and antioxidant defence system in normotensive Wistar Kyoto Rats (WKY) and SHR, as well as WKY+L-NAME and SHR+L-NAME was carried out. SHR developed hypertension by 8 weeks accompanied by increased in oxidative status markers TBARS and PCO levels, increased CAT, GR and GST activities, reduced GPx activity, enhanced GSH and GSSG level, reduced

GSH : GSSG ratio, NO level in heart. In WKY+L-NAME, the inhibition of NO synthase by L-NAME successfully developed hypertension by weeks 20 onwards. In this model there is an increased TBARS and PCO level, reduced GPx activity, enhanced GR and GST activities, increased GSSG level and reduced NO bioavailability. In SHR+L-NAME, the systolic blood pressure values exceed >200 mmHg, exhibit severe hypertensive condition. SHR+L-NAME showed an increased TBARS and PCO level, enhanced GR and GST activity, reduced GPx activity, increased GSSG and reduced NO level compared to SHR . In the second phase of study, the effect of Clonidine treatment on oxidant/antioxidant status in heart of SHR and SHR+L-NAME was assessed. Clonidine treatment reduced the SBP and increased the TAS level in SHR and SHR+L-NAME, reduced TBARS in SHR+L-NAME, reduced PCO level, GST activity and enhanced the level of GSH and GSH:GSSG ratio and CAT activity in SHR and SHR+L-NAME, reduced GSSG level in SHR+L-NAME, as well as increased NO level in SHR. In the third phase of study, the effect of Enalapril treatment on oxidant/antioxidant status in heart of SHR and SHR+L-NAME was assessed. It reduced the level of SBP, TBARS, PCO, GR and GST activities and increased the level of TAS, GSH, GSH:GSSG ratio and CAT activity in SHR and SHR+L-NAME, enhanced SOD activity in SHR, and reduced GSSG level in SHR+L-NAME. In conclusion, this study suggested that the oxidative stress play a role in the development and/or maintenance of hypertension and the imbalance in oxidant/antioxidant status in heart might leads to its damage in hypertensive rats. Antihypertensive drugs treatment such as Clonidine and Enalapril ameliorate the oxidative stress in heart of hypertensive rats and thereby these drugs might prevent its damage in addition to their role in reducing blood pressure.

CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Globally, nearly one billion people have hypertension; of these, two thirds are in developing countries. Hypertension kills nearly 8 million people worldwide every year and nearly 1.5 million people each year in the South-East Asia. (WHO, September, 2011). In addition, seven million premature deaths have been attributed to hypertension (WHO, 2002). In South-East Asia region, approximately one-third of adult population is this region has high blood pressure (WHO, September, 2011). The Sixth Reports of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC VI) (1997) define hypertension or high blood pressure is said to be present if the blood pressure is persistently at or above 140/90 mmHg. Hypertension is a global disease burden due to its high prevalence.

The World Health Organization (2002) reported that suboptimal blood pressure (>120 mm Hg SBP) is responsible for 62% of cerebrovascular disease and 49% of ischemic heart disease, with little variation by sex. In recent decades, it has become increasingly clear that the development of stroke, ischemic heart disease, and renal failure have been attributed by hypertension. Treating hypertension has been associated with 40% reduction in the risk of stroke and about 15% reduction in the risk of myocardial infarction (WHO, 2003).

Ministry of Health, Malaysia (2008) through its Clinical Practice Guidelines : Management of Hypertension reported that there is a positive relationship between systolic blood pressure (SBP), diastolic blood pressure (DBP) and the risk of developing cardiovascular, cerebrovascular and renal diseases. Therefore the main aim of identifying and treating high BP is to reduce these risks. Hence BP should be measured at every chance encounter. The classification of high BP, although arbitrary, is useful as clinicians must make treatment decisions based on the measured BP and the patients' associated cardiovascular/cerebrovascular risks and comorbidities. Table 1.1 provides a classification of BP for adults (age 18 and older) which was adopted from a similar classification that being used by the WHO-ISH and these criteria are for subjects who were not on any antihypertensive medication and who are not acutely ill.

Table 1.1 : Classification of blood pressure for adults aged 18 and above.

Category	Systolic (mmHg)		Diastolic (mmHg)	Prevalence in Malaysia*
Optimal	< 120	and	< 80	32%
Prehypertension	120-139	and/or	80-89	37%
Hypertension Stage 1	140-159	and/or	90-99	20%
Hypertension Stage 2	160-179	and/or	100-109	8%
Hypertension Stage 3	≥180	and/or	≥110	4%

* The prevalence of hypertension in Malaysia is according to the report by Lim *et al.*, 2000.

Various risk factors have been associated with hypertension, which include age, sex, race, physical activity and socioeconomic class. Vast majority of cases of uncontrolled hypertension are amongst individuals who are more than 60 years of age. (Thomas *et.al.*, 2005)

In recent decades, growing evidence indicate that imbalance of oxidant-antioxidant resulted in elevation of free radicals and their metabolites such as reactive oxygen species which plays an important role in the pathophysiology of hypertension (Zalba *et al.*, 2001). However, there is still a debate whether oxidative stress is a cause or a result of hypertension. Oxidative stress promotes vascular smooth muscle cell proliferation and hypertrophy and collagen deposition, leading to thickening of the vascular media and narrowing of the vascular lumen. In addition, increased oxidative stress may damage the endothelium and impair endothelium-dependent vascular relaxation as well as increases vascular contractile activity (Grossman, 2008). All these effects on the vasculature may explain how increased oxidative stress can cause hypertension.

As hypertension contributes to organ damage, antihypertensive drug treatment aims to reduce not only blood pressure values but also hypertension-induced organ damage including heart. Even though anti-hypertensive drug treatments have been

shown to reduce blood pressure and certain oxidative stress parameters, the studies concerned were not comprehensive as the antioxidant defence mechanism were not investigated in detail whereby only a few oxidative stress parameters were measured (Khanna *et al.*, 2008, Guillermore *et al*., 2004).

Therefore, this study attempts to examine the effect of antihypertensive drugs (Clonidine and Enalapril) on the reduction of blood pressure as well as oxidative stress markers and antioxidant status.

1.2 LITERATURE REVIEW

1.2.1 Free radicals and its formation

Free radical is an atom or molecule that have unpaired electron orbiting around central nucleus. Usually, an atom is composed of a central nucleus containing positively charged protons and neutral neutrons and electrons in pairs orbiting around it. Free radicals are generally unstable and highly reactive because the unpaired electrons tend to form pairs with other electrons from another radical which further forms a covalent bond or with non-radical molecule by various interaction leading to chain reaction.

Molecular oxygen (O_2) is essential for the survival of aerobic organisms. In aerobic organisms, oxygen is converted to water and this aerobic energy metabolism relies on oxidative phosphorylation, a crucial process by which the oxido-reduction

energy of mitochondrial electron transport is eventually converted to the high-energy phosphate bond of ATP (Novo and Parola, 2008)

An oxygen molecule (O_2) undergoes electron reduction when metabolized *in vivo* as follows:

Equation 1

$O_2 + e + H^+ \longrightarrow HO_2^{\cdot}$ (hydroperoxyl radical)

Equation 2

$HO_2^{\cdot} \longrightarrow H^+ + O_2^{\cdot -}$ (superoxide radical)

Equation 3

$O_2^{\cdot -} + 2H^+ + e \longrightarrow H_2O_2$ (hydrogen peroxide)

Equation 4

$H_2O_2 + e \longrightarrow OH^- + {}^{\cdot}OH$ (hydroxyl radical)

Equation 5

${}^{\cdot}OH + e + H^+ \longrightarrow H_2O$

(Gutteridge, 1995)

During this process, reactive oxygen metabolites are generated by the excitation of electrons secondary to addition of energy or interaction with transition elements. The reactive oxygen metabolites are continuously produced and highly reactive than the original oxygen molecule known as reactive oxygen species (ROS) (Apel and Hirt, 2004). ROS includes oxygen ions, free radicals and peroxides both inorganic and organic, which includes superoxide anions ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxides (H_2O_2), hydroperoxyl radical ($HO_2^{\cdot}$), peroxyl radical ($RO_2^{\cdot}$), alkoxyl radical ($RO^{\cdot}$), hypochlorite ($HOCl^{-}$) and hypochlorus acid (HOCl). There are also another group of free radicals, which are known as reactive nitrogen species (RNS). RNS includes nitric oxide (NO or $NO^{\cdot}$), nitrogen dioxide ($NO_2^{\cdot}$), peroxynitrite ($ONOO^{-}$), peroxynitrous acid (ONOOH), alkyl peroxinitrite (ROONO) and s-nitrosothiols (RSNO).

1.2.1.1 Superoxide anion ($O_2^{\cdot-}$)

Superoxide anion ($O_2^{\cdot-}$) is fairly abundant and approximately 2% of the oxygen consumed by cells is converted into $O_2^{\cdot-}$. Superoxide anion, $O_2^{\cdot-}$ is mainly formed *in vivo* by the electron transport chains in the mitochondria and microsomes through electron leakage, a phenomenon that occurs with an increase in O_2 utilization (Cui *et al*., 2004). Besides, oxygen also undergoes univalent reduction to form superoxide anion ($O_2^{\cdot-}$) by enzymatic action of nicotinamide adenine dinucleotide phosphate (NADH/NAD(P)H) oxidases and xanthine oxidases (XO). Oxygen can also become $O_2^{\cdot-}$ by non-enzymatic reaction with redox active compound such as

semiubiquinone of the mitochondrial electron transport chain. (Fridovich, 1978). Superoxide anion possesses different properties depending on the pH and environment. Due to its pKa of 4.8, superoxide anion can exist in the form of either $O_2^{\cdot -}$ or, at low pH , hydroperoxyl ($HO_2^{\cdot}$) (Halliwell and Gutteridge, 1999). Superoxide anion, $O_2^{\cdot -}$ is a weak oxidizing agent in aqueous solution and is able to oxidize molecules such as ascorbic acid and thiols. In contrary, $O_2^{\cdot\ -}$ is a much stronger reducing agent and can reduce several iron complexes such as cytochrome c and ferric-EDTA. (Gutteridge, 1995). Hydroperoxyl can therefore be considered an important species, although under physiological pH most of the superoxide is in the charged form. (Shiriska and Mastan, 2013). Superoxide anion is short lived owing to its rapid reduction to become hydrogen peroxide (H_2O_2) through the action of superoxide dismutases (SODs).

Equation 6 : $O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \longrightarrow H_2O_2 + O_2$

In biological tissues, $O_2^{\cdot -}$ can also undergo nonenzymatic transformation into H_2O_2 and singlet oxygen ($^1O_2$) (Steinback et. al, 2013)

1.2.1.2 Hydrogen peroxide (H_2O_2)

The result of the dismutation of superoxide radicals is the production of H_2O_2. However, H_2O_2 can be produced directly by the transfer of two electrons to oxygen by many enzymes such as urate oxidase, glucose oxidase, and D-amino acid oxidase produce (Gutteridge, 1995). The same report also reported that H_2O_2 is a weak oxidant

and reducing agent that are relatively stable in the absence of transition metal ions. Besides, H_2O_2 is uncharged which can easily diffuse across biological membranes, a non-radical potent oxidizing agent and able to oxidize or reduce several inorganic ions in aqueous solutions (Halliwell and Gutteridge, 1989, Gutteridge, 1995).

Damage occurs when H_20_2 interact with reduced form of transition metals such as Fe^{2+} or Cu^{+}. These process will further decompose the hydrogen peroxide to yield the highly reactive hydroxyl radical ($^{\cdot}OH$) via the Haber-Weiss of Fenton reaction (Cui *et al.*, 2004). Myeloperoxidase, a heme protein secreted by phagocytes, can amplify the oxidative potential of H_2O_2 involved in the formation of hypochlorous acid (HOCl) and singlet oxygen, ($^1O_2$) (Schraufstatter *et al.*, 1990, Tatsuzawa *et al.*, 1999). Unwanted H_2O_2 is removed from cells by the action of antioxidant enzymes either catalase or glutathione peroxidase through conversion to water molecules. (Griendling and FitzGerald 2013)

1.2.1.3 Hydroxyl radical ($^{\cdot}OH$)

The hydroxyl radical ($^{\cdot}OH$) is an extremely aggressive oxidant that indiscriminately attack most biological molecules at an almost diffusion controlled rate involving three main chemical reactions; hydrogen abstraction, addition reaction and electron transfer. (Halliwell and Gutteridge, 1989). Hydroxyl radical, $^{\cdot}OH$ is a potent oxidizing agent that can counter at a high rate with most organic and inorganic molecules in the cell, containing DNA, proteins, lipids, amino acids, sugars, and metals (Gutteridge,

1995). The main reaction for ·OH formation is known as the Fenton reaction. In this reaction, H_2O_2 react with transition metal such as Fe^{2+} or Cu^{+} to produce highly reactive hydroxyl radicals (·OH).

Equation 7 : $H_2O_2 + Fe^{2+} / Cu^{+} \longrightarrow {\cdot}OH + Fe^{3+} / Cu^{2+}$

Hydroxyl radical, ·OH can be produced when superoxide anion ($O_2^{\cdot -}$**)** and hydrogen peroxide react together in the iron-catalyzed reaction (Young and Woodside, 2001).

Equation 8 : $Fe^{3+} / Cu^{2+} + O_2^{\cdot -} \longrightarrow Fe^{2+} / Cu^{+} + O_2$

Equation 9: $Fe^{2+} / Cu^{+} + H_2O_2 \longrightarrow Fe^{3+} / Cu^{2+} + {\cdot}OH + OH^{-}$

These reactions produce nett reaction known as Haber-Weiss reaction, which leads to the production of hydroxyl radical *in vivo.*

Equation 10 : $O_2^{\cdot} + H_2O_2 \longrightarrow {\cdot}OH + OH^{-} + O_2$

1.2.1.4 Nitric Oxide ($NO^{\cdot}$)

Nitric oxide (NO•) is an abundant reactive radical that act as an important oxidative biological signaling molecule in a large variety of diverse physiological processes, including neurotransmission, blood pressure regulation, defense

mechanisms, smooth muscle relaxation and immune regulation (Patel *et al.*, 2000). Nitric oxide, NO is an uncharged lipophilic molecule that contains a single unpaired electron (NO•) which causes it to be reactive either as an electron donor (oxidant) or an electron acceptor (antioxidant) (Drew and Leeuwenburgh, 2002). Half-life of nitric oxide is only a few seconds due to rapid degradation by the superoxide anion. Superoxide anion is a major determinant of nitric oxide (NO) biosynthesis and bioavailability and which may modify endothelial function and result in vasoconstrictor. (Ceriello, 2008).

NO is produced within cells by the actions of a nitric oxide synthases which metabolize arginine to citrulline with the formation of NO• via the five-electron oxidative reaction. There are three distinct isoforms of nitric oxide synthase: neuronal (nNOS or NOS-1), inducible (iNOS or NOS-2), and endothelial (eNOS or NOS-3) (Drew and Leeuwenburgh, 2002). Nitric oxide synthase (NOS), and in particular the endothelial isoform of NOS (eNOS), is identified as an important source of superoxide (Martasek *et al*., 1998, Ferroni *et al*., 2004). eNOS can generate superoxide rather than NO in response to atherogenic stimuli has led to the concept of "NOS uncoupling". NOS uncoupling is said to be present when the activity of the enzyme for NO production is decreased, in association with an increase in NOS dependent superoxide production (Landmesser *et al*., 2003).

1.2.1.5 Peroxynitrite anion ($ONOO^-$)

Peroxynitrite anion ($ONOO^-$) is formed by the reaction of nitric oxide and the superoxide anion. Cells of the immune system produces $O_2^{\cdot -}$ and NO• during oxidative burst triggered during inflammatory processes which further produce peroxynitrite anion ($ONOO^-$), a potent oxidising agent that can cause DNA fragmentation and lipid oxidation (Carr *et al*, 2000)

Equation 11 : $NO\bullet + O_2^{\cdot -} \longrightarrow ONOO^-$

$ONOO^-$ is a powerful oxidant, which is able to damage many biological molecules, and decompose at acidic pH to release small amounts of hydroxyl radicals independent of metal catalysis (Equation 12) (Gutteridge, 1995)

Equation 12 : $ONOO^- + H^+ \longrightarrow {}^{\cdot}OH + NO_2$

Peroxynitrite may react directly with diverse biomolecules such as CO_2 to form highly reactive nitrous peroxocarboxylate ($ONOOCO_2^-$), or protonated as peroxinitrous acid (ONOOH). (Augusto *et al*, 2002). The protonated form of peroxynitrite (ONOOH) is a powerful oxidizing agent similar to OH•, which might cause depletion of sulfhydryl groups and oxidation of many molecules (Kohen and Nyska, 2002). This can also cause DNA damage including strand breaks, protein

oxidation and nitration of aromatic amino acid residues in proteins such as 3-nitrosotyrosine (Rubbo *et al.,* 2000). Under physiological conditions, ONOOH can react with other components present in high concentrations, such as H_2O_2 or CO_2, to form an adduct that might be responsible for many of the deleterious effects seen in biological sites (Czapski and Goldstein, 1995; Beckman and Koppenol, 1996).

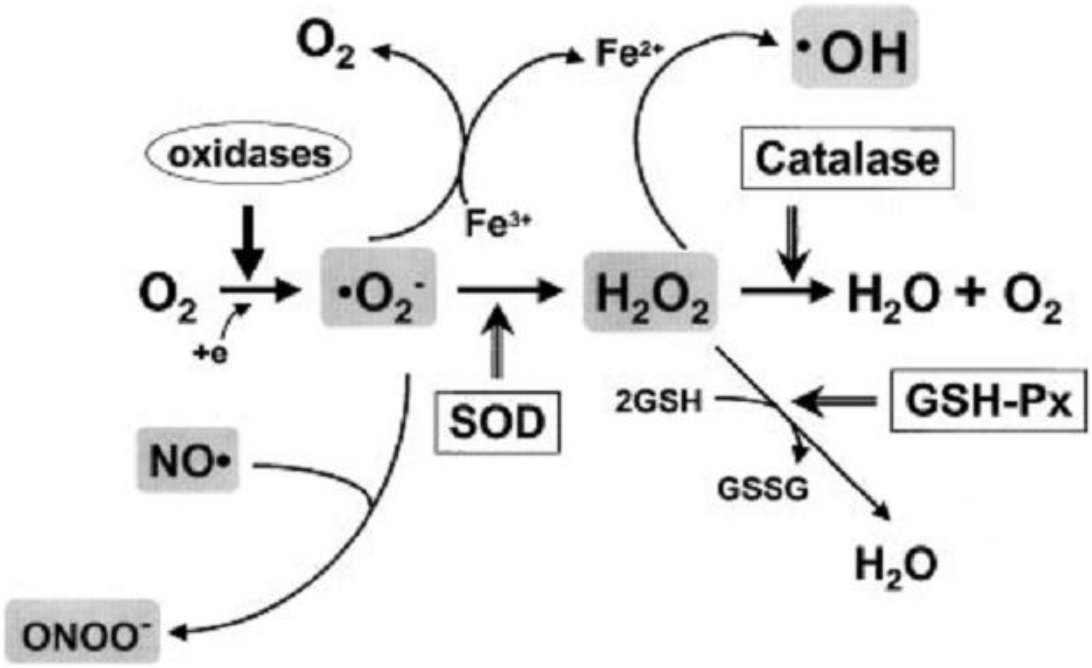

Figure 1.1 : Vascular ROS. Highlighted in gray are some of the most important ROS in vascular cells. Oxygen is converted to $O_2^{\cdot}$ by oxidases, which is further dismutated to H_2O_2 by superoxide dismutase (SOD). H_2O_2 can be converted to H_2O by enzymatic action of catalase or glutathione peroxidase (GSH-Px), or through transition metal binding reaction with Fe^{2+} to produced hydroxyl radical (·OH). In addition, $O_2^{\cdot}$reacts rapidly with nitric oxide (NO·) to form peroxynitrite ($OONO^-$). (Griendling and FitzGerald, 2003).

1.2.2 Oxidative damage to lipid, protein and DNA

ROS and RNS are formed through variety of event and pathways. Previous research by Beckman and Ames (1997) estimated that one human cell is exposed to approximately 1.5×10^5 oxidative hits per day from superoxide, hydroxyl radicals, hydrogen proxide and other such reactive species (Beckman and Ames, 1997). Free radical has high potential to oxidize the biomolecules such as lipid, protein and DNA, leading to damage of biomolecules, termed oxidative damage (Halliwell, 2007; Seven *et al*., 2008).

Cell membranes are the major site of lipid peroxidation as they contain polyunsaturated fatty acid residues of phospholipids that is sensitive to oxidation (Valko *et al*., 2005). Extensive lipid peroxidation in biological membranes causes alteration in fluidity, decreased membrane potential, increased permeability as well as cell rupture. A report by Valko *et al*., 2005 discussed that the overall lipid peroxidation process involved initiation, propagation and termination stages. The lipid peroxidation process was initially started with ROS abstracting a hydrogen atom from methylene group in the lipid. Furthermore, the fatty acid radical can react with oxygen that is present in the surrounding tissue formed lipo-peroxyl radicals ($ROO^{\cdot}$) during the propagation stage. $ROO^{\cdot}$ are very reactive, and can abstract another hydrogen from the neighboring fatty acid produce hydroperoxide (ROOH). ROOH, either being converted to unreactive fatty acid alcohols through reducion by glutathione peroxidases, or react with redox metals to produce reactive product such as aldehydes and epoxides. Lipid peroxidation process

was ended like other chain reaction termination process, which is by combination of two radicals to become non radicals or depletion of the substrate. Malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are examples of major aldehyde product of lipid peroxidation (Marnett, 1999). TBARS test are commonly used as indices of lipid peroxidation and peroxidative tissue injury. This test is predicated upon the reactivity of thiobarbituric acid (TBA) towards MDA (Janero, 1990).

Oxidation of protein may occur through several processes including oxidation of protein backbone, protein fragmentation or oxidation of amino acid side chains (Berlett and Stadman, 1997). Hydroxyl radical (·OH) abstracts the hydrogen atom of an amino acids residue to form carbon-centered radical in oxidative modification on polypeptide backbone. The carbon-centered radical reacts with oxygen to form alkylperoxyl radical intermediate, followed by alkylperoxide, alkoxyl radical and converted to hydroxyl protein derivative. Alkoxyl radicals, an intermediate product of protein backbone oxidation, may also cause protein fragmentation through cleavage of peptide bond by either diamide or α-amidation pathways. Furthermore, all amino acid residues of protein mainly cystein and methione are susceptible to oxidation of their side chains. Oxidation of amino acid residues and/or peptide backbone of proteins results in the generation of protein carbonyl group (Marnett *et al*., 2003). The presence of protein carbonyl has been used as a marker of protein oxidation (Levine *et al*., 1994).

ROS/RNS may also attack DNA bases or deoxyribose residues to produce damage bases or strand breaks (Hall *et al*., 1996; Cooke *et al*., 2003). ·OH is highly

reactive can attack the double bonds of DNA bases or abstract hydrogen atom from the methyl group of thymine and each of C-H bonds of 2'deoxyribose (Halliwell, 1991). Cellular DNA damage under prooxidant condition has also been reported to be mediated by iron. In fact, iron binds nucleic acids very well and DNA is a favored target of Fenton-mediated damage (Imlay, 2003). Guanine base is the most easily oxidized and serves as a major target of oxidative nucleic acid within the cell (Hall *et al*., 1996). Hydroxyl attack to the deoxyguanosine resulted in the formation of 8-hydroxy-2'-deoxyguanosine, which is the most used marker for DNA lesion (Shigenaga and Ames, 1991). The resultant damage to DNA bases may be a significant source of mutations that lead to cancer and other human pathology (Nicolic *et al*., 2006).

Whether ROS/RNS attacks these targets significantly depends upon the delicate balance between the level of oxidant and antioxidants. In fact, increase in ROS formation is a signal which activates redox homeostasis through upregulation of antioxidant enzymes activity to retain the redox balance (Young and Woodside, 2001).

1.2.3 Antioxidant defence system

Under normal physiological condition, the rate of oxidant formation is balanced by the rate of oxidant elimination though antioxidant defence mechanism. According to Halliwell and Gutteridge (1989), an antioxidant is any substance that when present at low concentration compared to that of an oxidizable substrate significantly delays or inhibits oxidation of that substrate. Cells are equipped with enzymatic and

nonenzymatic antioxidant systems to eliminate oxidant and maintain redox homeostasis (Trachootham *et al*., 2008).

1.2.3.1 Enzymatic antioxidants

The first line of antioxidant defence in cell is provided by antioxidant enzymes (Rodriguezl *et al*., 2004). The primary antioxidant defense mechanism is provided by three main antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase (Meydani, 2001). These antioxidant systems are widely distributed in nature, underlining their importance in preventing the damaging effects of reactive oxygen metabolites in biological systems with high specific cellular content (Sies, 1991). Another set of enzymatic antioxidants, glutathione reductase (GR) and glutathione-S-transferase (GST) which are the component of glutathione (GSH) system also plays a vital role in maintaining oxidant/antioxidant system (Lee *et al*., 2010).

1.2.3.1.1 Superoxide dismutase (SOD)

The first enzymatic reaction in the reduction pathway of oxygen occurs during the dismutation of two molecules of O_2 when they are converted to hydrogen peroxide (H_2O_2) and diatomic oxygen (Equation 6). The enzyme at this step is one of the three types of superoxide dismutase (SOD); CuZnSOD is present in the cytosol, MnSOD is located in the mitochondrial matrix, and extracellular SOD (EC-SOD) is present in interstitial spaces and extracellular fluids (Landis and Tower, 2005).

SOD dismutate $O_2^{\cdot -}$ by successive oxidation and reduction of the transition metal ion at the active site in a Ping Pong type mechanism with remarkably high reaction rates (Meier *et al*., 1998). The same report also reported that SOD are metalloproteins, containing Cu^{2+} , Ni^{3+} , Fe^{3+} or Mn^{3+} as active metal cofactor.

The first type of SOD, Cu/Zn-SOD has two identical subunits with molecular mass of approximately 32 kDa (Battistoni *et al*., 1996). Each subunit contains a metal cluster, an active site, constituted by a copper and a zinc atom bridged by a histamine residue (Battistoni *et al*., 1998). Cu/Zn-SOD is very stable and possess a compact structure that is highly resistant to denaturing agents such as urea, SDS and proteolytic enzymes. Moreover, several diseases are associated with defects in CuZnSOD (MacMillan-Crow and Cruthirds, 2001).

Mitochondrial Mn-SOD is a tetramer with molecular weight approximately 96 kDa, containing one manganese atom per subunit. This enzyme cycles from Mn^{3+} to Mn^{2+} and back to Mn^{3+} during the two step dismutations of superoxide (MacMillan-Crow *et al*., 1998). Mn-SOD greatly induced and depressed by cytokines, but moderately influenced by oxidants (Stralin and Marklund,1994).

Another type of SOD is extracellular SOD (EC-SOD) which is a secretory, tetrameric, copper and zinc containing glycoprotein, with a high affinity for certain glycosaminoglycans such as heparin and heparin sulphate (Mates *et al*.,

1999). In mammalian tissue, this type of SOD is coordinated by cytokines rather than as a response of individual cell to oxidants.

1.2.3.1.2 Catalase (CAT)

Catalase (EC 1.11.1.6) is a tetrameric enzyme consists of four identical tetrahedrally arranged subunits of 60 kDa which contributes to overall molecular weight of 240 kDa and contains a single ferriprotoporphyrin group per subunit (Aebi, 1980). It is abundantly located within cells in peroxisomes, which also contain most of the enzymes capable generating H_2O_2 (Schrader and Fahimi, 2006). CAT can react with hydrogen donors such as methanol, ethanol, formic acid, or phenols with peroxidase activity in addition to its main reaction to catalyze conversion of H_2O_2 to form water and molecular oxygen (Mates *et al*., 1999). Two stages are involved in this conversion :

Equation 13 : $CAT\text{-}Fe^{3+} + H_2O_2 \longrightarrow$ compound I

Equation 14 : compound I + $H_2O_2 \longrightarrow CAT\text{-}Fe^{3+} + 2H_2O + O_2$

CAT has high KM for its substrate and can remove H_2O_2 present in high concentrations (Kohen and Nyska, 2002). CAT has one of the highest turnover rates for all enzymes: one molecule of CAT can convert more than 6 million molecules of hydrogen peroxide to water and oxygen each minute. Although it has a very large

capacity to destroy H_2O_2, its affinity for H_2O_2 is low. Even though CAT is not essential for some cell types under normal conditions, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells (Mates *et al*., 1999).

1.2.3.1.3 Glutathione peroxidase (GPx)

Glutathione peroxidase (EC 1.11.1.19) consists of four identical subunits with overall molecular weight of 80 kDa. Each subunit contains a single selenocysteine residue, which is essential for enzyme activity (Tappel, 1978). GPx is the most abundant peroxidase present in both cytosol and mitochondria which catalyzes the reduction of H_2O_2 to water molecule in which glutathione (GSH) is oxidized to oxidized glutathione (GSSG) (Rodriguez *et al*., 2004).

In contrast to catalase, peroxidase possesses high affinity for H_2O_2 and the catalytic actions may takes place even in low concentration of H_2O_2 (Halliwell and Gutteridge, 1999). In addition, oxygen is not produced in the latter reaction which distinguishes the activity of peroxidase from catalase. However, the electron donors in these reactions are small molecules, such as glutathione or ascorbate (in plants). The removal of H_2O_2 through this reaction consumed two molecules of glutathione to remove one molecule of H_2O_2 (Kohen and Nyska, 2002).

Equation 15 : $2GSH + H_2O_2 \xrightarrow{\text{Peroxidase}} GSSG + 2H_2O$

Although GPx shares the same substrate with CAT which is H_2O_2, GPx alone can react effectively with lipid and other organic hydroperoxides. GPx is the major source of protection against low level of oxidative stress. (Kohen and Nyska, 2002).

1.2.3.1.4 Glutathione reductase (GR)

GR (EC 1.6.4.2) is a dimer of two identical subunits of molecular mass 50 kDa each. GR has a similar tissue distribution to glutathione peroxidase, which is mainly located in the cytosol (Himeno., 1993). GR is a flavoprotein, which uses NADPH as an electron donor for the reduction of GSSG (Gutterer *et al*., 1999). The oxidised form of glutathione (GSSG) may be converted back to the reduced form (GSH) by the activity of enzyme glutathione reductase (GR).

Equation 16 : $GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$

NADPH is usually formed by pentose phosphate pathway and required to replenish the supply of reduced glutathione (Champe, 2008). Any competing pathway that utilizes NADPH such as the aldose reductase pathway might lead to a deficiency of reduced glutathione and hence impair the action of glutathione peroxidase (Gutterer *et al*., 1999).

1.2.3.1.5 Glutathione S-transferase (GST)

Glutathione S-transferases (EC 2.5.1.18) are thought to play a physiological role in initiating the detoxication of potential alkylating agents, xenobiotics, chemotherapeutic agents, and some other pharmacologically active compounds (Manncrvick *et al*., 1985; Daniel, 1993). GST catalyzes the conversion of electrophilic compounds such as carcinogens and exogenous drugs with the presence of glutathione, and produce less toxic and more readily excreted metabolites (Manncrvick *et al*., 1985). The α, μ, π and θ are four classes of isoenzymes in GST superfamily which are encoded by different genes at different loci in the chromosomes and display peculiar structural and functional characteristics (Daniel, 1993).

Glutathione conjugates are metabolized by cleavage of glutamate and glycine residues, followed by acetylation of the resultant free amino group of cysteinyl residue, to produce a mercapturic acid. The mercapturic acid such as S-alkylated derivatives of N-acetylcysteine, is then excreted (Boyland and Chasseaud, 1969; Habiq *et al*., 1974).

The regulation of GST activity is complex as they exhibit sex, age, species and tumor specific pattern of expressions (Heyes and Pulford, 1995). GST activity may be regulated *in vivo* by ROS, because GST is one of the most potent inducers capable of generating free radicals by redox-cycling, as well as accumulation of H_2O_2 which result in induction of GST in mammalian cell (Arthur *et al*., 1987).

1.2.3.2 Non-enzymatic antioxidants

Another major group of antioxidant system of a cell is the non-enzymatic antioxidant. Non-enzymatic antioxidants are divided into two types, which are the chain breaking antioxidant and transition metal binding protein. As free radicals tend to interact with another molecule and generate another radical, chain breaking antioxidants will neutralize the radicals produced with the formation of stable byproduct (Gutteridge, 1995). Transition metal binding proteins such as ferritin, transferring, lactoferrin and ceruloplasmin also act as crucial components of the non-enzymatic antioxidant defence system by sequestering iron and copper so that they are not available to drive the formation of hydroxyl radicals (Kohen and Nyska, 2002). This section will focus on glutathione, an example of chain breaking antioxidant as this compound has a major impact on cellular respiration because it gathers and gives off hydrogen within the body and is part of the metabolic process.

Glutathione (GSH : γ-L-glutamyl-L-cysteinylglycine) is the most abundant intracellular non-protein thiol present in mammalian cells (Sies, 1999). GSH is now known to function directly or indirectly in many important biological phenomena, including the synthesis of proteins and DNA, transport, enzyme activity, metabolism, and protection of cells (Meister and Anderson, 1983). The location of GSH in the nucleus maintains the redox state of critical protein sulphydryls that are necessary for DNA repair and expression (Masella *et al.*, 2005).