ENDOTHELIAL PROTECTIVE ACTIVITY OF BOLDINE IN ANIMAL MODELS OF HYPERTENSION AND DIABETES MELLITUS

LAU YEH SIANG

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UNIVERSITY OF MALAYA
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2013
UNIVERSITI MALAYA

ORIGINAL LITERARY WORK
DECLARATION

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Registration/Matric No: MHA 100048
Name of Degree: Doctor of Philosophy

Field of Study: Pharmacology

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ABSTRACT

Free radical-induced oxidative stress is involved in the pathogenesis of a number of human diseases such as diabetes mellitus, hypertension and atherosclerosis. Endothelial dysfunction, usually assessed as decreased endothelium-dependent vasodilation, precedes clinically before obvious vascular pathologies and together with oxidative stress are major predictors of disease progression. Such endothelial dysfunction is observed early in the development of the pathology and is due, at least in part, to an excessive vascular formation of reactive oxygen species (ROS) in particular superoxide anion ($O_2^-$), which reduce nitric oxide (NO) bioavailability. Epidemiological and clinical studies have demonstrated that a growing list of natural products, as components of the daily diet or phytomedical preparations are a rich source of antioxidants and may improve vascular function by enhancing NO bioavailability.

In our preliminary studies, the crude extract of *Phoebe grandis* demonstrated significant protection of the endothelial cells against oxidative stress induced by the pro-oxidant β-NADH. Boldine ((S)-2,9-dihydroxy-1,10-dimethoxy-aporphine), the major compound in *Phoebe grandis*, exhibited the highest antioxidant activity, and thus, selected for further studies in animal models of hypertension and diabetes mellitus.

Spontaneously hypertensive rats (SHRs) and type 1 (streptozotocin-induced) diabetic rats were treated intraperitoneally either with vehicle or boldine (20 mg/kg) for 7 days while type 2 (db/db mice) diabetic mice were treated orally to investigate the chronic effects of boldine on vascular and endothelial functions. Repeated treatment with boldine significantly improved acetylcholine (ACh)-induced endothelium-dependent relaxation in isolated SHR aortas whilst sodium nitroprusside (SNP)-induced endothelium-independent relaxations remained unaltered. Furthermore, boldine
Abstract
treatment lowered aortic $O_2^-$ and peroxynitrite (ONOO$^-$) productions and downregulated the protein expression of the p47$^{phox}$ subunit of pro-oxidant enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the SHR aortas. This endothelial protective effect of boldine in hypertensive animals was achieved, at least in part, through the inhibition of NADPH-mediated $O_2^-$ production. Similarly, repeated treatment with boldine significantly improved the ACh-induced endothelium-dependent relaxation of the aorta in both type 1 and 2 diabetic animals but had no effect on endothelium-independent relaxations to SNP. In addition, boldine treatment effectively reduced the hyperglycaemia-induced oxidative stress level in the vascular wall of the diabetic rats and mice. This protective effect of boldine was further confirmed in primary rat/mouse aortic endothelial cells exposed to high glucose levels. In rat aortas, this endothelial protective role of boldine were correlated with increased NO levels and reduction of vascular ROS via inhibition of the NADPH oxidase subunits, p47$^{phox}$ and NOX2. In the db/db mouse type 2 diabetic model, repeated treatment with boldine normalized the overproduction of ROS, and this was associated with the restored level of eNOS phosphorylation and down-regulation of protein expression of angiotensin type 1 receptor (AT$_1$R) and oxidative stress markers (bone morphogenetic protein-4 and nitrotyrosine). Treatment with boldine, slightly decreased blood glucose levels in the type 1 diabetic rat but did not have a significant effect in the type 2 diabetic mice.

Taken together, it appears that boldine may exert positive effects on the endothelium via several mechanisms including mainly by protecting NO from degradation via inhibiting the excessive production and scavenging of $O_2^-$, and additionally by increasing NO bioavailability by upregulating activity of eNOS. The present study supports a complimentary therapeutic role of a natural product, boldine in improving endothelial dysfunction associated with hypertension and diabetes mellitus by interfering with the oxidative stress-mediated signalling pathway.
Stres oksidatif yang disebabkan oleh radikal bebas adalah berkaitan dengan patogenesis beberapa penyakit seperti diabetis, tekanan darah tinggi dan aterosklerosis. Disfungsi endotelial yang biasanya dinilai sebagai perencatan relaksasi ‘endothelium-dependent’ biasanya terjadi sebelum simptom patologi vaskular dan bersama stres oksidatif adalah ramalan utama untuk menilai progressi penyakit. Disfungsi endotelial selalunya berlaku pada peringkat awal penyakit dan disebabkan oleh pembentukan spesies oksigen reaktif (ROS) yang berlebihan seperti ‘superoxide anion’ (O$_2^-$) yang boleh mengurangkan kandungan nitrik oksida (NO) di dalam tisu vaskular. Kajian epidemiologi dan klinikal telah menunjukkan bahawa terdapat pelbagai produk semulajadi yang kaya dengan antioksidan dan boleh membaikpulih fungsi vaskular dengan meningkatkan kandungan NO di dalam tisu vaskular.

Dalam peringkat awal kajian kami, ekstrak mentah Phoebe grandis menunjukkan aktiviti yang baik dalam melindungi sel-sel endotelial terhadap tekanan oksidatif yang disebabkan oleh substrat β-NADH. Boldine ((S) -2,9-dihydroxy-1,10-dimethoxy-aporphine), kompaul utama yang ditemui dalam Phoebe grandis, telah mempamerkan aktiviti antioksidan yang tertinggi dan telah dipilih untuk kajian selanjutnya dalam model haiwan hipertensi dan diabetis.

Tikus hipertensi spontan (SHRs) dan tikus diabetis jenis 1 (diinduksi dengan streptozotocin) telah diberikan ‘vehicle’ (20% Tween-80 atau etanol) atau ‘boldine’ (20 mg/kg) secara intraperitoneal selama 7 hari manakala tikus diabetis jenis 2 (db/db) telah dirawat secara oral untuk menyiasat kesan kronik boldine ke atas fungsi vaskular. Rawatan berulang dengan boldine selama 7 hari mengembalikan relaksasi endotelium terhadap asetilkolin (ACh) dalam aorta SHR manakala relaksasi terhadap sodium
nitroprusside (SNP) tidak berubah dalam semua kumpulan. Tambahan pula, rawatan dengan boldine telah menurunkan pengeluaran radikal bebas $O_2^-$ dan peroxynitrik (ONOO⁻) dan mengurangkan ekspresi protein subunit, p47phox enzim pro-oksidan NADPH dalam aorta SHR. Penemuan ini mencadangkan bahawa perlindungan terhadap endotelium oleh boldine dalam penyakit darah tinggi, melibatkan sekurang-kurangnya perencatan pengeluaran radikal bebas oleh enzim NADPH oxidase. Kajian ini juga telah menunjukkan bahawa rawatan berulang dengan boldine meningkatkan relaksasi ‘endothelium-dependent’ dalam model haiwan diabetis jenis 1 dan jenis 2 tanpa mempunyai kesan ke atas relaksasi ‘endothelium-independent’. Di samping itu, rawatan berulang dengan boldine berkesan dalam mengurangkan stres oksidatif pada dinding vaskular yang disebabkan oleh hiperglisemia dan disahkan lagi di dalam sel-sel endotelium yang terdedah kepada glukosa yang tinggi. Dalam model tikus diabetis jenis 1, manfaat boldine adalah berkaitan dengan peningkatan tahap plasma nitrik oksida dan pengurangan ROS vaskular melalui perencatan protein subunit NADPH oksidant p47phox. Manakala, dalam model tikus diabetis jenis 2, rawatan boldine secara berulang menormalkan pengeluaran radikal bebas dan ianya melibatkan rencatan ekspresi protein reseptor-reserator AT1 dan penanda-penanda oksidatif (BMP4 dan nitrotyrosine). Dengan rawatan boldine, paras glukosa darah telah turun sedikit dalam model tikus diabetis jenis 1 tetapi tidak memberikan kesan yang signifikan dalam model tikus diabetis jenis 2.

Secara ringkasnya, boldine telah menunjukkan kesan-kesan yang positif terhadap endotelium melalui beberapa mekanisme-mekanisme termasuk mengurangkan pengeluaran dan memerangkap $O_2^-$. Tambahan pula, boldine juga menambahkan kandungan NO dengan meningkatkan aktiviti eNOS. Kajian ini menunjukkan sumbangan positif boldine sebagai produk semulajadi dalam mengurangkan komplikasi
Abstrak

kardiovaskular dalam penyakit darah tinggi dan diabetis adalah melalui perencatan mekanisme-mekanisme yang berkaitan dengan pengeluaran oksidan radikal yang menyebabkan stres oksidatif.
1. Academic Awards

1) First prize of poster presenter in the 9th Annual Scientific Meeting 2012 of the Malaysian Society of Hypertension, 10th - 12th February 2012, Kuala Lumpur, Malaysia.

2) Best abstract award at 25th Scientific Meeting of the Malaysian Society of Pharmacology and Physiology (MSPP), 25th - 26th May 2011, Universiti Putra Malaysia.

3) Research Grants Award by the Malaysia Society of Hypertension (Award amount: RM 10,000). Project title: ‘Endothelial protective activity of boldine in animal model of Hypertension’.

4) Best poster presenter in the 24th Scientific Meeting of the Malaysian Society of Pharmacology & Physiology, MSPP 2010, 2nd - 3rd June, Shah Alam, Malaysia.

5) National Science Fellowship (NSF, 2009-2012), Ministry of Science, Technology and innovations (MOSTI), Malaysia.

2. List of Publications

Original research article


### 3. Conference abstracts

**Oral presentation**


**Poster presentation**


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As the saying goes, Rome wasn’t built in a day. This thesis is a personal memorable journey for me filled with ups and downs, a sense of patience, lots of hard work and a deep commitment toward its successful completion. I have to say none of this could be made possible without the many wonderful people who shared their greatest passion, advice, support and encouragement with me throughout the past four years.

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<td>Symbol</td>
<td>Abbreviation</td>
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<td>--------------</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end product</td>
</tr>
<tr>
<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AT₁R</td>
<td>angiotensin type 1 receptor</td>
</tr>
<tr>
<td>AT₂R</td>
<td>angiotensin type 2 receptor</td>
</tr>
<tr>
<td>BH₄</td>
<td>tetrahydrobiopterin</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BMP4</td>
<td>bone morphogenetic protein-4</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium ion</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic Guanine MonoPhosphate</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>CYP</td>
<td>cytochrome P450</td>
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<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<td>DAF-FM</td>
<td>4-amino-5-methylamino-2',7'-difluorofluorescein</td>
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<tr>
<td>DCFH₂-DAG</td>
<td>dihydrodichlorofluorescein diacetate</td>
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<td>DECTA</td>
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</tr>
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<td>DHE</td>
<td>dihydroethidium</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DPI</td>
<td>diphenylene iodonium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EDC</td>
<td>endothelium-dependent contraction</td>
</tr>
<tr>
<td>EDCF</td>
<td>endothelium-derived contracting factor</td>
</tr>
<tr>
<td>EDHF</td>
<td>endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EDRF</td>
<td>endothelium-derived relaxing factor</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic Acid</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia (for example)</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>et al.</td>
<td>et alia (and other people)</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
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<tr>
<td>GADPH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>GSH</td>
<td>glutathione</td>
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<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HG</td>
<td>high glucose</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxidase</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>K$^+$</td>
<td>potassium ion</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>potassium dihydrogen orthophosphate</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N-Nitro-L-Arginine Methyl Ester</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LDLR$^-$</td>
<td>low density lipoprotein receptor knockout</td>
</tr>
<tr>
<td>Symbol</td>
<td>Abbreviation</td>
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<td>--------------------------------------------------------</td>
</tr>
<tr>
<td>LEC</td>
<td>lucigenin-enhanced chemiluminescence</td>
</tr>
<tr>
<td>L-NAME</td>
<td>Nω-Nitro-L-Arginine Methyl Ester</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NG</td>
<td>normal glucose</td>
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<tr>
<td>NOx</td>
<td>total nitrite and nitrate concentration</td>
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<tr>
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<tr>
<td>MAEC</td>
<td>mouse aortic endothelial cell</td>
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<tr>
<td>MDA</td>
<td>malondialdehyde</td>
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<td>min</td>
<td>minute</td>
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<td>mg</td>
<td>miligram</td>
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<tr>
<td>MgSO₄.7H₂O</td>
<td>magnesiussulphate heptahydrate</td>
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<tr>
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<td>manganese superoxide dismutase</td>
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<tr>
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<td>milinewton</td>
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<tr>
<td>MPG</td>
<td>methanolic <em>phoebe grandis</em> extract</td>
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<tr>
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<tr>
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<td>nitric oxide synthase</td>
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<tr>
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<td>oxygen</td>
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<tr>
<td>O₂⁻</td>
<td>superoxide anion</td>
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<tr>
<td>OCT</td>
<td><em>optimal cutting temperature</em></td>
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<td>Symbol</td>
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<tr>
<td>ONOO&lt;sup&gt;-&lt;/sup&gt;</td>
<td>peroxynitrite radical</td>
</tr>
<tr>
<td>OLETF</td>
<td>Otsuka Long-Evans Tokishima Fatty</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
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<td>phosphate buffered saline</td>
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<tr>
<td>Phe</td>
<td>phenylephrine</td>
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<td>Prostaglandin E&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>Prostaglandin F&lt;sub&gt;2α&lt;/sub&gt;</td>
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<td>Prostaglandin H&lt;sub&gt;2&lt;/sub&gt;</td>
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<tr>
<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostaglandin I&lt;sub&gt;2&lt;/sub&gt; or prostacyclin</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
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<tr>
<td>RAS</td>
<td>renin-angiotensin system</td>
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<tr>
<td>RAEC</td>
<td>rat aortic endothelial cell</td>
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<tr>
<td>RIPA</td>
<td>radio-immunoprecipitation</td>
</tr>
<tr>
<td>ROS</td>
<td>radical oxygen species</td>
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<tr>
<td>RPM</td>
<td>revolutions per minute</td>
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<tr>
<td>SBP</td>
<td>systolic blood pressure</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of mean</td>
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<tr>
<td>SHR</td>
<td>spontaneously Hypertensive Rats</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
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<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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<tr>
<td>STZ</td>
<td>streptozotocin</td>
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<tr>
<td>Tris-HCl</td>
<td>Tris(hydroxymethyl)aminomethane hydrochloride</td>
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<tr>
<td>TP</td>
<td>thromboxane receptor</td>
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<tr>
<td>TXA</td>
<td>thromboxane</td>
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<td>Symbol</td>
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<tr>
<td>U/ml</td>
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<td>μg</td>
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<td>V</td>
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<td>vs.</td>
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<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar Kyoto</td>
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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
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CHAPTER I

INTRODUCTION

1.1 Endothelial dysfunction

The endothelium, a monolayer of cells that lines the inner wall of blood vessel is particularly important in the regulation of vascular homoeostasis. Through the release of various local mediators, the endothelium modulates vascular tone, vascular growth, inflammation, cellular adhesion, and smooth muscle cell proliferation (Deanfield et al., 2007; Nedeljkovic et al., 2003). Under normal physiological condition, the healthy endothelial cells maintain the balance between vasodilation and vasoconstriction. Nitric oxide (NO) is the most important vasodilator released by endothelium (Fenster et al., 2003; Vanhoutte et al., 2009). Conversely, in diseased conditions, endothelium-dependent vasoconstriction becomes more prominent and endothelial dysfunction is exacerbated in the presence of various vasoconstrictors, including endothelins, angiotension II, cyclooxygenase-derived prostanoids and superoxide anion (O$_2^-$) (Radenkovic et al., 2013). Typically, the hallmark of endothelial dysfunction is impaired endothelium-dependent vasodilation due to attenuated NO release and increases in endothelium-derived contracting factors (Cai & Harrison, 2000; Lerman & Burnett, 1992; Radenkovic et al., 2013). Cardiovascular risk factors commonly associated with endothelial dysfunction are obesity, smoking, aging, hypercholesterolemia, hypertension and hyperglycaemia (Brunner et al., 2005; Versari et al., 2009). Endothelial dysfunction is associated with various cardiovascular diseases including atherosclerosis, hypertension, coronary artery disease, chronic heart failure and diabetes mellitus (Bonetti et al., 2003; De Vriese et al., 2000; Luscher et al., 1987; Schafer et al., 2004; Thambyrajah et al., 2001).
1.2 The role of oxidative stress in hypertension and diabetes mellitus

Nitric oxide is synthesized from L-arginine by endothelial NO synthase (eNOS), one of the 3 major forms of NOS presently identified. Increased degradation by reactive oxygen species (ROS) and reduced activation of eNOS due to deficiency of essential substrates or cofactors may account for the loss of NO and decreased NO bioavailability (Cai & Harrison, 2000; Vanhoutte et al., 2009). Several studies in cell culture, animal models and human vessels have shown that oxidative stress is the single most important mechanism implicated in endothelial dysfunction. One of the major ROS strongly implicated in the pathogenesis of endothelial dysfunction is the superoxide anion (O$_2^-$), free radicals formed from the reaction of oxygen with single electron (Dong et al., 2012; Guzik & Harrison, 2006; Montezano & Touyz, 2012). These O$_2^-$ can further interact with other molecules to generate “secondary” ROS such as hydrogen peroxide (H$_2$O$_2$) (Valko et al., 2007). There are two important enzymatic sources of O$_2^-$: 1) nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex which catalyses the reduction of molecular oxygen using NADPH substrate as an electron donor. 2) Uncoupling of eNOS, resulting in O$_2^-$ formation instead of NO due to deficiency of the substrate, L-arginine and co-factor tetrahydrobiopterin (BH$_4$) (Nedeljkovic et al., 2003). Increased O$_2^-$ could potentially inactivate NO released by the endothelial cells and in turn form a highly reactive molecule, peroxynitrite (ONOO$^-$) (Jay et al., 2006).

Diabetes mellitus and high blood pressure are known major risk factors for cardiovascular diseases (CVD) and their incidences continue to increase globally. Recent evidence has shown that excessive oxidative stress caused by increased production of ROS, particularly O$_2^-$ and its derivatives, may account for the endothelial dysfunction observed in the early stages of these diseases (Johansen et al., 2005b; Li et al., 2004; Rizzoni, 2002; Tian et al., 2011). Reactive oxygen species may modify
endothelial function directly by activating several signalling cascades and redox-sensitive transcription factors leading to modification of important roles of many functional genes in vascular cells such as upregulation of adhesion molecules to platelets and leukocytes, and decreasing the bioavailability of NO or increasing oxidation of low density lipoprotein (Griendling et al., 2000). Accumulating evidence suggests that NADPH oxidase is the primary source of excessive $O_2^-$ generator in both animal models of hypertension and diabetes, including angiotensin II-induced hypertension, genetic hypertension, renovascular hypertension and type 2 diabetic db/db mice (Fenster et al., 2003; Tian et al., 2011; Zalba et al., 2001). Reactive oxygen species are considered to play important pathological roles in cardiovascular diseases due to their abilities to alter the function of specific cellular proteins and enzyme as well, which eventually leads to alternation on the expression of pro-inflammatory molecules and impaired endothelium-dependent relaxation (Griendling et al., 2000).

1.3 Pharmacological therapies to improve endothelial dysfunction

Endothelial dysfunction is a systemic cardiovascular disorder and it is reversible with several non-pharmacological and pharmacological therapies. Regular physical exercise is an important non-pharmacological intervention that has been demonstrated to reduce oxidative stress and improve endothelial function in animal models of hypertension and in hypertensive patients (Higashi et al., 1995; Higashi & Yoshizumi, 2004; Yen et al., 1995). Pharmacological agents that have been primarily known to achieve vascular protection includes angiotensin converting-enzyme (ACE) inhibitors, angiotension II receptor (ARB) blocker, calcium blockers, statins and antioxidants (Esper et al., 2000; Keegan et al., 1995; Koh et al., 2007; Schiffrin et al., 2002; van de Ree et al., 2001). A growing body of evidence has demonstrated that the major pathophysiologic processes of endothelial dysfunction such as loss of NO bioavailability, oxidation of LDL and the
vascular inflammatory response are all modulated by oxidant stress. Therefore, therapeutic approaches associated with antioxidants have gained considerable attention in recent year to ameliorate oxidant stress in vascular cells. Antioxidants such as vitamin C and E have been shown to effectively improve endothelial function in several animal models and in clinical studies (Bohm et al., 2007; Heitzer et al., 1999; Keegan et al., 1995; Mullan et al., 2005). Vitamin C has been reported to inhibit LDL oxidation, reduce monocyte adhesion to endothelial cell, decrease inactivation of NO and prevent homocysteine-induced impairment of vascular function (Chambers et al., 1999; Heitzer et al., 1996; Wu et al., 2007). Two weeks of vitamin E treatment was shown to decrease P-selectin in patients with hypercholesterolemia, suggesting attenuation of endothelial activation (Davi et al., 1998). However, the beneficial effects of antioxidants on cardiovascular diseases in clinical trials are limited and inconsistent. For example, long-term studies using vitamins have not shown any positive effect on endothelium function (Gilligan et al., 1994; Elliott et al., 1995). Furthermore, high concentrations of the vitamin E have been reported to worsen endothelial function (Keaney et al., 1994). Therefore, the optimal dose of an antioxidant must also be titrated and investigated in order to achieve an optimal beneficial effect.

Apart from vitamins, a class of naturally occurring compounds called polyphenols, contained largely in fruits, vegetables, red wine and chocolate, have been found to effectively ameliorate endothelial function in peripheral large arteries (Grassi et al., 2005; Schramm et al., 2012). It is found that polyphenols such as flavanoids, apart from their well-know superoxide scavenging activity, exert their protective effect by inhibition of NADPH oxidase (Al-Awwadi et al., 2005). Resveratrol, a red wines polyphenol, has been shown to prevent platelet aggregation via inhibition of cyclooxygenase-1 (COX-1) to protect endothelial cells from oxLDL-induced oxidative
stress by direct ROS scavenging and inhibition of NADPH oxidase (NOX) activity (Chow et al., 2007; Pandey & Rizvi, 2009; Szewczuk et al., 2004). The polyphenol has also been demonstrated to improve endothelial function by enhancing NO signalling pathway in vascular endothelial cells in several human and rat arteries (Rush et al., 2007; Zhang et al., 2009).

1.4 Aims of the study

Interventions that can ameliorate endothelial dysfunction may offer new potential therapeutic opportunities for treating these chronic diseases which are closely associated with oxidative stress. Several studies have demonstrated that administration of antioxidants decreased the development of endothelial dysfunction in animal models of hypertension and diabetes mellitus (Keegan et al., 1995; Ting et al., 1996). The first objective of the present study is to determine the antioxidant activities and evaluate the endothelial protective effect of a local Malaysian plant, Phoebe grandis on the isolated rat thoracic aorta. Amongst the major compounds identified from this plant, boldine (Figure 1.1) exhibited the most potent antioxidant activity and the greatest protection against endothelial dysfunction induced by β-NADPH, an enzymatic generator of O$_2^-$. Boldine has been extensively reported earlier as a potent ‘natural’ antioxidant and possesses several health-promoting properties like anti-inflammatory, anti-tumour promoting, anti-diabetic, and cyto-protective. Therefore, boldine was subsequently selected for further investigations in hypertensive and diabetic animals in vitro and in vivo.

Figure 1.1: Structure of boldine ((S)-2,9-dihydroxy-1,10-dimethoxy-aporphine)
In addition to its antioxidant effects, boldine has been reported to promote vascular smooth muscle relaxations (Ivorra et al., 1993b) and however, there have been no reports on the antihypertensive effects of the alkaloid. The second objective of this thesis is to examine the effectiveness of boldine in the treatment of endothelial dysfunction in spontaneously hypertensive rats (SHR), the most studied animal experimental model of hypertension.

Similar to hypertension, increased oxidative stress due to hyperglycaemia may participate in endothelial dysfunctions in animal models of diabetes. As a third objective, this thesis examines the therapeutic effect of acute and repeated treatment of boldine on high glucose-induced oxidative stress and endothelial function in the isolated aorta of streptozotocin (STZ)-induced diabetic rats.

For the fourth objective, this thesis describes the endothelial protective effects of boldine treatment in db/db mice, a type 2 diabetic animal model by inhibiting Ang II-mediated BMP4-dependent oxidative stress cascade. To further elucidate the mechanisms of the endothelial protective effects of boldine, this thesis describes actions of the aporphine alkaloid on NADPH oxidase activity, eNOS expression, free radical formation and nitric oxide levels in vitro and in vivo. The findings from these experiments are expected to address our main hypothesis whether boldine could protect the endothelium in chronic diseases such as hypertension and diabetes.
2.1 Endothelium

The endothelium is a complex organ system which lines the inner surface of the entire vascular system and acts as an interface between blood and smooth muscle cells of the blood vessel wall (Luscher & Noll, 1995; Mas, 2009). The vascular endothelium plays a pivotal role in the modulation of normal vascular tone by releasing short-lived vasodilators and vasoconstrictors known as endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs) respectively (Furchgott & Vanhoutte, 1989; Vanhoutte & Tang, 2008). Besides controlling vascular tone, the endothelium also regulates haemostasis, thrombosis and inflammatory responses by secreting a variety of procoagulant, anticoagulant, fibrinolytic and inflammatory factors (Disse et al., 2009). Under normal condition, endothelial cells favourably release anticoagulant and EDRFs rather than other substances. Endothelium-derived relaxing factors such as NO, prostaglandins and endothelium-derived hyperpolarizing factors (EDHFs), promotes vasodilation via stimulation of intracellular guanosine 3',5'-cyclic monophosphate (cGMP) on smooth muscle cells (Perrella et al., 1991; Vanhoutte & Scott-Burden, 1994). However, in pathophysiological condition, the phenotype of endothelial cells is modified to facilitate vasoconstriction, inflammation, and thrombotic events instead of regulating normal vascular tone (Pratico, 2005).

2.2 Endothelium-derived relaxing factors (EDRFs)

Endothelium-derived relaxing factor is a relaxing substance that was serendipitously discovered by Furchgott and Zawadski in 1980 during experiments examining acetylcholine (ACh)-induced relaxation in intact rat thoracic aortas (Deedwania, 2000).
The release of EDRF is triggered in response to shear stress (a physical stimulation in vivo), neurotransmitters, autacoids, platelet products and hormones which then readily diffuses to adjacent vascular smooth muscle cells and causes relaxation (Vanhoutte & Miller, 1989). The endothelium produces at least 3 types of EDRFs which are NO, endothelium-derived hyperpolarizing factors (EDHFs) and prostacyclin (Vanhoutte & Scott-Burden, 1994). Vanhoutte et al. (1994) reported that there are differences in EDRFs released in the various vascular beds, e.g. large arteries mainly rely on NO and smaller arteries on EDHFs for relaxations.

Nitric oxide is the primary EDRF molecule released in most vascular beds and it was universally designated as the ‘molecule of the year’ in 1992. Nitric oxide is released in response to endogenous vasodilators, such ACh and bradykinin, to control several physiological processes including thrombosis formation in platelets, vasodilatation, vascular remodeling and smooth muscle cell proliferation (Mitchell et al., 2008). Endogenous vasodilators stimulate the release of intracellular calcium (Ca^{2+}) in endothelial cells and thereby activating NO synthesis by eNOS which catalyzes the oxidation of L-arginine to NO and L-citrulline (Palmer et al., 1988; Stuehr, 2004). Tetrahydrobiopterin (BH4) is an essential cofactor to retain the dimer formation of a functional eNOS (Fleming & Busse, 1999). After NO is synthesized, it diffuses rapidly to the vascular smooth muscle cells (VSMCs), where it activates guanylate cyclase and the synthesis of cyclic guanosine-3’,5’-monophosphate (cGMP) from guanosine triphosphate (GTP) (Vallance & Chan, 2001). Cyclic GMP is an important intermediate second messenger which binds and activates protein kinase G (PKG) to reduce calcium influx and inhibit Ca^{2+}-dependent muscle contraction (Koenigsberger et al., 2005) (Figure 2.1).
Figure 2.1: A general diagram illustrating the mechanism of vascular smooth muscle relaxation mediated by nitric oxide (NO). NO is synthesized by the endothelial nitric oxide synthase (eNOS) in response to endogenous vasodilators, eg, acetylcholine (ACh) and bradykinin, and the activation of calcium (Ca\(^{2+}\)) released from the endoplasmic reticulum into cytoplasm. NO rapidly diffuses into vascular smooth muscle cells to produce cGMP and causes vasodilatation (Reproduced from Boulanger and Vanhoutte (1997)).
2.3 Endothelium-derived contracting factors (EDCFs)

Under certain conditions, the endothelial cells also can produce several diffusible substances others than EDRFs to cause the vasoconstriction in VSMCs which are known as EDCFs, e.g, cyclooxygenase (COX)-derived prostanoids, ROS, endothelin-1 and angiotensin II (Tang & Vanhoutte, 2010). Endothelium-dependent contraction can also be initiated by receptors-mediated agonists such as A23187, ACh and thrombin which leads to high accumulation of intracellular Ca^{2+} concentration in endothelial cells, a major trigger of EDCFs events (Wong & Vanhoutte, 2010). Cyclooxygenase was identified as the major enzyme involved in the arachidonic metabolism, leading to the production of endoperoxides from arachidonic acid. Endoperoxides is then further transformed into several prostanoids including prostacyclin (PGI\(_2\)), thromboxane A\(_2\) and various prostaglandins and ROS is formed as a by-product (Wong & Vanhoutte, 2010). Endothelial dysfunction may occur due to the imbalance between EDCFs and EDRFs and always resulting from the overproduction of EDCFs (Wong & Vanhoutte, 2010) (Figure 2.2). This enhancement of the vascular tone has been demonstrated in a number of cardiovascular diseases and related complications (Matsumoto et al., 2007; Shi et al., 2007; Qu et al., 2010).

2.4 Reactive oxygen species (ROS) and oxidative stress

Reactive oxygen species (ROS) is a highly bioactive molecule or chemical species formed by incomplete reduction of oxygen, e.g, O\(_2^-\), OH, peroxyl radical (RO\(_2\)), alkoxy radical (RO). These molecules possess varying oxidizing potencies. Other molecules like hydrogen peroxide (H\(_2\)O\(_2\)), hypochlorous acid (HOCl), ozone (O\(_3\)), and singlet oxygen (\(\cdot\)O\(_2\)) also possess oxidizing capability (Leonarduzzi et al., 2010). ROS has been reported to either directly or indirectly involve in the activation of EDCFs by triggering COX signalling pathway in the vascular smooth muscle cells and therefore increasing
vasoconstriction as well as reducing NO bioavailability (Wong & Vanhoutte, 2010). In the past few decades, $\text{O}_2^-$ has been identified as a ‘primary’ ROS which can further interact with other molecules to generate ‘secondary’ ROS like $\text{H}_2\text{O}_2$ or react rapidly with NO to form $\text{ONOO}^-$(Valko et al., 2007).

Oxidative stress is defined as overproduction of pro-oxidant molecules such as reactive oxygen and nitrogen species, which can cause oxidative damage to bio-molecules (lipids, proteins, DNA, RNA) and organs (Uttara et al., 2009). An increase in oxidative stress is typically attributed to either an excessive over production of ROS or decreased endogenous antioxidant activity. Such an imbalance plays a critical contributory role in the pathophysiology of endothelial dysfunction.

2.4.1 NADPH oxidase and pro-oxidant enzymes

Nicotinamide adenine dinucleotide phosphate oxidase is a multi-component enzyme that comprises of a membrane-bound cytochrome b558 ($\text{p}22^{\text{phox}}$ and $\text{gp}91^{\text{phox}}$) and other regulatory cytosolic proteins ($\text{p}47^{\text{phox}}$, $\text{p}67^{\text{phox}}$, $\text{p}40^{\text{phox}}$ and Rac) which has been found in membranes of vascular cells including endothelial cells, vascular smooth muscle cells and fibroblasts (Forstermann, 2008; Ray & Shah, 2005). Nicotinamide adenine dinucleotide phosphate oxidase appears to be the primary source of endothelial superoxide and it can potentially influence the generation of ROS by other oxidant enzymes like xanthine oxidase and mitochondrial enzymes. This promotes NOS uncoupling which leads to $\text{O}_2^-$ cascades and decreased bioavailability of NO (Ray & Shah, 2005). The other sources of ROS generating systems include arachidonic acid metabolizing enzymes, eg, COX, lipoxygenase, CYP epoxygenase (Li & Shah, 2004). An increase of oxidative stress is associated with the up-regulation of oxidant enzymes
which later contribute to cardiovascular complication in hypertension and diabetes mellitus (Forstermann, 2008).

2.4.2 Angiotension II type 1 receptor (AT1R) and NADPH oxidase

Angiotensin II (Ang II) is a bioactive product of renin-angiotensin system (RAS) and is known as a potent vasoconstrictor that has pro-inflammatory, mitogenic and profibrotic actions. There are two subtype receptors for Ang II, denoted as angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R) (Burnier & Brunner, 2000). It is well documented that Ang II produces vasoconstriction by acting on the AT1R and this receptor subtype is found in both VSMCs and endothelial cells (Higuchi et al., 2007). Upon activation by Ang II, AT1R in the plasma membrane interacts with several heterotrimeric G protein subunits such as Gq/11, Gi, G12 and G13 and thereby activate phospholipase C to form second messengers including inositol trisphosphate (IP3) and diacylglyceryl (DAG) (Higuchi et al., 2007). Eventually, this leads to the phosphorylation of the voltage-sensitive calcium channels, whereby calcium influx is enhanced, followed by an increase of vascular tone.

There are also growing evidences indicating that AT1R not only activates the classical receptor-coupled calcium signalling pathway but also NADPH oxidase-dependent pathway (Nguyen Dinh Cat et al., 2012). Ang II is known as one of the major regulator of vascular NADPH oxidase to produce O2− upon stimulation of AT1R, which can potentially increase oxidative stress cascades in the vascular endothelial cells and has been shown to increase NADPH-dependent O2− generation in cultured human umbilical vein endothelial cells (HUVECs) and VSMCs (Griendling et al., 1994). Furthermore, it has been reported that treatment with losartan, AT1R blocker, significantly improved ACh-induced vasodilatations and reduced the up-regulation of NADPH oxidase and
oxidative stress in both hypertensive and diabetic animal models (Bayorh et al., 2005; Fukui et al., 1997), supporting that Ang II induced NADPH-dependent ROS generation via AT₁R.

### 2.4.3 ROS and vascular tone

It is well established that ROS can regulate many physiological functions of the endothelium. Reactive oxygen species is known to affect vasorelaxation, in which $\text{O}_2^-$ reacts with NO to generate the oxidative cascade and reduces the NO bioavailability in the blood vessel walls, leading to impairment of endothelium-dependent relaxations (Taniyama & Griendling, 2003). They also exerts a direct vasoconstrictive effect on VSMCs or indirectly, activates the release of EDCFṣ to the VSMCs through arachidonic acid metabolism and the latter activates endoperoxides-thromboxane A₂ prostanoid (TP) receptors in VSMCs to evoke vasoconstriction (Taniyama & Griendling, 2003) (Figure 2.2).
Figure 2.2: Schematic diagram showing the overproduction of ROS leads to vasoconstriction and consequently to endothelial dysfunction in vascular vessel. The source of ROS includes NADPH oxidase, cytochrome P450 oxidase, xanthine oxidase, cyclooxygenase (COX) and uncoupling of eNOS. ROS causes an increase in intracellular calcium level and the activation of arachidonic acid metabolism, which eventually results in vascular smooth muscle contraction. The production of ROS also decreases NO bioavailability through the rapid reaction between superoxide anion (O$_2^-$) and NO to result in peroxynitrite (ONOO$^-$) formation and eNOS uncoupling (Reproduced from Wong & Vanhoutte, 2010 and Vanhoutte, 2002).
2.4.4 Bone morphogenic protein-4 (BMP4) and oxidative stress

Bone morphogenetic protein-4 (BMP4) is multi-functional growth factors which belongs to the transforming growth factor-β superfamily and is found in calcified atherosclerotic plaques (Dhore et al., 2001). Studies have showed that BMP4 plays a role in vascular inflammation (Miriyala et al., 2006; Sorescu et al., 2003). Previous studies have demonstrated that BMP4 is up-regulated following exposure to disturbed flow and oxidative condition in cultured endothelial cells triggering NADPH-dependent ROS generation and inflammatory responses (Sorescu et al., 2004). Recently, a study by Bostrom et al., (2011) have also showed that high glucose promotes BMP4 expression in cultured human aortic endothelial cells that is associated with the vascular calcification. Moreover, BMP4 has been increasingly reported to impair endothelial function in mouse aortas either by increased ROS formation through NADPH oxidase or cyclooxygenase-2 (Miriyala et al., 2006), suggesting the possibility that BMP4 may play a pivotal role in vascular disease such as hypertension, diabetes and atherosclerosis. However, BMP4 only exerts its pro-oxidant, pro-hypertensive and pro-inflammatory effect in the systemic arteries such as aorta, carotid or coronary arteries, whereas pulmonary arterial endothelial cells are resistant to the adverse effect induced by BMP4 (Csiszar et al., 2008).

2.2 Cellular antioxidant system

The degree of oxidative stress is dependent on the fine balances between the generation of ROS and the antioxidant systems. Antioxidant is a reducing agent that plays a crucial role in protecting the cells against the excessive ROS formation by reacting with the ROS and therefore minimizing their action (Gomes et al., 2012; Nordberg & Arner, 2001). Generally, cellular antioxidant system can be divided into two groups, enzymatic and non-enzymatic. The important enzymatic antioxidants include (1) catalase (CAT),
responsible to degrade H₂O₂ into H₂O (2) glutathione peroxidase (GPx), protects membrane lipids, proteins and nucleic acids from oxidation by reducing H₂O₂ into H₂O and (3) superoxide dismutase (SOD), converts O₂⁻ into O₂ and H₂O₂. These enzymatic antioxidants are usually located in different cellular compartments such as cytosol and mitochondria (Gomes et al., 2012). The non-enzymatic antioxidant compound includes vitamin C, vitamin E, lipoic acid, ubiquinones, carotenoids and polyphenol derived from dietary sources (Uttara et al., 2009).

2.6 Endothelial dysfunction

Hypertension and diabetes mellitus affects the health of millions worldwide and are major risk factors for cardiovascular disease, the number one killer globally. There are increasing evidences demonstrating excessive oxidative stress may account for the endothelial dysfunction in the early stages of these diseases. Among the major cause of the endothelial dysfunction is the overproduction of ROS such as O₂⁻. The O₂⁻ may either bind or inactivate the NO released from the endothelium or combine with the later to form another toxic radical, ONOO⁻ (Li & Shah, 2004). In addition to their important physiological roles in the body, ROS are implicated in the pathogenesis of many acute and chronic diseases. In the cardiovascular system, these oxygen radicals may decrease the bioavailability of EDRFs such as NO or alter the function of cellular proteins, nucleic acids and lipids in cardiac membranes, leading to endothelial dysfunctions and cell death (Li & Shah, 2004).

2.6.1 Endothelial dysfunction in hypertension

Hypertension defined as elevated blood pressure, can result when total peripheral vascular resistance is increased (Carretero & Oparil, 2000; Virdis et al., 2011). The risk factors that contribute to the increase of blood pressure include obesity, insulin
resistance, high alcohol intake, high salt intake (in salt-sensitive patients), aging and stress (Virdis et al., 2011). There are two forms of hypertension: primary hypertension and secondary hypertension. Primary or essential hypertension is seen in almost 90% of the patient presenting with hypertension with an unknown cause. It is classified as secondary hypertension if the increase in blood pressure is secondary to renal disease, endocrine disorders, or other identifiable causes (Acelajado & Calhoun, 2010). Impaired endothelium-dependent relaxations have been well documented in several cardiovascular diseases such as hypertension and atherosclerosis (Ajay et al., 2006a; Benndorf et al., 2007; Jang et al., 2000b) Similarly, endothelial dysfunctions are also a common feature in various animal models of hypertension including spontaneously hypertensive rats, salt-induced hypertension and renovascular hypertension (Benndorf et al., 2007; Heitzer et al., 1999; Hermann et al., 2003; Lockette et al., 1986).

De Champlain et al (2004) demonstrated a greater sensitivity of the vascular tissue of SHRs to oxidative stress and it is attributed to the excess production of vascular ROS such as O$_2^{-}$. The exaggeration of ROS production in vascular tissues not only decreased the NO bioavailability but also reduced the antioxidant capacity of the smooth muscle cells. Of note, almost all experimental models of hypertension have been associated with the excessive oxidative stress production. For example, Ang II- and diet-induced animal models, as well as genetically modified hypertensive rat models have been reported to exhibit increased vascular oxidative stress via the activation of NADPH oxidase (Lee & Griendling, 2008; Touyz & Briones, 2011). Furthermore, an up-regulation of NADPH subunit (p22$^{phox}$ or p47$^{phox}$) have been demonstrated in aortas of hypertensive rat models whilst NADPH knockout animal attenuated the development of hypertension, suggesting that NADPH oxidase is probably one of the major enzymatic sources of O$_2^{-}$ (Dikalova et al., 2005; Fukui et al., 1997; Zalba et al., 2000).
Hypertensive patients are also reported to have reduced antioxidant capacity with lower expression of SOD, catalase and GSH peroxidase in the whole blood and peripheral mononuclear cells from hypertensive patients (Redon et al., 2003). Elevated blood pressure have been also reported in animal model in which GSH synthesis was inhibited and extracellular SOD gene deleted (Welch et al., 2006).

Excess $O_2^-$ leads to greater vasoconstriction by the altering in the cellular signal transduction system characterized by an enhanced production of IP$_3$ and a reduced cGMP (De Champlain et al., 2004). It is worth noting that apart from reduced endothelium-dependent relaxations, endothelium-dependent contraction (EDC) also significantly contributes to the development of endothelial dysfunctions in hypertension (Vanhoutte & Tang, 2008).

2.6.2 Endothelial dysfunction in diabetes mellitus

Diabetes mellitus presents a growing health and socioeconomic problem especially in developing countries like Malaysia. The hyperglycaemia in diabetes may result from poor glucose utilization or defective insulin secretion, insulin resistance or both (Wei et al., 2003). There are two types of diabetes mellitus: type 1 and type 2. Type 1 diabetes is caused by pancreatic β-islet cell failure with resulting insulin deficiency, encompasses 5-10 % of diabetes diagnosed. While type 2 diabetes, accounting for almost 90 % of diabetics are characterized by insulin resistance (Jay et al., 2006). Reactive oxygen species generated by hyperglycaemia are implicated in the microvascular and macrovascular complications in diabetic patients. These microvascular complications include nephropathy and retinopathy, while macrovascular complications include atherosclerotic cardiovascular diseases (Johansen et al., 2005b).
Endothelial dysfunction has been demonstrated in both type 1 and type 2 diabetes, which is associated with decreased level of EDRFs following increased destruction by ROS (De Vriese et al., 2000). Glucose oxidation is believed to be a main source of ROS generation in diabetes followed by glycation and protein kinase C (PKC) activation (Maritim et al., 2003; Wiernsperger, 2003). Similar to hypertension, excessive oxidative stress has been associated with the increased production of $\text{O}_2^-$ via NADPH oxidase and accounts for the endothelial dysfunction in the early stage of the disease (Heitzer et al., 2001). Impaired endothelium-dependent vasodilatations had been observed in several animal models of type 1 and type 2 diabetes including streptozotocin (STZ)-induced diabetes, $db/db$ mice, Otsuka Long-Evans Tokushima Fatty (OLETF) rats and Goto-Kakizaki (GK) rats (Gupte et al., 2010; Kim et al., 2002; Tian et al., 2011; Woodman & Malakul, 2009).

There are increasing evidences showing that the increased production of ROS interferes with NO-cGMP signaling (De Vriese et al., 2000, Van den Oever et al., 2010). In both diabetic patients and animal models, elevated generation of oxidative stress has been linked to the up-regulation or activation of oxidant enzymes such as NADPH oxidase and impaired of antioxidant enzymes system such as SOD, catalase and glutathione peroxidase, leading to excess ROS production (Guzik et al., 2006; Guzik et al., 2004; Maritim et al., 2003; Olukman et al., 2010). Nicotinamide adenine dinucleotide phosphate-induced $\text{O}_2^-$ reacts rapidly with NO to form the toxic $\text{ONOO}^-$, which in turn uncouples eNOS by oxidizing the essential NOS redox-sensitive co-factor $\text{BH}_4$ and cause eNOS to initiate $\text{O}_2^-$ cascades (Woodman & Malakul, 2009; Zou et al., 2004). In consequence, reduced NO bioavailability leads to impaired endothelium-dependent relaxations. It has been recently demonstrated that $\text{O}_2^-$ scavengers like tempol and SOD reversed the endothelial dysfunction in STZ-induced diabetic and $db/db$ mice (Moien-
Afshari et al., 2008; Serizawa et al., 2011). In addition, several antioxidants such as vitamin C and vitamin E has also been reported to prevent the development of endothelial dysfunction in diabetic patients and STZ-induced diabetic animals, suggesting the protective effect of antioxidant in reversing endothelial dysfunction in diabetic patients and animal models (Keegan et al., 1995; Ting et al., 1996).

2.6.3 Therapeutic strategies for treating endothelial dysfunction

Impaired endothelium-dependent relaxation with reduced NO bioavailability and increased oxidative stress are the most common features of the diseases associated with the cardiovascular events. Numerous studies have evaluated both non-pharmacological and pharmacological interventions to improve and reverse endothelial function in cardiovascular diseases (Table 2.1). Regular exercise is an example of non-pharmacological interventions that has been shown to improve endothelial function by up-regulating eNOS protein expression and phosphorylation (Hambrecht et al., 2003). Others non-pharmacological interventions including lifestyle and dietary modifications have also been shown to decrease insulin resistance, increase adiponectin levels and improve endothelial function in clinical studies (Kim et al., 2006; Milan et al., 2002; Yang et al., 2001). For example, Sasaki et al. (2002) reported that 2 weeks of caloric restriction in dietary significantly resulted in weight loss and improvement of endothelial-dependent vasodilation through an increased release of NO in obese hypertensive patients.

The pharmacological strategies, which include the use of ACE inhibitors, statins, ARB blockers and antioxidants, have been known to improve endothelial function, ameliorate oxidative stress and limit cardiovascular risk in diseases. The effects of pharmacological treatment on endothelial function and cardiovascular risk are summarized in Table 2.1.
Table 2.1: Non-pharmacological and pharmacological therapies for treating endothelial dysfunction from the reviewed clinical studies

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Patient condition</th>
<th>Result in endothelial function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-pharmacologic therapy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regular aerobic exercise</td>
<td>Young, old sedentary</td>
<td>↑ EDV</td>
<td>(DeSouza et al., 2000)</td>
</tr>
<tr>
<td>Weight loss</td>
<td>Premenopausal obese women</td>
<td>↑ EDV</td>
<td>(Ziccardi et al., 2002)</td>
</tr>
<tr>
<td>Caloric restriction and weight loss</td>
<td>Obese hypertensive patients</td>
<td>↑ EDV</td>
<td>(Sasaki et al., 2002)</td>
</tr>
<tr>
<td><strong>Pharmacologic therapy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statins (Atorvastatin or cerivastatin)</td>
<td>Hypercholesterolemic patients</td>
<td>↑ EDV</td>
<td>(John et al., 2005)</td>
</tr>
<tr>
<td>Flavanoids</td>
<td>Coronary artery disease, hypertensive patients</td>
<td>↑ EDV</td>
<td>(d'El-Rei et al., 2013; Duffy et al., 2001)</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Hypercholesterolemic patients and diabetes mellitus</td>
<td>↑ EDV</td>
<td>(Regensteiner et al., 2003; Ting et al., 1997)</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>Diabetes mellitus</td>
<td>↑ EDV</td>
<td>(Economides et al., 2005; Regensteiner et al., 2003)</td>
</tr>
<tr>
<td>ARB blocker (Telmisartan)</td>
<td>Hypertension and impaired glucose tolerance</td>
<td>↑ EDV</td>
<td>(Perl et al., 2010)</td>
</tr>
<tr>
<td>β-receptor blocker (Nebivolol)</td>
<td>Cardiac syndrome X</td>
<td>↓ High-sensitivity C-reactive protein</td>
<td>(Kayaalti et al., 2010)</td>
</tr>
<tr>
<td>ACE blocker (Zofenopril)</td>
<td>Mild hypertensive patients</td>
<td>↑ EDV</td>
<td>(Pasini et al., 2007)</td>
</tr>
</tbody>
</table>

Abbreviations: EDV, endothelium-dependent vasodilation
2.7 Boldine

In recent years, interests in the use of ‘natural’ antioxidants in the treatment of oxidative stress-related diseases including hypertension and diabetics have grown exponentially. Boldine ((S)-2,9-dihydroxy-1,10-dimethoxy-aporphine) is a aporphine alkaloid found abundantly in the leaves and bark of Chilean boldo tree (*Peumus boldus* Molina), a widely distributed tree native to central region of Chile (O’Brien *et al.*, 2006). Over a past decade, boldine was also found as one of the major alkaloid in the bark of a local tree in northern part of Peninsular Malaysia (Mukhtar *et al.*, 1997). Boldine-containing herbal teas are gaining popularity in South America and its usage has been extended to some European countries for further pharmaceutical processing into boldine-containing concentrates. Boldine has been extensively reported earlier as a potent ‘natural’ antioxidant and possesses several health-promoting properties like anti-inflammatory, anti-tumour promoting, anti-diabetic, and cyto-protective. These activities of boldine have been attributed to its ability to scavenge reactive free radicals (O’Brien *et al.*, 2006).

Boldine has also been reported to protect the red blood cells against free radical-induced haemolytic damage, even in micro molar concentrations (Jimenez *et al.*, 2000); inhibit spontaneous autoxidation of brain membrane lipids and prevent lipid peroxidation in human liver microsomal (Kringstein & Cederbaum, 1995); protect lysozyme inactivation against AAPH-derived peroxyl radicals damage (Cassels *et al.*, 1995; Speisky *et al.*, 1991) and to scavenge hydroxyl radicals (Jang *et al.*, 2000a). In addition, boldo extracts (*Peumus boldus*) containing boldine was found to effectively inhibit lipid peroxidation in erythrocytes (Schmeda-Hirschmann *et al.*, 2003). Boldine has been shown in several studies to have anti-inflammatory properties via its ability in interfering with the free radical generation (Backhouse *et al.*, 1994; Milian *et al.*, 2004).
In low density lipoprotein receptor knockout (LDLR\(^{-/-}\)) mice, treatment with boldine for 12 weeks decreased atherogenic lesion formation and inhibited oxidation of low density lipoprotein (LDL) without altering plasma cholesterol, triglycerides, LDL and HDL levels (Santanam et al., 2004). In the STZ-induced diabetic model, treatment with boldine for 8 weeks decreased malondialdehyde (MDA) and carbonyls in liver, kidney and pancreas mitochondria and normalize the elevated Mn-SOD and GSH-peroxidase activity in pancreas mitochondrial (Jang et al., 2000). It has also been reported to exert a concentration-dependent muscle relaxing effects in intestinal and uterus by directly interfering with the nicotinic ACh receptor (Ivorra et al., 1993b). Furthermore, it has been shown to block the \(\alpha_1\)-adrenoceptor in rat (Ivorra et al., 1993a) and guinea pig aorta (Chulia et al., 1996).

In addition, boldine has been indicated to have low toxicity. Boldine given in drinking water (100 mg/kg) prevented oxidative mitochondrial damage and possess anti-diabetic effect (Jang et al., 2000a). Boldine (20 mg/kg, i.p) also exhibited both free radical scavenging and antinociceptive activities in mice (Zhao et al., 2006). Relatively high doses are required to cause side effects, toxicity or lethality in several mammalian species, for example, doses of 500 and 1000 mg/kg were required to cause death of mice and guinea pigs respectively (Kreitmair, 1952; O’Brien et al., 2006).

Epidemiologic studies have demonstrated that dietary and supplemental intake of antioxidants has reduced the coronary artery diseases or cardiovascular events. Therefore, the present research explores the therapeutic potential of ‘natural’ antioxidant boldine, an aporphine alkaloid on endothelial function in animal models of hypertension and diabetes mellitus. This research works should address the following aspects:
1) The endothelial protective effect of a local Malaysian plant, *Phoebe grandis* on the isolated rat thoracic aortas exposed to β-NADH and pyrogallol-induced $\text{O}_2^-$ *in vitro*.

2) A novel role of boldine as a potent antioxidant in treating endothelial dysfunction in hypertensive and diabetic animals.

3) To identify several oxidative stress markers, in particularly a major source of $\text{O}_2^-$ generator NADPH oxidase, in hypertensive and diabetic aortas.

4) To clarify the protective role of boldine on endothelial function and its underlying mechanism by evaluating vascular reactivity, NO levels, ROS production, activity of eNOS, NADPH oxidase-mediated and Ang II-mediated $\text{O}_2^-$ production.
CHAPTER III

REACTIVE OXYGEN SPECIES-INDUCED IMPAIRMENT OF ENDOTHELium-DEPENDENT RELAXATIONS IN RAT AORTIC RINGS: PROTECTION BY METHANOLIC EXTRACTS OF PHOEBE GRANDIS

3.1 Abstract

Generation of reactive oxygen species (ROS) plays a pivotal role in the development of cardiovascular diseases. The present study describes the effects of the methanolic extract of *Phoebe grandis* (MPG) stem bark on ROS-induced endothelial dysfunction *in vitro*. Endothelium-dependent (acetylcholine, ACh) and -independent relaxation (sodium nitroprusside, SNP) was investigated on isolated aorta of Sprague Dawley (SD) rat in the presence of the β-NADH (enzymatic superoxide inducer) and MPG extract. Superoxide anion (O$_2^-$) production in aortic vessels was measured by lucigenin chemiluminescence. Thirty minutes incubation of the rat aorta *in vitro* with β-NADH increased superoxide radical production and significantly inhibited ACh-induced relaxation. Pre-treatment with MPG (0.5, 5 and 50 μg/ml) restored the ACh-induced relaxation ($R_{max}$: 92.29 ± 2.93%, 91.02 ± 4.54% and 88.31 ± 2.36%, respectively) in the presence of β-NADH. Methanolic extract of *Phoebe grandis* was ineffective in reversing the impaired ACh-induced relaxation caused by pyrogallol, a non-enzymatic superoxide generator. Superoxide dismutase (a superoxide scavenger), however, reversed the impaired ACh relaxation induced by both β-NADH and pyrogallol. Methanolic extract of *Phoebe grandis* also markedly inhibited the β-NADH induced generation of the superoxide radicals. Furthermore, MPG scavenged peroxyl radicals generated by tBuOOH (100 μM). These results indicate that MPG may improve the endothelium dependent relaxation to ACh through its scavenging activity as well as by inhibiting the NADH/NADPH oxidase induced generation of O$_2^-$.
3.2 Introduction

The vascular endothelium plays a pivotal role in regulating normal vascular tone and maintaining uninterrupted blood flow in the vessels (Balakumar et al., 2009). Under normal conditions, the endothelium regulates vascular homeostasis by releasing a variety of factors that act locally in the blood vessel wall and lumen, such as nitric oxide (NO), prostacyclin and endothelin. Nitric oxide released from the endothelium in response to various vasoactive factors such as ACh plays a key role in the maintenance of smooth muscle relaxation. Vascular endothelial dysfunction may be defined as impairment in endothelium dependent vasodilatation and alteration in the normal properties of endothelium (Stehouwer, 2004). The vascular dysfunction results in reduced activation of endothelial nitric oxide synthase (eNOS), and reduced generation and bioavailability of NO (Calles-Escandon & Cipolla, 2001). Among the factors contributing to the endothelial dysfunction is the overproduction of reactive oxygen species (ROS) such as superoxide anion (O$_2^-$) which binds and inactivates NO released from the endothelium (Berry et al., 2001).

Phoebe grandis (Nees) Merr is a local Malaysian timber tree of some 20 m in height with yellowish brown flowers (Mukhtar et al., 1997). Mukhtar et al. (2009) recently demonstrated that the stem bark of Phoebe grandis contains several known isoquinoline alkaloids: (-)-8,9-dihydrolinarisine, boldine, norboldine, lauformine, scortechine A and scortechine B and a novel oxoproaporphine; (-)-grandine A. Two of the aporphine alkaloids, boldine and norboldine have been demonstrated to have several pharmacological actions including anti-inflammatory, anti-cancer, anti-diabetic and potent antioxidant activities (O'Brien et al., 2006; Zhao et al., 2006). Plants rich in antioxidants are much sought out for their therapeutic potential, particularly in the prevention of cardiovascular diseases such as atherosclerosis, heart failure and hypertension. Several studies with these pytochemicals, including green tea, showed improved endothelial function by inhibiting O$_2^-$ production.
or scavenging the O$_2^-$ (Ajay *et al.*, 2006b; Nakagawa & Yokozawa, 2002; Romero *et al.*, 2009; Vera *et al.*, 2007). In the present study, we investigated the endothelial protective effect of the methanolic extract of *Phoebe grandis* (MPG) on the NADH/NADPH oxidase induced oxidative stress in the isolated rat thoracic aorta.

3.3 Materials and methods

3.3.1 Chemicals and drugs

Dichlorofluorescin, tert-butylhydroperoxide (tBuOOH), ACh, SNP, β-NADH, SOD, pyrogallol, DECTA and DPI were purchased from Sigma Aldrich Chemicals. HepG2 was purchased from ATCC (American Type Culture Collection). Chemicals used for Krebs solution preparation were purchased from BDH.

3.3.2 Preparation of methanolic extract of *Phoebe grandis* (MPG)

Bark of *Phoebe grandis* (Nees) Merr. (Lauraceae) were collected at Sik, Kedah (1994) by G. Perromat (Institut de Chimie des Substances Naturelles, CNRS, Gif sur Yvette, France). Identification was made by Dr K. M. Kochummen (Forest Research Institute of Malaysia, Kepong, Malaysia). Voucher specimens (KL 4318) are deposited at the Laboratoire de Phanerogamie, Museum National d’Historie Naturelle in Paris, the Herbarium of Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia and the Herbarium of the Forest Research Institute, Kepong, Malaysia. A total of 11.5 g of crude extract was obtained from the bark (1 kg). Crude product underwent column chromatography on silica gel with CH$_2$Cl$_2$ containing increasing amount of methanol and subsequent purification by preparative thin layer chromatography.
3.3.3 Cell based intracellular anti-oxidant assay of MPG extract

The intracellular antioxidant activity of the MPG extract was evaluated against the formation of intracellular ROS in HepG2 cells after treatment with t-BuOOH (tert butyl hydroperoxide), a compound used to induce oxidative stress. The cells were seeded in 96 wells plate at $3 \times 10^4$ cells/well (Black plate with transparent bottom) and incubated for 24 h at 37 °C in 5% CO$_2$. The next day, various concentrations of MPG extract was added into the wells and incubated again for another 1 h. The MPG extracts were dissolved in DMSO and the final concentration of DMSO (0.2%) used were not toxic to the cells or affect the assay. Next, cells were washed with phosphate buffered saline (PBS) and followed with the incubation with 10 μM dichlorofluorescin (DCF) for 60 min. Then, cells were washed and incubated with 100 μM tBuOOH for 60 min to induce oxidative stress. The plate was read at 485/535 nm. Results were expressed as a percentage inhibition of control.

3.3.4 Preparation of aortic rings

Twelve weeks old male Sprague-Dawley (SD) rats were obtained from the University of Malaya animal house and housed in well ventilated room at ambient temperature. They were provided with normal rat chow and tap water ad libitum. All experiments were reviewed and approved by the University of Malaya Animal Care and Ethics Committee (Ethics number: FAR/27/01/2010/0112/LYS). The rats were killed by cervical dislocation and aorta from the thoracic region was excised and cleared from any adherent fat and connective tissue with extra care to avoid any damage to the endothelium. The thoracic aorta was cut into small rings (3-5 mm in width) and suspended in a 5 ml organ bath containing Krebs physiological salt solution (pH 7.4) of the following composition (mM): NaCl 118, KCl 4.7, CaCl$_2$·2H$_2$O 2.5, KH$_2$PO$_4$ 1.2, MgSO$_4$·7H$_2$O 1.2, glucose 11.7, NaHCO$_3$ 25.0, and EDTA 0.026. The tissue-bath
solution was aerated continuously with 95% oxygen and 5% carbon dioxide at 37 °C. Isometric tension (g) was measured using a force displacement transducer connected to a Mac Lab recording system (ADI Instruments, Australia). The aortic rings were allowed to equilibrate for 20 min under resting tension of 1 g before any initiation of experimental protocols. Each experiment was conducted with a minimum of 5-8 numbers of rats.

3.3.5 Pharmacological studies

After equilibration, the rings were repeatedly stimulated with KCl solution (high K⁺, 80 mM) for 4 min at 10 min intervals until two consecutive equal contractions were reached. Following washout of high K⁺ responses, vascular relaxation study was performed by doing cumulative concentration-response curves to the endothelium-dependent and -independent relaxant agonists, acetylcholine (ACh, 0.1 nM to 10 µM) and sodium nitroprusside (SNP, 0.01 nM to 1 µM), respectively. To test the relaxation responses to ACh and SNP, the aortic rings were pre-contracted with phenylepherine, Phe (1 µM). To investigate the involvement of O₂⁻ on vascular relaxation, the aortic rings were pre-incubated for 30 min in the presence of β-NADH (300 µM, induces O₂⁻ through NADH/NADPH oxidase) prior to conducting concentration curve to ACh and SNP. To investigate the scavenging ability of MPG extract, various concentration of the extract (0.5, 5 or 50 µg/ml) was incubated with β-NADH for 30 min prior to ACh and SNP concentration response study. In other experiments, pyrogallol (10 µM) was incubated with the aorta to generate the O₂⁻ independent of NADH/NADPH oxidase. Superoxide dismutase (SOD, 50 U/ml), a superoxide scavenger was used as a positive control. Pre-incubation with β-NADH, SOD and pyrogallol did not affect the resting tension of the aortic tissue. Indomethacin (10 µM) was added in all experiments to exclude the influence of prostaglandin.
3.3.6 Measurement of superoxide anion

Lucigenin-enhanced chemiluminescence assay is performed as described previously by Chan et al. (2003) with some modification. The aortic rings were pre-incubated at 37 °C in Krebs-Hepes buffer containing diethylythiocarbamic acid (DETCA, 10 mM) and β-NADH (0.3 mM) and either vehicle (20% Tween 80), MPG extract (0.005 μg/ml - 50 μg/ml) or diphenylene iodonium (DPI, NAD(P)H oxidase inhibitor) for 45 min. The rings were then transferred to a 96-well plate in luminometer (Plate CHAMELEON™, Hidex, Finland). The background photon was measured previously for 20 min in the presence of 5 μM of lucigenin and Krebs-Hepes buffer. The output of chemiluminescence was then measured for 20 min. All of the samples were dried in a 65 °C oven for 48 h. The results are expressed as counts per milligram dry weight tissue (i.e., count/ mg).

3.3.7 Calculations and statistical analysis

The concentrations indicated in the text or in the figures represent the final tissue-bath concentrations of respective drugs. The responses were recorded as mean ± standard error of the mean (SEM) and ‘n’ indicates number of rats used for each set of data. Statistical evaluation of the data for pharmacological studies and chemiluminescense assay was performed by unpaired Student's t-test when comparing means of two groups and one-way analysis of variance (ANOVA) and Dunnett post hoc test for more than two group comparisons. A value of P < 0.05 was considered statistically significant.
3.4 Results

3.4.1 Effect of MPG extract on tBuOOH-induced intracellular oxidative stress

In the present study, the MPG extract protected the cells against the oxidative stress effect of ROS generated by tBuOOH (100 µM). At the highest concentration, MPG (3 mg/ml) inhibited almost completely the oxidative stress induced by tBuOOH (Figure 3.1).

Figure 3.1: Effect of various concentrations Phoebe grandis (stem bark) methanolic extract on oxidative stress induced by tBuOOH (100 µM). Quercetin (0.3 mM) was used as positive control.
3.4.2 Effect of MPG extract on vascular relaxations

Concentration dependent vasorelaxation was observed with both ACh and SNP. Maximal relaxation induced by ACh at 10 μM and SNP at 0.1 μM was 93.36 ± 3.82% and 105.09 ± 3.87%, respectively (Figure 3.2A and 3.2B).

Presence of β-NADH attenuated ACh induced relaxation ($R_{\text{max}}$: 66.64 ± 1.25%) and reduced the responses to SNP (1-100 nM) without marked effects on maximal relaxation ($R_{\text{max}}$: 98.67 ± 3.94%) (Figure 3.2). Presence of β-NADH slightly decreased the sensitivity of ACh and SNP (Table 3.1).

Pre-incubation with MPG extract alone or the vehicle (0.1% DMSO), did not affect the vascular responses to ACh (Appendix A). MPG extract also did not affect the resting tension of the aortic rings or the KCl-induced contraction. In the presence of β-NADH, pre-incubation with different concentrations of MPG extracts (0.5, 5 and 50 μg/ml) significantly improved the ACh induced relaxations ($R_{\text{max}}$: 92.29 ± 2.93%, 91.02 ± 4.54% and 88.31 ± 2.36%, respectively) (Figure 3.2A). In the presence of β-NADH, pre-incubation of 50, 5 or 0.5 μg/mL MPG extract did not significantly affect the SNP maximal relaxation compared to the control (Figure 3.2B). The different concentrations of the extract only slightly reversed the decreased sensitivity of ACh and SNP which was seen in the presence of β-NADH (Table 3.1).

In the presence of SOD and β-NADH, ACh-induced relaxation was significantly improved compared with the group with β-NADH alone. The improvement in ACh relaxation was similar to those observed with 0.5 μg/ml extract (Figure 3.3A). ACh-induced relaxation was impaired in the presence of 10 μM pyrogallol with maximal relaxation at 1 μM ACh 30.33% compared to control 93.36%. The $\text{pEC}_{50}$ of ACh
induced relaxation in the presence of pyrogallol was not significantly altered compared to the control (-6.30 ± 0.68 vs -7.18 ± 0.13, respectively). Pre-incubation of 0.5 μg/ml extract did not alter the impaired relaxation caused with pyrogallol (pEC50, -6.76 ± 0.23). However, pre-incubation of the aorta with SOD, markedly reduced the inhibitory effects of pyrogallol on the ACh-induced relaxation of the aorta (pEC50, -6.67 ± 0.34) (Figure 3.4).
Figure 3.2: Effect of 0.5 μg/ml, 5 μg/ml and 50 μg/ml Phoebe grandis (stem bark) methanolic extract on (A) ACh- and (B) SNP-induced relaxation in rat aortic rings induced oxidative stress by β-NADH. Results are mean ± SEM (n = 5-6). * P < 0.05 compared to control, # P < 0.05 compared to β-NADH.
Figure 3.3: Effect of SOD, a superoxide scavenger and 0.5 µg/ml Phoebe grandis (stem bark) methanolic extract in the presence of β-NADH on (A) ACh and (B) SNP- induced relaxation in rat aortic rings. Results are mean ± SEM (n = 5-6). * P < 0.05 compared to control, # P < 0.05 compared to β-NADH.
Figure 3.4: Effect of SOD, a superoxide scavenger and 0.5 μg/ml Phoebe grandis (stem bark) methanolic extract in the presence of pyrogallol (non-enzymatic superoxide inducer) on ACh-induced relaxation in rat aortic rings. Results are mean ± SEM (n= 5-6). * P < 0.05 compared to control, # P < 0.05 compared to pyrogallol.
Table 3.1: Agonist sensitivity (pEC$_{50}$) and % maximum response (R$_{\text{max}}$) value for ACh- and SNP-induced relaxation in rat aortic rings pre-treated with various concentrations of MPG extract or SOD in the presence of β-NADH or pyrogallol. Results are mean ± SEM (n = 4-6). * P < 0.05 compared to control; # P < 0.05 compared to β-NADH

<table>
<thead>
<tr>
<th></th>
<th>ACh</th>
<th>SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC$_{50}$ (log M)</td>
<td>R$_{\text{max}}$ (%)</td>
</tr>
<tr>
<td>Control</td>
<td>7.29 ± 0.13</td>
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</tr>
<tr>
<td>0.5 μg/ml extract</td>
<td>7.49 ± 0.19</td>
<td>92.96 ± 2.93 #</td>
</tr>
<tr>
<td>5 μg/ml extract</td>
<td>7.42 ± 0.16</td>
<td>91.02 ± 4.54 #</td>
</tr>
<tr>
<td>50 μg/ml extract</td>
<td>6.94 ± 0.34</td>
<td>88.31 ± 2.36 #</td>
</tr>
<tr>
<td>β-NADH</td>
<td>6.99 ± 0.16</td>
<td>66.64 ± 1.25 *</td>
</tr>
<tr>
<td>β-NADH + 0.5 μg/ml extract</td>
<td>7.34 ± 0.27</td>
<td>91.30 ± 2.49 #</td>
</tr>
<tr>
<td>β-NADH + SOD</td>
<td>7.48 ± 0.18</td>
<td>84.00 ± 6.72 #</td>
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<tr>
<td>Pyrogallol</td>
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</tr>
<tr>
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<td>6.76 ± 0.23</td>
<td>35.22 ± 1.63 *</td>
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<tr>
<td>Pyrogallol + SOD</td>
<td>6.67 ± 0.34</td>
<td>60.66 ± 5.30 #</td>
</tr>
</tbody>
</table>
3.4.3 Effect of MPG extract on β-NADH-mediated vascular superoxide production

Measurement of $O_2^-$ by lucigenin-enhanced chemiluminescence assay demonstrated that MPG dose-dependently reduced $O_2^-$ production induced by β-NADH (Figure 3.5). Without the presence of β-NADH, the vascular $O_2^-$ production from isolated rat aortic ring was 100 ± 50 counts/mg. In the presence of β-NADH, the superoxide radical production increased to 350 ± 50 counts/mg. Diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, significantly decreased the superoxide radical production induced by β-NADH (200 ± 25 counts/mg). The vehicle (0.1% DMSO) used to dissolve the extract did not have any effect on the $O_2^-$ production induced by β-NADH. The MPG extract dose-dependently reduced the $O_2^-$ stimulated by β-NADH with a significant decrease observed from the concentration 0.5 μg/ml to 50 μg/ml.
Figure 3.5: Effect of various concentration of *Phoebe grandis* (stem bark) methanolic extract on superoxide production induced by β-NADH in rat aortic rings detected by chemiluminescence assay. Superoxide production was inhibited in the presence of diphenyleneiodonium (DPI, 5 µM), an NADPH oxidase inhibitor. Results are mean ± SEM (n=5-7). * P < 0.01 compared to vehicle control.
3.5 Discussion

The results from the present study shows that the stem bark of MPG exhibited: (1) a marked cellular antioxidant activity at the concentration range of 1-3 mg/ml and (2) improved endothelium-dependent relaxation by inhibiting NADH/NADPH oxidase stimulated superoxide production. In the cell-based assay, the intracellular antioxidant effects of the MPG extract protected the cells against the ROS, i.e. peroxyl radicals, induced by tBuOOH. One of the active components of MPG extract is boldine, an aporphine alkaloid, and known to possess potent peroxyl radical scavenging and poor superoxide scavenging functions (O'Brien et al., 2006). The cell-based assay reaffirms that the MPG extract posses free radical scavenging activity. Improved endothelium-dependent relaxation to ACh in response to oxidative stress induced by β-NADH and decreased tissue superoxide levels, suggests that MPG is able to scavenge peroxyl radicals as well as reduce the generation of superoxide radicals produced by intracellular NADH/NADPH oxidase. Accumulating evidence indicates that the generation of ROS is closely associated with the development of many cardiovascular diseases (Cai & Harrison, 2000). β-NADH has been commonly used to stimulate NADH/NADPH oxidase activity in vitro. Previous studies have indicated increased superoxide generation in vascular vessel preparations in response to application of exogenous NADH (Brandes et al., 1997; Didion & Faraci, 2002; Lund et al., 2000). In the present study, pre-incubation of the tissues with β-NADH markedly reduced the endothelium-dependent relaxation to ACh in the isolated rat aorta and partly the endothelium-independent relaxation to SNP. Stimulation of M3-muscarinic receptor by ACh releases NO from the endothelium which then diffuses into adjacent smooth muscle cells and leads to soluble guanylate cyclase (sGC) activation, cyclic GMP elevation and ultimately to vascular smooth muscle relaxation (Murad, 1986). SNP breaks down spontaneously to yield NO, thereby causing endothelium-independent
vasodilatation by the same effectors mechanism as NO released from endothelium (i.e. activation of sGC) (Murad, 1986). Increased production of O$_2^-$, leads to reduce released of NO and/or inactivation of NO released from the endothelium, and ultimately attenuating the ACh-induced relaxations and partly SNP-induced relaxation. Pre-treatment with MPG significantly prevented β-NADH-induced attenuation of ACh relaxation in the rat aorta, suggesting the extract may increase the bioavailability of NO from the scavenging effects of the oxygen radicals. Furthermore, treatment with the extract in β-NADH treated aortas partly improved the relaxant responses and the sensitivity to endothelium-independent NO donor, SNP. This is indirect evidence that the beneficial effect of MPG on ACh-induced relaxation resides mainly improving the upstream endothelial NO bioavailability, since the extract only partly attenuated the downstream NO signal transduction pathway. Several studies have repeatedly shown that treatment with antioxidants improved endothelium-dependent relaxation in animal models of oxidative stress such as in spontaneously hypertensive rats (Machha & Mustafa, 2005; Vera et al., 2007). Many medicinal plants have also been found to cause endothelium-dependent relaxation in vascular tissues. Many are related to the balance between NO and O$_2^-$ (Achike & Kwan, 2003). In the present study, the MPG extract protected the cells against the oxidative stress effect of reactive oxygen radicals generated by tBuOOH. Boldine, an aporphine alkaloid is found in high concentrations in Phoebe grandis, and is known to possess potent antioxidative and free radical scavenging functions (Schmeda-Hirschmann et al., 2003). Thus, antioxidant actions of MPG may have increased the bioavailability of endothelium-derived NO, subsequently increasing the ACh-dependent relaxation. Both MPG and O$_2^-$ scavenger (superoxide dismutase, SOD) improved ACh-induced relaxation in β-NADH pre-treated tissues with an essentially similar magnitude. This indicates that MPG may be scavenging the β-NADH-induced superoxide productions in the rat aorta. However, in the presence of
pyrogallol, MPG failed to restore the NO-dependent relaxations. Pyrogallol, a pro-oxidant auto-oxidizes in tissue bath medium to generate extracellular $O_2^-$, while β-NADH stimulates release of intracellular $O_2^-$ from NADH/NADPH oxidase activity (Didion & Faraci, 2002; Marklund & Marklund, 1974). This is suggesting that the protective effect of the extract does not involve scavenging of $O_2^-$ but somewhat affects the intracellular NADH/NADPH oxidase activity in endothelial cells or in the vascular smooth muscle cells. These hypotheses are further supported in which measurement of $O_2^-$ by lucigenin chemiluminescence assay demonstrated that MPG dose-dependently decreased $O_2^-$ production induced by β-NADH. This suggests that MPG may inhibit NADH/NADPH oxidase stimulated $O_2^-$ production in a similar manner to apocynin (Stolk et al., 1994).

3.6 Conclusion

In summary, results from the present study showed that MPG extract may have improved the magnitude of endothelial dependent and independent relaxations through its antioxidant activity (peroxyl radical scavenging) as well as inhibiting NADH/NADPH induced $O_2^-$ productions in endothelial cells or vascular smooth muscle cells.
CHAPTER IV

THE APORPHINE ALKALOID BOLDINE IMPROVES ENDOTHELIAL FUNCTION IN SPONTANEOUSLY HYPERTENSIVE RATS

4.1 Abstract

Boldine, a major aporphine alkaloid found in Chilean boldo tree, is a potent antioxidant. Oxidative stress plays a detrimental role in the pathogenesis of endothelial dysfunction in hypertension. In the present study, we investigated the effects of boldine on endothelial dysfunction in hypertension using spontaneously hypertensive rats (SHR), the most studied animal model of hypertension. SHR and their age-matched normotensive Wistar-Kyoto (WKY) rats were treated with boldine (20 mg/kg per day) or its vehicle, which served as control, for 7 days. Control SHR displayed higher systolic blood pressure (SBP), reduced endothelium-dependent aortic relaxation to acetylcholine (ACh), marginally attenuated endothelium-independent aortic relaxation to sodium nitroprusside (SNP), increased aortic $O_2^-$ and $\text{ONOO}^-$ production, and enhanced $p47^{\text{phox}}$ protein expression as compared to control WKY rats. Boldine treatment significantly lowered SBP in SHR but not in WKY. Boldine treatment enhanced the maximal relaxation to ACh in SHR, but had no effect in WKY, whereas the sensitivity to ACh was increased in both SHR and WKY aortas. Boldine treatment enhanced sensitivity, but was without effect on maximal aortic relaxation responses, to sodium nitroprusside in both WKY and SHR aortas. In addition, boldine treatment lowered aortic $O_2^-$ and $\text{ONOO}^-$ production and downregulated $p47^{\text{phox}}$ protein expression in SHR aortas, but had no effect in the WKY control. These results show that boldine treatment exerts endothelial protective effects in hypertension and is achieved, at least in part, through the inhibition of NADPH-mediated superoxide production.
4.2 Introduction

Hypertension is the most important risk factor for cardiovascular disease, which is the leading cause of death and illness throughout the world (Lip et al., 2000). Endothelial dysfunction, which is mostly manifested as impairment in endothelium-derived nitric oxide (NO)-dependent vasodilation of blood vessels, represents a major risk factor for hypertension (Armitage et al., 2009; Gewaltig & Kojda, 2002). Therefore, interventions that can restore endothelial function are likely to improve clinical outcome in hypertensive subjects. One such intervention that received much attention in recent times is supplementation with “natural” antioxidants, confounded by the fact that increased production of reactive oxygen species (ROS) plays a critical role in the pathogenesis of endothelial dysfunction in hypertension (Ferroni et al., 2006; Schulz et al., 2008).

Boldine, an aporphine alkaloid, is a potent “natural” antioxidant found majorly in leaf and bark of the Chilean boldo (Peumus boldus Molina) tree (O'Brien et al., 2006; Speisky & Cassels, 1994). In earlier studies, boldine has been demonstrated to have anti-inflammatory, antipyretic, anti-diabetic, anti-atherogenic, anti-platelet, anti-tumour promoting, and cytoprotective effects, which stem from its potent antioxidant actions (O'Brien et al., 2006). For instance, treatment with boldine decreased artherogenic lesion formation and inhibited oxidation of low density lipoprotein in LDLR−/− mice (Santanam et al., 2004). Apart from its antioxidant effects, boldine can also promote vascular smooth muscle relaxation (Ivorra et al., 1993b). However, to the best of our knowledge, no studies at this point in time have demonstrated the effects of boldine in hypertension. This study, therefore, evaluated the effectiveness of boldine in the treatment of endothelial dysfunction in spontaneously hypertensive rats (SHR), the most studied experimental model of hypertension. Our own interest in boldine arose from the
observation that 1) it is widely found in the bark of local tree *Phoebe grandis* and 2) boldine-containing herbal teas are widely consumed around the world, in particular in South America (O'Brien *et al*., 2006).

### 4.3 Materials and methods

#### 4.3.1 Drugs and chemicals

Acetylcholine chloride (ACh), serotonin hydrochloride, bis-N-methylacridinium nitrate (lucigenin), diethyldithiocarbamate acid (DETCA), diphenyliodonium (DPI), β-nicotinamide adenine dinucleotide phosphate (NADPH), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), boldine, uric acid and Tris-base were purchased from Sigma chemicals company (St. Louis, MO, USA). Bovine serum albumin (BSA) and Tween-20 were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Sodium nitroprusside (SNP) and Kreb’s salts were purchased from BDH Limited and BDH Laboratory Supplies (Poole, UK), respectively.

#### 4.3.2 Animals and experimental protocol

Male spontaneously hypertensive (SHRs) and Wistar-Kyoto (WKYs) rats (17-18 weeks old) were obtained from the University of Malaya Animal Unit, and all the experimental procedures were approved by the University of Malaya Animal Care and Ethics Committee. The animals were housed in a well ventilated room (temperature: 24 ± 1°C), and had free access to standard rat chow and tap water. The rats were randomised to receive boldine vehicle (20% tween 80), which served as control, or boldine (20 mg/kg). The treatments were given by intraperitoneal injection, once daily for 7 days. To avoid the possible involvement of acute effects of the treatment, all the post-treatment experiments were conducted at least 24 h after the final treatment.
4.3.3 Non-invasive measurement of blood pressure

Systolic blood pressure was measured prior to and after the treatment period by tail-cuff plethysmography (NIBP machine, IITC Inc. CA, USA). Blood pressure values for individual rats were obtained from the average of eight consecutive measurements and were monitored in the morning at the same period of the time of the day.

4.3.4 Measurement of \textit{ex vivo} vascular function

After measurement of blood pressure, the rats were anaesthetized with a single intraperitoneal dose of pentobarbitone sodium (60 mg/kg body weight) and sacrificed by cervical dislocation. Thereafter, the descending thoracic aorta was rapidly removed and cleaned of fat and connective tissues. The aortas were sectioned into small rings (3-5 mm in width) and placed in jacketed organ baths (one ring in each bath) containing 5 ml of Krebs physiological salt solution (KPSS) composed of (mM): NaCl 118.2, NaHCO\textsubscript{3} 25.0, KCl 4.7, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4}.7H\textsubscript{2}O 1.2, glucose 11.7, CaCl\textsubscript{2}.2H\textsubscript{2}O 2.5 and EDTA 0.026. The bath solution was maintained at 37 °C and aerated continuously with a mixture of 95% O\textsubscript{2} and 5% CO\textsubscript{2}. The rings were connected to isometric force-displacement transducers (Grass Instrument Co., Quincy, MA, USA) and the output was amplified and recorded continuously using the Mac Lab recording system (AD Instruments, Sydney, Australia). The rings were equilibrated for 45 min under 1.0 g resting tension. After the equilibration period, the contractile responses of aortic rings were tested for viability by the repeated addition of high KCl solution (high K\textsuperscript{+}, 80 mM). Once reproducible contractions were obtained with high K\textsuperscript{+}, the aortic rings were contracted with serotonin (30 µM) and the concentration-response curves to endothelium-dependent and -independent vasodilators, acetylcholine (ACh, 0.1 nM to 10 µM) and sodium nitroprusside (SNP, 0.01 nM to 1 µM), respectively, were recorded. Acetylcholine or sodium nitroprusside was added cumulatively at 3-min intervals.
between successive doses. The choice of serotonin over more conventionally used phenylephrine to test the relaxation responses to ACh and SNP was prompted by earlier investigations which demonstrated that boldine may interact with $\alpha_1$-adrenoceptors and Ca$^{2+}$ channels (Chulia et al., 1996; Ivorra et al., 1993a).

4.3.5 Measurement of superoxide anion production

Levels of $O_2^-$ production from aortas isolated from different groups of rats were measured using the lucigenin-enhanced chemiluminescence (LEC) method with a luminometer (CHAMELEON™ V, Hidex, Turku, Finland) (Woodman & Malakul, 2009). After clearing of fat and connective tissues, aortic rings were rinsed in Krebs-HEPES buffer [composition in mM: NaCl 99.0, NaHCO$_3$ 25, KCl 4.7, KH$_2$PO$_4$ 1.0, MgSO$_4$.7H$_2$O 1.2, glucose 11.0, CaCl$_2$.2H$_2$O 2.5 and Na-HEPES 20.0] and incubated for 45 min at 37 °C in the presence and absence of diphenylene iodonium (DPI, 5 μM), a NADPH oxidase inhibitor, in Krebs-HEPES buffer containing 1 mM diethylthiocarbamic acid (DETCA) and 0.1 mM $\beta$-nicotinamide adenine dinucleotide phosphate (NADPH). After the incubation period, the rings were washed with Krebs-HEPES buffer and transferred to a 96-well plate with one ring in each well containing of 300 μl Krebs-HEPES buffer with lucigenin (5 μM) and NADPH (0.1 mM). The plate was immediately loaded into a luminometer and the output of LEC was recorded. Thereafter, the rings were dried for 48 h at 65 °C and weighed. The levels of superoxide generation were normalized to milligrams dry weight of tissue.
4.3.6 Measurement of peroxynitrite production

Levels of \( \text{ONOO}^- \) production from aortas isolated from different groups of rats were measured using the luminol-enhanced chemiluminescence method with a luminometer (CHAMELEON™ V, Hidex, Turku, Finland) (Radi et al., 1993). This method is similar to that used for superoxide detection (section 4.3.5) with the exceptions that 1) luminol (100 µM) is used instead of lucigenin and 2) aortic rings were incubated in Krebs-HEPES buffer containing 1 mM DETCA and 0.1 mM NADPH in the presence and absence of uric acid (250 mM), a scavenger of \( \text{ONOO}^- \).

4.3.7 Measurement of \( p47^{phox} \) protein expression

Aortas isolated from different groups of rats were freeze-dried in liquid nitrogen and stored at -80 ºC until analyzed. Aortas were homogenized in ice-cold 1X RIPA buffer (Santa Cruz Biotechnology, CA, USA) by using gentleMACS™ dissociator (Miltenyi biotec Inc., Bergisch Gladbach, Germany). The lysates were then centrifuged and supernatants were collected for Western blotting. Protein concentrations of the supernatant were determined by Bradford assay. For each sample, 30 µg of total tissue protein was separated in 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel and transferred to nitrocellulose membranes at 100 V for 90 min. The blots were blocked for non-specific binding with 3% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature with gentle shaking. It was then washed three times with TBS-T and incubated overnight at 4 ºC with primary mouse monoclonal antibody (1:500 dilution, Santa Cruz Biotechnology, CA, USA). The membranes were washed three times for 5 min in TBS-T and incubated with secondary goat anti-mouse antibody conjugated to horseradish peroxidise for 1 h at room temperature. After extensive washing of the membrane, the bands were detected using 3,3′,5,5′-Tetramethylbenzidine (TMB) liquid substrate system (Sigma, St. Louis, MO,
USA). The membrane image was captured under Gel DOC XR system (Bio-Rad Laboratories, Hercules, CA, USA) and densitometric analysis was performed using Quantity One® 1-D analysis software. The p47phox protein expression levels were normalized to α-actin and data are expressed as a percentage of the values in WKY control group.

4.3.8 Statistical analysis

All results are presented as mean ± standard error of mean (SEM) for number (n) of rats in each experimental group. Concentration-response curves were fitted to a sigmoidal curve using non-linear regression with the aid of the statistical software GraphPad Prism version 4, USA. Agonist sensitivity (pEC\textsubscript{50}) and maximal response (R\textsubscript{max}) for each group were obtained from the curves. The observed responses were analyzed for statistical significance using Student’s t-test for unpaired observations and the one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test for multiple value comparison (Prism 2.0, GraphPad Software, USA). A value of \( P < 0.05 \) was considered statistically significant.

4.4 Results

4.4.1 Effect of boldine treatment on blood pressure

Table 4.1 summarizes the mean ± SEM values for systolic blood pressure in different groups of animals. Systolic blood pressure was elevated in SHR animals compared with WKY animals. Boldine treatment (20 mg/kg per day, i.p) had no significant effect on systolic blood pressure of WKY rats, but significantly lowered it in SHR animals.
Table 4.1: Pre- and post-treatment systolic blood pressure (SBP) among vehicle- (control) or boldine-treated Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR)

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY-control</td>
<td>104.6 ± 2.37</td>
<td>110.8 ± 1.87</td>
</tr>
<tr>
<td>WKY-boldine</td>
<td>107.1 ± 3.40</td>
<td>111.6 ± 2.91</td>
</tr>
<tr>
<td>SHR-control</td>
<td>190.2 ± 2.92 *</td>
<td>192.4 ± 1.97</td>
</tr>
<tr>
<td>SHR-boldine</td>
<td>193.5 ± 1.23</td>
<td>166.9 ± 1.44 #</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM (n =6–7)

* P < 0.001 compared with WKY-control

# P < 0.001 compared with SHR-control
4.4.2 Effect of boldine treatment on vascular function

Figure 4.1 depicts the relaxation responses to ACh (panel A) and SNP (panel B) in aortic rings isolated from vehicle (control)- or boldine-treated WKY and SHR animals. Aortic rings from control SHR in general showed lesser relaxation responses to ACh and SNP compared to respective responses in control WKY aortic rings (Figure 4.1 and Table 4.2). Aortic rings from boldine-treated WKY and SHR animals demonstrated enhanced sensitivity to ACh and SNP compared to equivalent responses in respective control rats (Table 4.2). Maximal relaxation response to ACh was markedly increased in aortic rings obtained from boldine treated SHR compared with control SHR aortic rings, but remain essentially similar in the control and boldine-treated WKY rings (Table 4.2). Maximal relaxation response to SNP remains unaltered in aortic rings from boldine treated WKY and SHR animals compared with equivalent responses observed in respective control rats (Table 4.2).
Figure 4.1: Chronic boldine treatment (20 mg/kg) improves endothelial function in aorta of SHR rats. (A) The representative traces showing the impairment of ACh-induced endothelium-dependent relaxation in SHR aorta that were restored by chronic treatment of boldine (B) The summarized graph shows that chronic boldine treatment improved ACh-induced endothelium-relaxation in SHR rat aorta (C) SNP-induced endothelium-independent relaxations were not altered by all groups of treatment. Data are expressed as mean ± SEM (n= 6-7). # P < 0.001 compared to WKY-control, * P < 0.001 compared to SHR-control.
Table 4.2: Agonist sensitivity (pEC$_{50}$) and % maximum response (R$_{max}$) of endothelium-dependent and -independent vasodilators acetylcholine (ACh) and sodium nitroprusside (SNP), respectively, in aortic rings isolated from control and boldine-treated Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR)

<table>
<thead>
<tr>
<th>Group</th>
<th>ACh</th>
<th>SNP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pEC$_{50}$</td>
<td>R$_{max}$ (%)</td>
</tr>
<tr>
<td></td>
<td>(log M)</td>
<td></td>
</tr>
<tr>
<td>WKY- control</td>
<td>6.52 ± 0.48</td>
<td>88.44 ± 1.85</td>
</tr>
<tr>
<td>WKY- boldine</td>
<td>7.68 ± 0.21 *</td>
<td>90.38 ± 3.05</td>
</tr>
<tr>
<td>SHR - control</td>
<td>7.96 ± 0.12 *</td>
<td>40.60 ± 5.64 *</td>
</tr>
<tr>
<td>SHR- boldine</td>
<td>8.28 ± 0.13 †</td>
<td>94.67 ± 5.28 †</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM (n = 6–7)

* P < 0.001 compared with WKY-control

# P < 0.05 compared with WKY-control

†P < 0.001 compared with SHR-control
4.4.3 Effects of boldine treatment on superoxide anion production

Levels of $O_2^-$ generation in aortic rings from vehicle or boldine-treated WKY and SHR rats are shown in Figure 4.2. Superoxide levels were increased in control SHR aortas compared with control WKY aortas, and this increase was totally abolished in the presence of DPI, a NADPH oxidase inhibitor (Figure 4.2). There were no significant differences in superoxide generation between control and boldine-treated WKY rat aortas. Aortas from SHR treated with boldine demonstrated significant reduction in superoxide generation compared with control SHR aortas. There were no significant difference observed in superoxide generation from control and boldine-treated WKY and SHR aortas in the presence of DPI (Figure 4.2).

4.4.4 Effects of boldine treatment on peroxynitrite production

Levels of ONOO$^-$ generation in aortic rings from vehicle- or boldine-treated WKY and SHR rats are shown in Figure 4.3. Peroxynitrite levels were increased in control SHR aortas compared with control WKY aortas, and this difference was completely abolished in the presence of ONOO$^-$ scavenger, uric acid (Figure 4.3). There were no significant differences in ONOO$^-$ generation between control and boldine-treated WKY rat aortas. Aortas from SHR treated with boldine demonstrated significant reduction in ONOO$^-$ generation compared with control SHR aortas. There was no significant difference observed in ONOO$^-$ generation from control and boldine-treated WKY and SHR aortas in the presence of uric acid (Figure 4.3).
Figure 4.2: Levels of superoxide anion ($O_2^-$) generation in aortic rings from control and boldine treated Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Results are shown as mean ± SEM (n = 8-10), # $P < 0.001$ compared to WKY-control, * $P < 0.01$ compared to SHR-control.
Figure 4.3: Levels of peroxynitrite (ONOO^{-}) generation in aortic rings from control and boldine treated Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR).

Results are mean ± SEM (n = 4-6). # P < 0.01 compared to WKY-Control, * P < 0.01 compared to SHR-Control.
4.4.5 Effects of boldine treatment on p47<sup>phox</sup> protein expression

The p47<sup>phox</sup> protein expression was significantly higher in control SHR aortas as compared to control WKY aortas (Fig 4.4). Boldine treatment significantly reduced p47<sup>phox</sup> protein expression in SHR but had no effect in WKY (Fig 4.4).

![Image of Western Blot](image.png)

**Figure 4.4**: Protein expression levels of p47<sup>phox</sup> in aortic rings from control and boldine treated Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR). Results are mean ± SEM. (n = 4) of densitometric values normalized to corresponding α-actin and expressed as a percentage of WKY-control. # P < 0.05 compared to WKY-Control, * P < 0.01 compared to SHR-Control.
4.5 Discussion

The present study investigated, for the first time, the effects of boldine treatment (20 mg/kg per day) on endothelial dysfunction in hypertension. The novel findings of the present study are that boldine treatment improved impaired endothelial-dependent vasorelaxation in SHR aortas. In addition, boldine treatment also decreased $O_2^-$ and $\text{ONO}O^-$ levels and $p47^{\text{phox}}$ protein expression in SHR aortas. Furthermore, boldine treatment decreased systolic blood pressure in SHR. Collectively, these findings suggest an apparent protective role for boldine in the treatment of endothelial dysfunction in hypertension.

The present results demonstrated that boldine treatment significantly reduced mean SBP in SHR (Table 4.1). While the possibility of a direct vasodilator effect of boldine could be entertained in this phenomenon, this appears unlikely as blood pressure measurements were recorded at least 24 h after the final treatment with boldine. Scavenging of endothelium-derived NO by increased $O_2^-$, leading to inadequate NO concentrations and to increased formation of $\text{ONO}O^-$, can contribute to the diminished role of NO in the regulation of blood pressure in hypertension (Endemann & Schiffrin, 2004). Indeed, despite some paradoxical findings, several studies have shown that treatment with antioxidants such as ascorbic acid reduced blood pressure in humans and in animal models of hypertension including SHR (Akpaffiong & Taylor, 1998; Fujii et al., 2003; Laursen et al., 2001; Virdis et al., 2004). In the present study, boldine treatment reduced $O_2^-$/$\text{ONO}O^-$ production in SHR, suggesting that by inhibiting/decreasing $O_2^-$, boldine reduced blood pressure in SHR. A superoxide decreasing role for boldine in reduced blood pressure in SHR also gains ground from the finding that boldine treatment failed to alter blood pressure in normal WKY rats in
which the vascular $O_2^-$ production is expected to be too little (or even negligible) to effect the regulatory functions of endothelium-derived NO.

Impaired endothelium-dependent relaxations have been observed in different animal models of hypertension including SHR (Lyle & Griendling, 2006; Schulz et al., 2008; Suzuki et al., 1995; Ungvari et al., 2004). Consistent with these reports, the results of the present study demonstrated that endothelium-dependent relaxation responses to acetylcholine were significantly lesser in SHR aortas compared to their WKY counterparts (Figure 4.1A and 4.1B). Present results also demonstrate that treatment with boldine significantly improved endothelium-dependent relaxation responses to acetylcholine in SHR aortas (Figure 4.1A and 4.1B), indicating improvement in endothelial function in these hypertensive animals. One important mechanism for impaired endothelium-dependent relaxations in SHR is increased production of $O_2^-$ (Schulz et al., 2008). Superoxide anion reacts with endothelium-derived NO to form ONOO⁻, leading to impaired endothelium-dependent relaxations in hypertension (Schulz et al., 2008). Boldine is a potent antioxidant, as evidenced by inhibition of $O_2^-$ and other ROS in earlier studies (Estelles et al., 2005; Jang et al., 2000; O’Brien et al., 2006). For example, in an earlier study, we have shown that boldine inhibited peroxyl radical formation in cell-free xanthine/xanthine oxidase superoxide generation system (Lau et al., 2010). In the present study, boldine treatment significantly attenuated $O_2^-$ and ONOO⁻ production in SHR aortas (Figure 4.2 and Figure 4.3). In addition, boldine treatment also decreased p47phox protein expression in SHR aortas. It is well known that p47phox regulates the activity of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidases, which play a major role in increased $O_2^-$ production in hypertensive vasculature. NADPH oxidase is a multicomponent enzyme that comprises a membrane-bound cytochrome b558 (p22phox and gp91phox) and regulatory cytosolic
proteins (p47phox and p67phox) which has been found in membranes of all vascular cells including endothelial cells, vascular smooth muscle cells and fibroblasts (Forstermann, 2008; Guzik & Harrison, 2006). By inhibiting NADPH-mediated production of O2− in both endothelial cells and smooth muscle cells, NO bioavailability can be improved, which helps in vasorelaxation. Putting together, these observations suggest that boldine treatment improves endothelial function in SHR at least in part through inhibition of NADPH-mediated O2− production and subsequently improving endothelial NO bioavailability. In support to this, boldine treatment enhanced total plasma nitrite/nitrate (NOx) levels in SHR but not in WKY rats (Appendix B).

Interestingly, in the present study we found that aortic rings from boldine-treated WKY rats demonstrated higher sensitivity to acetylcholine-induced relaxation compared to control WKY aortas. This improvement in WKY aortas, as argued above, cannot be attributed to antioxidant activity of boldine. This is because boldine treatment had no effect on O2− and ONOO− production in WKY aortas (Figure 4.2 and Figure 4.3). In addition, normotensive WKY tissues produce little or no free radicals (Ajay et al., 2006a; Katusic, 1996). In earlier studies, boldine had been reported to elicit smooth muscle-relaxing effects, which may well contribute to improved relaxation to acetylcholine observed in the present study (Ivorra et al., 1993a). However, with the vascular reactivity studies performed at least 24 h after the final day treatment, this possibility is unlikely. On the other hand, however, boldine treatment improved the sensitivity of WKY aortic rings to the endothelium-independent vasodilator, SNP (Figure 4.1B), thus supporting the view that boldine improved the sensitivity of WKY aortas to ACh. Both ACh and SNP cause smooth muscle relaxation by the same effect or mechanism with the exception that acetylcholine-induced relaxation requires release of NO from the endothelium whereas SNP breaks down spontaneously to release NO
independently of the endothelium (Hansen & Nedergaard, 1999; Murad, 1986). Once released, NO diffuses into adjacent smooth muscle cells and leads to soluble guanylate cyclase (sGC) activation, cGMP elevation and ultimately to vascular smooth muscle relaxation (Hansen & Nedergaard, 1999; Murad, 1986). Putting these observations together, it appears that the effects of boldine not only reside upstream of the NO-sGC-cGMP cascade (i.e., preservation of NO bioavailability) but also reside downstream the NO-sGC-cGMP cascade (i.e., enhancement in the cGMP accumulation and NO bioactivity). The direct measurement of cGMP production which we were unable to perform in this study will be useful in further clarifying these assumptions.

Lastly, in the present study, despite normalizing oxidative stress parameters, boldine treatment failed to completely normalize the elevated blood pressure in SHR. The exact mechanism(s) of this discrepancy is unclear; but, however, it is noteworthy to mention that several oxidative stress-independent factors perse alterations in sympathetic nervous system can also contribute to development of hypertension in SHR. On the other hand, it is also important to note that, whereas all the above observations suggest the possibility that by decreasing $O_2^-$ boldine reduced blood pressure and improved endothelium-dependent relaxations in SHR, present findings neither support nor rule out a role for other actions of boldine perse anti-inflammatory actions in its observed effects.

4.6 Conclusion

In summary, the present results show that boldine treatment improves endothelial function in SHR in part by inhibiting NADPH-mediated superoxide production. The present results point to a potential therapeutic use for boldine in the management of elevated blood pressure and endothelial dysfunction in hypertension.
CHAPTER V

BOLDINE PROTECTS ENDOTHELIAL FUNCTION IN HYPERGLYCAEMIA-INDUCED OXIDATIVE STRESS THROUGH AN ANTIOXIDANT MECHANISM

5.1 Abstract

Increased oxidative stress is involved in the pathogenesis and progression of diabetes. Antioxidants are therapeutically beneficial for oxidative stress-associated diseases. Boldine ([s]-2,9-dihydroxy-1,10-dimethoxyaporphine) is a major alkaloid present in the leaves and bark of the boldo tree (*Peumus boldus* Molina), with known antioxidant activity. This study examined the protective effects of boldine against high glucose-induced oxidative stress in rat aortic endothelial cells (RAEC) and its mechanisms of vasoprotection related to diabetic endothelial dysfunction. In RAEC exposed to high glucose (30 mM) for 48 h, pre-treatment with boldine reduced the elevated ROS and nitrotyrosine formation, and preserved nitric oxide (NO) production. Pre-incubation with β-NAPDH reduced the acetylcholine-induced endothelium-dependent relaxation; this attenuation was reversed by boldine. Compared with control, endothelium-dependent relaxation in the aortas of streptozotocin (STZ)-treated diabetic rats was significantly improved by both acute (1 µM, 30 min) and chronic (20 mg/kg/daily, i.p., 7 days) treatment with boldine. Intracellular $O_2^-$ and $ONOO^-$ formation measured by DHE fluorescence or chemiluminescence assay were higher in sections of aortic rings from diabetic rats compared with control. Chronic boldine treatment normalized ROS over-production in the diabetic group and this correlated with reduction of NAD(P)H oxidase subunits, NOX2 and p47phox. The present study shows that boldine reversed the increased ROS formation in high glucose-treated endothelial cells and restored
endothelial function in STZ-induced diabetes by inhibiting oxidative stress and thus increasing NO bioavailability.

5.2 Introduction

Endothelial dysfunction is correlated with hypertension, arteriosclerosis, diabetes and chronic heart failure (Schulz et al., 2008). It is defined as impairment of endothelium-dependent relaxation and the major factor contributing to this condition is the compromised nitric oxide-cyclic GMP (NO-cGMP) signalling (Armitage et al., 2009). Excessive production of reactive oxygen species (ROS) interferes with NO signalling and thus plays a pivotal role in the development of vascular complications in diabetes (Armitage et al., 2009).

Over the past few decades, hyperglycaemia-induced oxidative stress has been increasingly known as a hallmark in diabetic vasculature through several mechanisms such as activation of protein kinase C, polyol pathway and formation of advanced glycation end-product (Choi et al., 2008). Hyperglycaemia causes the excessive ROS formation, particularly $O_2^-$, a radical that result from the reaction of oxygen with a single electron (Jay et al., 2006; Johansen et al., 2005a). NAD(P)H oxidase, a multi-subunit enzymatic complex, is the major enzymatic sources of superoxide generation in vascular cells (Kalinowski & Malinski, 2004). Excess $O_2^-$ can react with nitric oxide, forming the toxic ONOO-, which in turn uncouples enzymatic nitric oxide synthase (eNOS) by oxidizing the essential NOS redox-sensitive co-factor tetrahydrobiopterin and causes eNOS to produce more $O_2^-$ (Schulz et al., 2008). This continuous cascade of events reduces the bioavailability of NO and eventually leads to endothelial dysfunction in diabetes (Li et al., 2003).
Boldine ((S)-2,9-dihydroxy-1,10-dimethoxy-aporphine) is an aporphine alkaloid found abundantly in the leaves/bark of boldo (Peumus boldus Molina), a widely distributed tree native to Chile. Boldine is also one of the major alkaloids in the bark of a local tree of Phoebe grandis found in the Northern part of Peninsular Malaysia (Yeh-Siang et al., 2011). Boldine has been reported to have several pharmacological activities, such as anti-inflammatory, antipyretic, antidiabetic, antiatherogenic, antiplatelet, antitumor promoting and cytoprotective effects (O'Brien et al., 2006). The action of boldine has been attributed to its antioxidant activity as it prevents lipid peroxidation in human liver microsomes (Kringstein & Cederbaum, 1995), and scavenges hydroxyl radicals (Jang et al., 2000). In addition, boldo extracts (Peumus boldus) has been demonstrated in several studies to have anti-inflammatory properties via its ability to interfere with the generation of free radical (Backhouse et al., 1994; Milian et al., 2004). The methanolic extract of Phoebe grandis of which boldine, is a major compound, was shown to effectively improve the endothelium-dependent relaxations that were diminished by oxidative stress (Yeh-Siang et al., 2011). Although the antioxidant activities of boldine have been extensively studied, it has not been correlated with the hyperglycaemia-induced oxidative stress and improving endothelial dysfunction in diabetic animals. Therefore, this study aims to investigate the effect of boldine in abating high glucose-induced ROS formation and improving endothelial dysfunction in streptozotocin-induced diabetic rats.

5.3. Materials and methods

5.3.1 Chemicals and materials

Acetylcholine (ACh) chloride, sodium nitroprusside (SNP), serotonin hydrochloride, bis-N-methylacridinium nitrate (lucigenin), diethylthiocarbamic acid (DETCA), diphenylene iodonium (DPI), β-NADPH, 5-amino-2,3-dihydro-1,4-phthalazinedione
(luminol), boldine, uric acid, D-(-)-glucose, D-mannitol, triton-X, Hepes, citrate buffer, Tween-80 and Tris-base were purchased from Sigma Chemicals Company (St Louis, MO, USA). Bovine serum albumin (BSA) and Tween-20 were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Kreb’s salts were purchased from BDH Limited and BDH Laboratory Supplies (Poole, UK), respectively. RPMI 1640 media, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco (invitrogen, CA, USA). Tempol was purchased from Tocris (Bristol, UK).

5.3.2 Primary culture of rat aortic endothelial cells
Primary aortic endothelial cells were isolated from adult Sprague Dawley (SD) rats and cultured as previously described (Tian et al., 2012b). Briefly, the aorta was isolated from the abdominal cavity of the rat after CO\(_2\) inhalation. The fat and connective tissues of the rat aorta was cleaned and placed in sterile ice-cold PBS. The aorta was then digested with filtered collagenase type 1A (Sigma, MO, USA) solution at 37 °C for 10 min with gentle shaking. After incubation the cells were centrifuged at 1500 rpm for 10 min and re-suspended in RPMI-1640 containing 10% FBS plus 100 U/ml penicillin and 100 μg/ml streptomycin. After 1 h incubation at 37 °C, the medium was removed and replaced to eliminate the smooth muscle cells. The endothelial cells were incubated in a humidified atmosphere containing 5% CO\(_2\) at 37 °C until the cells reached 80% confluence. The identity of RAECs was confirmed by a positive immunofluorescence staining of PECAM-1 (Santa Cruz, CA, USA).

5.3.3 Measurement of intracellular ROS generation
The amount of intracellular ROS generation was measured with CM-H\(_2\)DCFDA fluorescein (Invitrogen, CA, USA) dye that performed under Olympus FV1000 laser scanning confocal system (Olympus, America Inc., Melville, NY, USA). Intracellular
ROS was detected once CM-H₂DCFDA oxidized to a fluorescent DCF product within the cell. In brief, the isolated primary rat endothelial cells were seeded on circular cover slip and the cells were incubated in normal glucose (NG, 5 mM glucose and 25 mM mannitol as osmotic control of HG) or co-treated with or without boldine (1 µM) in high glucose condition (HG, 30 mM) for 48 h. Tempol (100 µM), a superoxide dismutase (SOD) mimetic compound was added in the co-cultured as a positive control. At the end of treatment, the cells seeded on the circular cover slips were rinsed twice with normal physiological saline solution (NPSS in mM: NaCl 140, KCl 5, CaCl₂ 1, MgCl₂ 1, glucose 10 and HEPES 5) and then incubated at 37 °C for 20 min with CM-H₂DCFDA (1 µM). The fluorescence intensity was analyzed using Olympus Fluoview version 1.6 software.

5.3.4 Detection of nitrotyrosine by immunofluorescence

The confluent cells seeded on coverslip were fixed with 4% formaldehyde for 30 min. The cells were then permeabilized with 0.01% Triton-X 100 and blocked in 5% normal donkey serum (Jackson ImmunoResearch Laboratories, PA, USA) for 1 h. The cells were incubated with mouse anti-nitrotyrosine (1:20; Milipore, MA, USA) overnight at 4 °C. After that, the cells were washed with PBS and incubated with Alexa® Fluor 488-conjugated goat-anti-mouse secondary antibody (1:500; Invitrogen, CA, USA) for 2 h. At the end of incubation, the cells were stained with propidium iodide (1:3000; Sigma, MO, USA) to visualize the nucleus. The images were captured under Olympus FV1000 laser scanning confocal system (Olympus, America Inc., Melville, NY, USA) and the immunofluorescence intensity was analyzed using Olympus Fluoview version 1.6 software.
5.3.5 Detection of NO production in cultured endothelial cells

The experiment was performed as previously described (Yuen et al., 2011). Basically, the confluent endothelial cells were seeded on the coverslip and followed by high glucose treatment as described in previous section. At the end of treatment, the cells were rinsed with NPSS and incubated with 10 μM DAF-FM diacetate (Invitrogen, CA, USA) for 10 min at 37 °C. NO productions in response to ACh (10 μM) were measured by the level of fluorescence intensity change which were detected under Olympus Fluoview FV1000 laser scanning confocal system (Olympus America, Inc., Melville, NY, USA) mounted on an inverted IX81 Olympus microscope with excitation at 495 nM and emission at 515 nM. The real-time changes in intracellular fluorescence intensity were measured for 15 min and the results were presented as a ratio of fluorescence relative to intensity (F₁/F₀) before and after addition of ACh.

5.3.6 Induction of diabetes and chronic treatment

Male Sprague-Dawley (SD) (9-10 weeks old) were obtained from the University of Malaya Animal Unit, and housed in a well-ventilated room (temperature: 24 ± 1 °C), and had free access to standard rat chow (Specialty Feeds Pty Ltd, Glen Forrest, Australia) and tap water. All the experimental procedures were approved by the University of Malaya Animal Care and Ethics Committee. Diabetes mellitus were induced in 10-11 weeks SD rats (200-250 g) by injecting a single dose of 60 mg/kg streptozotocin (STZ) freshly dissolved in 0.1 M citrate buffer intraperitoneally. Blood glucose levels were measured using an Accu-check monitor (Roche, Mannheim, Germany) 3 days after diabetes induction. The animals were considered diabetic if the blood glucose level exceeds 17 mmol/l. After 8 week of induction, the rats were injected intraperitoneally with boldine (20 mg/kg/day) or vehicle (20% Tween 20) for 7 days. Body weights and blood glucose levels were recorded.
5.3.7 Measurement of lipids

Total cholesterol, triglyceride, HDL and non HDL were determined using the assay kit purchased from BioAssay System, CA, USA (EnzyChrom™ AF Cholesterol Assay Kit; EnzyChrom™ Triglyceride Assay kit; EnzyChrom™ AF HDL and LDL/VLDL Assay Kit, E2HL-100). All assays were done according to the manufacture instructions.

5.3.8 Preparation of aortic rings

At the end of 7 days treatment, the rats were anesthetized with single intraperitoneal dose of pentobarbitone sodium (60 mg/kg body weight). The descending thoracic aorta was isolated and cleaned from surrounding fat and connective tissues. The aorta was cut into rings segments, 3-5 mm long and placed in oxygenated Krebs physiological salt solution (KPSS in mM: NaCl 119, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, glucose 11.7, and CaCl₂·2H₂O 2.5) and some tissues were snap frozen in liquid nitrogen and stored in -80 °C for protein analysis. The fresh aortic rings were maintained at 37 °C and stretched to optimal tension of 9.82 mN in a Multi Wire Myograph System (Danish Myo Technology, Aarhus, Denmark) and continuously oxygenated with 95% O₂ and 5% CO₂, and the changes of isometric tension in response to different drugs were recorded using the PowerLab LabChart 6.0 recording system (AD Instruments, Australia). The rings were equilibrated for 45 min before being repeatedly stimulated with high KCl solution (high K⁺, 80 mM) three times at 4 min intervals to prime the tissues until two consecutive equal contractions were attained. The presence of functional endothelium in pre-contracted aortic rings was confirmed by a relaxant response to acetylcholine (ACh, 10 µM). Thereafter, concentration-relaxation curves of the endothelium-dependent relaxant, (ACh, 0.1 nM - 10 µM) and of the endothelium-independent relaxant, sodium nitroprusside (SNP, 0.01 nM - 1 µM) were carried out on 5-HT-precontracted aortic rings.
5.3.9 In situ detection of vascular superoxide production by laser confocal fluorescence microscopy

The amount of the in situ vascular superoxide formation was determined with using dihydroethidium (DHE, invitrogen, CA, USA) dye and Olympus FV1000 laser scanning confocal system (Olympus, America Inc., Melville, NY, USA) as described previously (Tian et al., 2011). Briefly, aortic rings from the respective groups were frozen in OCT compound (Sakura Finetek, Netherland) and 10 μm frozen cross sections were obtained. The sections were incubated in dark for 15 min in normal physiological saline solution containing 5 μM DHE fluorescence dye (NPSS: NaCl 140, KCl 5, CaCl₂ 1, MgCl₂ 1, glucose 10 and HEPES 5 mM). The fluorescence intensity was measured at excitation/emission of 488/605 nm to visualize the signal. The images were analysed using the Olympus Fluoview version 2.0 software.

5.3.10 Vascular superoxide and peroxynitrite production

Lucigenin-enhanced chemiluminescence method was used to estimate the vascular superoxide production as previously described (Lau et al., 2012a; Woodman & Malakul, 2009). Briefly, aortic rings from each of the vehicle- and boldine-treated ring segments from the SD and STZ-induced diabetic rats was pre-incubated for 45 min at 37 °C in 2 ml of Krebs-HEPES buffer (in mM: NaCl 99.0, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.0, MgSO₄·7H₂O 1.2, glucose 11.0, CaCl₂·2H₂O 2.5 and Na-HEPES 20.0) in the presence of diethylthiocarbamic acid (DETCA, 1 mM) to inactivate superoxide dismutase and β-nicotinamide adenine dinucleotide phosphate (NADPH, 0.1 mM) as a substrate for NADPH oxidase. Diphenylene iodonium (DPI; 5 μM) was added for the positive control as an inhibitor of NADPH oxidase. Prior to measurement, a 96-well Optiplate was filled with 300 μl of Krebs-HEPES buffer containing lucigenin (5 μM) and NADPH (0.1 mM) per well and loaded into the Hitex plate CHAMELEON™ V
(Finland) in luminescent detection mode to measure the background photo emission over 20 min. At the end of measurement, the rings were dried for 48 h at 65 °C and weighed. The data were expressed as average counts per mg of vessel dry weight.

Levels of ONOO⁻ production from aortas of different groups of rats were measured using the luminol-enhanced chemiluminescence method (CHAMELEON™ V, Hidex, Finland) as described (Lau et al., 2012a; Laursen et al., 2001). This method is similar to that used for superoxide detection with the exceptions that 1) luminol (100 μM) is used instead of lucigenin and 2) aortic rings were incubated in Krebs-HEPES buffer containing 1 mM DETCA and 0.1 mM NADPH in the presence and absence of uric acid (250 mM), a scavenger of ONOO⁻.

5.3.11 Total nitrates/nitrites measurement

The NO levels were measured by using Griess reagent kit (Sigma-Aldrich). Total nitrite and nitrate were determined as described by Ansari et al. (2007) with slight modification. Briefly, all the nitrates in plasma were converted into nitrites using aspergillus nitrate reductase (20 mU) in the presence of FAD (0.11 mM) and NADPH (100 μM). The incubation was carried out at 37 °C for 90 min in the dark. Equal volume of blood sample and 1X Griess reagent (Sigma, MO, USA) were mixed and the absorbance of the sample is read at 540 nm after 15 min. The reading was compared to the sodium nitrites standard curve.

5.3.12 Western blot

Aortas and cells were homogenized and lysed in ice-cold RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The lysates were then centrifuged at 20,000 g for 20 min and supernatants were collected for Western blotting. Protein concentrations
of the supernatant were determined by modified Lowry assay (Bio-Rad Laboratories, Hercules, CA, USA). For each sample, 10-30 μg of total tissue protein was separated in 7.5-13% sodium dodecyl sulphate polyacrylamide gel and transferred onto nitrocellulose membranes. The non-specific binding was blocked with 5% non-fat milk or 3% bovine serum albumin in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature with gentle shaking. After washing in TBS-T, the blots were incubated with either primary mouse monoclonal antibody of nitrotyrosine (1:500, Milipores), p47phox (1:500, Santa Cruz), NOX-2 (1:1000, Abcam, UK), p67phox (1:500, Santa Cruz) or Rac1 (1:1000, Abcam, UK) overnight at 4 °C. The next day, the membranes were washed three times for 5 min in TBS-T and incubated with respective secondary antibodies conjugated to horseradish peroxidase for 2 h at room temperature. The membranes were developed with Amersham™ ECL plus Western Blotting detection system (Amersham, Buckinghamshire, UK). The membrane image was captured under the ChemiDoc-It® Imaging system (UVP, Cambridge, UK) and densitometric analysis was performed using Quantity One 1-D analysis software. The respective protein expression levels were normalized to housekeeping protein α-actin or GAPDH and data are expressed as a percentage of the values in the control group.

5.3.13 Data analysis

Results represent mean ± SEM from n rats. Concentration–relaxation curves were analysed by non-linear regression curve fitting using GraphPad Prism 4.0 software (San Diego, CA). Statistical significance were determined by one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test for multiple value comparison. A value of P < 0.05 was considered statistically significant.
5.4. Results

5.4.1 Boldine reduces high glucose-stimulated ROS formation

Incubation with high glucose (30 mM, HG) for 48 h markedly increased the ROS formation in cultured rat aortic endothelial cells (RAEC) as reflected by the intensity of DCF fluorescence staining when compared with mannitol control (NG). Co-treatment with boldine (1 μM) or tempol (100 μM) reduced the high glucose-stimulated ROS rise (Figure 5.1A and 5.1B). Western blotting results revealed that treatment with either boldine or tempol inhibited the high glucose-stimulated up-regulation of NADPH subunits, NOX2 and p47phox (Figure 5.1C and 5.1D) without affecting the expression of other subunits p67phox and Rac1 (Appendix C) in RAEC.
Figure 5.1: Boldine reversed high glucose stimulated-ROS production in rat aortic endothelial cells (RAEC). Confluent RAEC was cultured in the medium containing normal glucose (NG) or high glucose (HG, 30 mM), or co-treated with either boldine (1 μM) or tempol (100 μM) for 48 h. (A) Representative images showing the ROS elevation as measured by CM-H₂DCF-DA fluorescence under various treatments. (B) Summarized value of results presented in A. The elevated expression of NOX2 (C) and p47\textsuperscript{phox} (D) was reduced by co-treatment of boldine or tempol. Results are mean ± SEM of 3 experiments using RAECs from different rats. NG: normal glucose (5 mM glucose + 25 mM mannitol as osmotic control). * P < 0.05, ** P < 0.01 compared with HG, # P < 0.01, ## P < 0.001 compared with NG.
5.4.2 Boldine attenuates high glucose-induced increases in nitrotyrosine formation

High glucose stimulation (30 mM, 48 h) elevated formation of nitrotyrosine (an index for increased oxidative stress) in RAEC compared with normal glucose-treated endothelial cells; such increase was inhibited by treatment with boldine or tempol (Figure 5.2A and 5.2B). Western blotting showed that treatment with boldine and tempol reversed the high glucose-induced up-regulation of nitrotyrosine protein expression in RAEC (Figure 5.2C).

5.4.3 Boldine increases NO bioavailability under high glucose stimulation

The stimulated NO production in RAEC in response to ACh as revealed by the time-dependent increase in the DAF fluorescence intensity was significantly lower after high glucose stimulation compared with normal glucose (Figure 5.3A and 5.3B). The effect of high glucose was reversed by co-treatment with boldine or tempol in RAEC (Figure 5.3).
Figure 5.2: Boldine reduced high glucose-stimulated nitrotyrosine formation in RAEC. (A) Representative images and summarized results (B) showing the increased nitrotyrosine formation in high glucose-treated RAEC was reversed by the co-treatment with boldine. (C) Western blot showing that the co-treatment with boldine normalized the increased nitrotyrosine formation in high glucose-treated RAEC. Results are mean ± SEM of 3 experiments using RAECs from different rats. * P < 0.05, ** P < 0.01 compared with HG, # P < 0.05, ## P < 0.01 compared with NG.
Figure 5.3: Boldine reversed the diminished NO productions in high glucose-treated RAEC. (A) Representative images showing the basal level (0 min) and ACh (10 μM)-stimulated NO productions at 15 min in the four treatment groups. (B) Boldine restored the NO production in high glucose-treated RAEC. Results are mean ± SEM of 3 experiments using RAEC from different rats. * P < 0.01 compared with NG, # P < 0.01 compared with HG.
5.4.4 Body weight, plasma blood glucose and lipid profile

At the end of treatment, body weight decreased significantly in STZ-induced diabetic rats compared to control rats. The plasma glucose level of diabetic rats was significantly greater than that of control rats. In diabetic rats, there was a slight decrease in blood glucose level after boldine treatment for 7 days (Table 5.1). STZ-treated rats exhibited a significantly higher plasma triglyceride level compared with control and this level was unaffected after 7-day treatment with boldine, while total plasma cholesterol, HDL and non-HDL levels were similar in all groups of rats (Table 5.2).

Table 5.1: Effect of boldine treatment on mean body weight and blood glucose level

<table>
<thead>
<tr>
<th>Strain</th>
<th>Body weight (g)</th>
<th>Blood glucose (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before treatment</td>
<td>After treatment</td>
</tr>
<tr>
<td>Control</td>
<td>231.1 ± 7.8</td>
<td>372.2 ± 13.9</td>
</tr>
<tr>
<td>Control + boldine</td>
<td>230.0 ± 7.1</td>
<td>376.7 ± 10.0</td>
</tr>
<tr>
<td>Diabetes</td>
<td>228.6 ± 6.9</td>
<td>157.1 ± 7.6</td>
</tr>
<tr>
<td>Diabetes + boldine</td>
<td>238.6 ± 6.9</td>
<td>179.4 ± 12.0</td>
</tr>
</tbody>
</table>

Sprague Drawly rats and STZ-induced diabetic rats were treated with boldine (20 mg/kg/daily, i.p.). Results are mean ± SEM of 7-9 animals. * P < 0.001 compared with control, # P < 0.05 compared with diabetes.
Table 5.2: Effect of boldine on lipid profiles

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + boldine</th>
<th>Diabetes</th>
<th>Diabetes + boldine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/ml)</td>
<td>0.60 ± 0.09</td>
<td>0.58 ± 0.03</td>
<td>0.71 ± 0.04</td>
<td>0.72 ± 0.07</td>
</tr>
<tr>
<td>Triglyceride (mg/ml)</td>
<td>0.92 ± 0.02</td>
<td>0.85 ± 0.07</td>
<td>1.94 ± 0.28 *</td>
<td>1.65 ± 0.32 *</td>
</tr>
<tr>
<td>HDL (mg/ml)</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.10 ± 0.01</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>non-HDL (mg/ml)</td>
<td>0.47 ± 0.05</td>
<td>0.52 ± 0.03</td>
<td>0.61 ± 0.04</td>
<td>0.51 ± 0.07</td>
</tr>
</tbody>
</table>

Effect of chronic boldine treatment (20 mg/kg/daily, i.p.) on levels of total cholesterol, triglyceride, HDL and non-HDL in vehicle- or boldine-treated Sprague Dawley (control) rats and STZ-induced diabetic rats. Results are mean ± SEM of 6 rats. * P < 0.05 compared with control.
5.4.5 Boldine reverses β-NADPH-induced impairment of endothelium-dependent relaxations in rat aortas

Pre-incubation of aortic rings with β-NADPH attenuated the acetylcholine-induced endothelium-dependent relaxations. Boldine concentration-dependently (0.01- 1 µM) rescued the impaired relaxations (Figure 5.4A and 5.4B). The vascular oxidative stress induced by β-NADPH was confirmed with the addition of SOD, a superoxide scavenger in functional study. Pre-treatment with SOD prevented the β-NADPH-induced impairment of relaxations (Figure 5.4C). By contrast, endothelium-independent relaxations to SNP were comparable in all treatment groups and this suggests the relaxing sensitivity of vascular smooth muscle cells to NO remains unchanged (Figure 5.4D).
Figure 5.4: Boldine reversed the β-NADPH-induced endothelial dysfunction in rat aortas. (A) Representative traces showing the β-NADPH-induced impairment of ACh-evoked endothelium-dependent relaxation was improved by acute 30-min treatment with boldine which exhibited a concentration-dependent benefit (B). (C) SOD (50 U/ml) produced the same vascular protection as boldine (1 µM) in improving ACh-induced relaxations. (D) SNP-induced endothelium-independent relaxations were similar in all treatment groups. Results are mean ± SEM of experiments from 6 different rats. *P<0.01, **P<0.001 compared to β-NADPH, # P<0.001 compared with control.
5.4.6 Boldine improves endothelium-dependent relaxation in diabetic rat aortas

Endothelium-dependent relaxations to ACh were significantly less in diabetic than in normal rat aortas (Figure 5.5A). Acute 30 min treatment with boldine (1 µM) restored the relaxations to the level seen in non-diabetic rat aortas (Figure 5.5A). Again neither STZ treatment nor boldine treatment modulated SNP-induced endothelium-independent relaxations (Figure 5.5D). The acute beneficial effect of boldine can be confirmed by chronic boldine treatment. STZ-induced diabetic rats were administered with boldine (20 mg/kg, i.p) for 7 days and aortas were then harvested for functional and biochemical assays. Chronic treatment with boldine significantly improved ACh-induced relaxations in diabetic rat aortas (Figure 5.5C) without affecting endothelium-independent relaxations to SNP (Appendix D). The improvement of endothelial function in boldine-treated rats was accompanied by the restoration of the lost plasma nitrates/nitrites level in diabetic rats (Figure 5.5D).
Figure 5.5: Acute and chronic boldine treatment benefited endothelial function in STZ-treated diabetic rats. Acute 30-min treatment of boldine (1 µM) restored ACh-induced endothelium-dependent relaxations (A) but not SNP-induced endothelium-independent relaxations (B) in diabetic rat aortas. Chronic treatment with boldine improved ACh-induced aortic relaxations (C) and the total plasma nitrates/nitrites (D) in STZ-induced diabetic rats. Results are mean ± SEM of experiments from 6-7 different rats. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with diabetes, # P < 0.01, ## P < 0.001 compared with control.
5.4.7 Boldine reduces *in situ* vascular superoxide formation and inhibits the elevated expression of p47\textsuperscript{phox} in diabetic rat aortas

The intracellular superoxide formation measured by DHE fluorescence was higher in aortic rings from diabetic rats compared with control rats. Seven-day treatment of diabetic rats with boldine reduced the ROS accumulation in the vascular wall of diabetic rat aortas (Figure 5.6A and 5.6B). Boldine attenuated the increased level of the superoxide-generating p47\textsuperscript{phox} in the diabetic rat aorta (Figure 5.6C).

5.4.8 Boldine reduces vascular superoxide and peroxynitrite in diabetic rat aortas

Production of O$_2^\cdot$ and ONOO$^-$ in the diabetic rat aortas was significantly greater than controls (Figure 5.7). This increased superoxide production was abolished by DPI, a NADPH oxidase inhibitor. Uric acid, a direct ONOO$^-$ scavenger reduced the ONOO$^-$ level in all groups. Chronic treatment with boldine attenuated the excess aortic generation of O$_2^\cdot$ and ONOO$^-$ in the diabetic rat aortas (Figure 5.7).
Figure 5.6: Chronic boldine treatment reduced the oxidative stress level in diabetic rat aortas. The elevated DHE fluorescence intensity (A and B) and p47<sub>phox</sub> level (C) in STZ-induced diabetic rat aortas were markedly reduced by administration with boldine. Results are mean ± SEM of 3-4 separate experiments. * P < 0.05, ** P < 0.01 compared with diabetes, # P < 0.001 compared with control.
Figure 5.7: Chronic treatment with boldine reduced the production of superoxide anion, $\text{O}_2^-$ (A) and peroxynitrite, ONOO$^-$ (B) in aortic rings from STZ-induced diabetic rats. The NADPH oxidase inhibitor diphenyleneiodonium (DPI, 10 μM) and the ONOO$^-$ scavenger uric acid (250 mM) abolished the generation of $\text{O}_2^-$ and ONOO$^-$, respectively. Results are mean ± SEM of 6 separate experiments. * P < 0.001 compared with diabetes, # P < 0.001 compared with control.
5.5. Discussion

The major findings of the present study include that (i) treatment with boldine effectively reduces high glucose-stimulated ROS and nitrotyrosine formation in rat aortic endothelial cells; (ii) acute treatment with boldine reverses the impaired endothelium-dependent aortic relaxations induced by β-NAPDH and in STZ-induced diabetic rats; and (iii) the in vitro effect of boldine is confirmed by in vivo treatment as chronic boldine administration improves endothelium-dependent relaxations in aortas from STZ-treated rats. This endothelial cell protection appears to correlate with the increase of NO bioavailability following boldine treatment. Collectively, the present study shows for the first time that boldine treatment attenuates the diabetes-associated oxidative stress and endothelial dysfunction through restoring the NO bioavailability.

It has previously been shown in the STZ-induced diabetic rats, 8-week treatment with boldine decreased mitochondrial malondialdehyde and carbonyls in the liver, kidney and pancreas, and normalized the elevated Mn-SOD and GSH-peroxidase activity in mitochondria of the pancreas (Jang et al., 2000). We have also showed that chronic treatment with boldine restored endothelial function in hypertensive rats through the inhibition of NAD(P)H oxidase (Lau et al., 2012a). However, there is little information concerning the vasoprotective effect of boldine in metabolic stress-related cardiovascular diseases, in particular endothelial dysfunction in diabetes. The present study provides novel evidence that boldine treatment is effective in reducing oxidative stress and thus restoring endothelial function in STZ-induced diabetic rats.

We first examined the effect of boldine against high glucose-induced ROS over-production in tissue culture. High glucose exposure markedly increases ROS generation in cultured rat aortic endothelial cells (RAEC) which is reversed by boldine at a low
concentration of 1 µM but high glucose did not affect the glutathione antioxidant system in RAEC (Appendix E). High glucose-stimulated ROS elevation is causally associated with increased expression of NADPH oxidase subunits including NOX2 and p47phox, which is also reduced by boldine. Superoxide anion reacts with NO to form ONOO− and the latter further lowers bioavailability of NO (Maritim et al., 2003). We confirmed the contributory role of ONOO− by detecting an elevated level of nitrotyrosine in high glucose-treated RAEC resulting from tyrosine nitration mediated by ONOO− radicals. The increased O2− may explain a low NO level in RAEC exposed to high glucose. Co-treatment with boldine or tempol, equi-effectively reduces the elevated nitrotyrosine formation and increases the NO formation in endothelial cells. It is possible that ROS-inhibiting effect of boldine or tempol is sufficient to restore the diminished NO production in high glucose-treated endothelial cells without affecting the eNOS expression (Appendix F), thus improving endothelial function.

ROS derived from NAD(P)H oxidase plays a key role in vascular endothelial dysfunction in diabetes (Wong et al., 2010c). In this study acute exposure to boldine effectively reversed the β-NAPDH-induced impairment of endothelium-dependent relaxations, thus indicating that the anti-oxidative (reduction of ROS) activity of boldine helps to preserve the bioavailability of NO. This finding is further supported by the improvement of endothelial function observed in isolated diabetic aortas that has been given acute and chronic treatment of boldine. The present study, for the first time, reveals the mechanism of vasoprotection of boldine in diabetic aortas is mediated through reducing ROS and increasing NO bioavailability. Further evidence is provided by the findings that 1) elevated O2 production in aortas of STZ-induced diabetic rats is reversed by chronic boldine treatment and 2) boldine suppressed the upregulated NOX2 and p47phox in the STZ-treated rat aortas and augmented the plasma levels of NO.
metabolites. In contrast to the changes in endothelial function, the sensitivity of vascular smooth muscle to NO remained unaltered as SNP-induced endothelium-independent relaxation is comparable in all treatment groups (Figure 4D and 5B). The present results are in line with earlier reports of overexpression of p47\textsuperscript{phox} in high glucose-stimulated ROS generation in human coronary artery endothelial cells, vascular smooth muscle cells, and in diabetic rat arteries (Liu et al., 2007; Serizawa et al., 2011; Zheng et al., 2010). Taken together, the present study demonstrates a vascular protective effect of boldine under hyperglycaemic conditions and the concomitant diabetic vasculopathy.

Boldine has been shown to block α\textsubscript{1}-adrenoceptors in arteries from rats (Ivorra et al., 1993a) and guinea pigs (Chulia et al., 1996). This α\textsubscript{1}-adrenoceptor blocking activity is unrelated to the endothelial cell protection conferred by boldine based on our observation that boldine at 1 µM used in the present study did not inhibit contraction triggered by phenylephrine, an α\textsubscript{1}-adrenoceptor agonist (Appendix G) and this study used another contractile agent, serotonin instead of phenylephrine. In addition, the vascular benefits of chronic treatment with boldine is unlikely to be associated with favourable modulation of metabolic parameters as boldine only slightly reduces plasma glucose levels (P < 0.05) without affecting lipid profile in diabetic rats.

ROS has been implicated in the pathogenesis of β-cell destruction and liver injury in diabetes (Kakkar et al., 1998). Streptozotocin causes DNA fragmentation of pancreatic β-cell by stimulation of ROS generation in vitro and in vivo (Takasu et al., 1991). Jang et al (2000) reported that the plasma glucose lowering effect of boldine was associated with its cytoprotective action on pancreatic β-cell and the prevention of peroxidation products formation. Boldine also attenuates the STZ-induced MDA formation, carbonyl formation and thio oxidation in the pancreas homogenates (Jang et al., 2000).
The slight glucose lowering effect by boldine observed in this study may be related to its cytoprotective effect against the oxidative damage in pancreatic β-cell as reported by Jang et al (2000) although other contributing factors cannot be ruled out.

5.6 Conclusion

In summary, both *in vitro* (acute) and *in vivo* (chronic) treatments with boldine augment endothelial function through restoring the NO bioavailability in diabetic rats, thus indicating that boldine could be a potentially effective herb-derived ingredient in inhibiting oxidative stress and thus preserving endothelial function in hyperglycaemic or diabetic conditions.
CHAPTER VI

BOLDINE IMPROVES ENDOTHELIAL FUNCTION IN DIABETIC DB/DB MICE THROUGH INHIBITION OF ANGIOTENSINII-MEDIATED BMP4 OXIDATIVE STRESS CASCADE

6.1 Abstract

Boldine is a potent natural antioxidant present in the leaves and bark of Chilean boldo tree. The present study investigated the endothelial protective effect of boldine in arteries of db/db diabetic mice and in cultured mouse aortic endothelial cells receiving high glucose treatment. Vascular reactivity was studied in mouse aortas. Reactive oxygen species (ROS) production, angiotensin type 1 receptor (AT1R) localization and protein expression of oxidative stress markers in the vascular wall were evaluated by DHE fluorescence, lucigenin enhanced-chemiluminescence, immunohistochemistry and Western blot, respectively. The effect of boldine was also examined in high glucose (30 mM)-treated primary mouse aortic endothelial cells. Both oral treatment (20 mg/ kg/day, 7 days) and incubation in vitro with boldine (1 μM, 12 h) enhanced endothelium-dependent aortic relaxations of db/db mice. Boldine reversed the impaired relaxations induced by high glucose or angiotensin II (Ang II) in non-diabetic mouse aortas while it reduced the ROS overproduction and increased the phosphorylation of eNOS in db/db mouse aortas. The elevated expression of oxidative stress markers such bone morphogenic protein 4 (BMP4), nitrotyrosine and AT1R was reduced in boldine-treated db/db mouse aortas. The Ang II-stimulated BMP4 expression was also inhibited by treatment with boldine, tempol, noggin and losartan. Boldine inhibited high glucose-stimulated ROS production and restored the lost phosphorylation of eNOS in mouse aortic endothelial cells. Boldine is effective to reduce oxidative stress and thus to improve endothelium-dependent relaxations in aortas of diabetic mice largely through
inhibiting ROS over-production associated with Ang II-mediated BMP4-dependent mechanisms.

6.2 Introduction

Type 2 diabetes, a common metabolic disorder, is characterized by hyperglycemia and hyperinsulinaemia which impair functions of both macro- and micro-circulation, and thus increases risks for developing hypertension and atherosclerosis (Senador et al., 2009; Tranche et al., 2005). Excessive oxidative stress or increased production of reactive oxygen species (ROS) damage endothelial function as an early pathological event leading to cardiovascular diseases (Heitzer et al., 2001). For example, increased formation of NADPH oxidase-dependent superoxide anion has been observed in diabetic animals including db/db mice, diet-induced obese mice, Otsuka Long-Evans Tokishima Fatty (OLETF) rats, and Goto-Kakizaki (GK) rats (Gupte et al., 2010; Kim et al., 2002; Tian et al., 2011). Activation of angiotensin II (Ang II) type 1 receptor (AT1R) plays a critical role in mediating endothelial dysfunction through AT1R-dependent NADPH-derived ROS over-production in arteries of db/db diabetic mice (Tian et al., 2011), while Ang II is a potent vasoconstrictor with pro-inflammatory, mitogenic and profibrotic properties.

Bone morphogenetic protein (BMP), a member of transforming growth factor-β superfamily, activates Smads as the immediate downstream molecules upon binding to BMP receptors (Chen et al., 2004). The isoforms of BMP family include BMP2, BMP4 and BMP7 which are up-regulated in diabetes and act as pro-inflammatory regulators in blood vessels (Bostrom et al., 2011; Nett et al., 2006). BMP4 impairs endothelial function in mouse aortas either by increased ROS formation through NADPH oxidase or up-regulation of cyclooxygenase-2 (Miriyala et al., 2006; Wong et al., 2010a). An
elevated expression of BMP4 and NAPDH oxidase in db/db mice suggests a positive involvement of this redox-sensitive pro-inflammatory mechanism in diabetes (San Martin et al., 2007). Therefore, natural products which improve endothelial function in diabetes by favorable modulation of redox-sensitive mechanisms could be potentially useful for treating diabetic vasculopathy.

Boldine ((S)-2,9-dihydroxy-1,10-dimethoxy-aporphine) is an aporphine alkaloid derived from benzylisoquinoline family and found to be the major alkaloid in the leaves and bark of Chilean boldo tree (Peumus boldus Molina) (O’Brien et al., 2006). Boldine possesses a potent anti-oxidative property (Cassels et al., 1995). Although the pharmacological effects of boldine were reported a decade ago, it remains to be elucidated whether its antioxidant activity benefits vascular function in type 2 diabetic mouse model. Therefore, the present study investigated the hypothesis that in vivo and in vitro treatment with boldine ameliorates endothelial dysfunction in diabetic db/db mice by inhibiting Ang II-mediated BMP4-dependent oxidative stress cascade.

6.3 Material and methods

6.3.1 Chemicals
Acetylcholine chloride (ACh), sodium nitroprusside (SNP), boldine, N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), phenylephrine, angiotension II (Ang II) were purchased from Sigma (St. Louis, MO, USA). Tempol was purchased from Tocris (Avonmouth, UK). Noggin and BMP4 were purchased from R&D System, Inc (Minneapolis, MN, USA). Losartan was purchased from Cayman (Ann Arbor, MI, USA). Noggin and BMP4 were dissolved in PBS plus 0.1% BSA and 4 mM HCl, respectively. Losartan was dissolved in DMSO. Boldine was dissolved in DMSO for in
vitro study or ethanol (20%) for oral feeding. Other drugs were dissolved in double distilled water.

6.3.2 Animals and experimental protocol

Male diabetic mice (C57BL/KSJ background) lacking the gene encoding for leptin receptor (db/db), heterozygote (db/m+) and non-diabetic C57 mice were purchased from the Laboratory Animal Service Center of Chinese University of Hong Kong (CUHK). The experimental procedures were approved by the CUHK Animal Experimentation Ethics Committee. Mice were maintained in a well-ventilated holding room at constant temperature of 24 ± 1°C and received normal chow and tap water ad libitum. The db/db mice (16-17 weeks old) were randomly assigned to control (vehicle), boldine, and tempol treatment group, and they were treated daily with vehicle (20% ethanol, 0.8 ml/kg), or boldine (20 mg/kg/day) or tempol (20 mg/kg/day) by oral administration for 7 days. At the end of the treatment period, mice were sacrificed by CO₂ inhalation.

6.3.3 Artery preparation

The thoracic aorta was isolated, cleaned of surrounding connective tissues, and cut into several ring segments, 2 mm each in length. Rings were suspended in a Multi Wire Myograph System (Danish Myo Technology, Aarhus, Denmark) and bathed in oxygenated Krebs solution containing (in mM) NaCl 119, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.2, MgSO₄.7H₂O 1.2, glucose 11.7, and CaCl₂.2H₂O 2.5. Some arteries were snap-frozen in liquid nitrogen and stored in -80 °C for later processing. All rings were maintained at 37 °C, stretched to an optimal baseline tension of 3 mN, and continuously oxygenated by 95% O₂ and 5% CO₂. The changes of isometric tension were recorded by a PowerLab LabChart 6.0 recording system (AD Instruments, Australia).
6.3.4 Experimental protocol

After 30 min equilibration, rings were first contracted by 60 mM KCl and washed in Krebs solution three times before phenylephrine (1 μM) was added to induce a stable contraction. Concentration-response curves for both endothelium-dependent relaxations in response to acetylcholine (ACh, 3 nM to 10 μM) and endothelium-independent relaxations to sodium nitroprusside (SNP, 1 nM to 10 μM) were obtained.

6.3.5 Detection of ROS formation in en face endothelium and cryostat section of mouse aortas

The oxidative stress level in en face endothelium and cryostat section of mouse aortas was assessed by confocal microscopy using dihydroethidium (DHE) dye. The aortic segments and cryostat sections (5 μm) of mouse aortas were pre-incubated in DHE (5 μM, invitrogen, CA, USA) for 15 min in normal physiological saline solution (NPSS: NaCl 140, KCl 5, CaCl₂ 1, MgCl₂ 1, glucose 10 and HEPES 5 mM) (Tian et al., 2012a). At the end of incubation period, the aortic DHE dye was washed away and the fluorescence intensity at one optical section of the rings was visualized using Olympus FV1000 laser scanning confocal system (Olympus, America Inc., Melville, NY, USA). The fluorescence intensity was measured at 515 nm excitation and 585 nm emission and the images were analyzed using Olympus Fluoview software (version 2.0).

6.3.6 Detection of vascular superoxide formation

The amount of superoxide anion formation was determined using lucigenin-enhanced chemiluminescence method (Lau et al., 2013). Briefly, isolated mouse aortic rings were pre-incubated for 45 min at 37 °C in 2 ml of Krebs–HEPES buffer (in mM: NaCl 99.0, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.0, MgSO₄·7H₂O 1.2, glucose 11.0, CaCl₂·2H₂O 2.5 and Na-HEPES 20.0) in the presence of diethylthiocarbamic acid (DETCA, 1 mM) to
inactivate superoxide dismutase (SOD) and β-nicotinamide adenine dinucleotide phosphate as NADPH oxidase substrate (0.1 mM). The rings were then transferred into vials containing 300 ul of Krebs-HEPES buffer containing 10 µM lucigenin. Repeated measurements were made over 10 min in 1 min intervals using luminometer (GloMax® 20/20 Luminometer, WI, USA). The data were expressed as average counts per mg of tissue dry weight.

6.3.7 Detection of AT$_1$R by immunohistochemical staining

The localization of AT$_1$R in mouse aortas was determined by immunohistochemistry (Wong et al., 2010a). Briefly, aortic rings were fixed in 4% paraformaldehyde at 4 °C overnight and preceded to dehydration and embedding in paraffin on the following day. The paraffin block was cut into 5-µm thick sections on microtome (Leica Microsystems, Germany), followed by re-hydration. Sections were then treated with 1.4% H$_2$O$_2$ in absolute methanol for 30 min at room temperature to block the activity of endogenous peroxidase. To avoid false negative staining, sections were boiled in 0.01 M sodium citrate buffer (pH 6) for 15 min to unmask antigenic sites in the specimens. After washes in PBS, sections were blocked with 5% donkey serum (Jackson Immunoresearch, PA, USA) for 1 h and incubated with primary mouse monoclonal antibody AT$_1$R (1: 50, Abcam, Cambridge, UK) overnight in a humidified chamber at 4 °C. At the end of incubation period, sections were incubated with biotin-SP conjugated goat anti-mouse secondary antibodies (1:200, Jackson Immunoresearch, PA, USA) for 1 h at room temperature and then for 30 min with streptavidin-HRP conjugate (1:200, Zymed laboratory, CA, USA). Sections were washed in PBS for three times and colors were developed using 3,3’-diamonobenzidine (DAB) peroxidase substrate kit (Vector laboratory, CA, USA). The nuclei were counterstained with haematoxylin and the section without primary antibody served as negative control. Images were captured
using Leica DMRBE microscope coupled to SPOT-RT cooled CCD color digital camera and SPOT Advanced software (Version 3.5.5, Diagnostic Instruments, MI, USA).

6.3.8 Organ culture of isolated aortas

The isolated aortic rings were cultured in Dulbecco’s Modified Eagle’s Media (DMEM, Gibco, Gaithersberg, MD) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin. Aortas from db/db mice were incubated for 12 h in the presence or absence of boldine (1 μM in 0.001% DMSO), tempol (SOD mimetic, 100 μM), noggin (BMP4 antagonist, 100 ng/ml) or losartan (AT₁R blocker, 3 μM). Aortic rings from C57 mice were incubated in normal glucose (NG, 5 mM glucose and 25 mM mannitol as osmotic control of high glucose), high glucose (30 mM) and co-treatment with either boldine (1 μM) or tempol (100 μM) for 36 h in a 5% CO₂ incubator at 37 °C. In another set of experiments, rings were treated with Ang II (0.5 μM) for 24 h and thereafter transferred to wire myographs for functional examinations.

6.3.9 Primary culture of mouse aortic endothelial cells

Primary mouse aortic endothelial cells (MAECs) were isolated from two male mice (C57BL/6J at age of 5-6 weeks) (Tian et al., 2012a). In brief, the aorta was isolated from C57 mice after single intraperitoneal injection of pentobarbital sodium (40 mg/kg) and perfusion of heparin (100 U/ml) to the circulation from the left ventricle. The aortas were dissected in sterile ice-cold PBS to remove adipose and connective tissues, incubated for 10 min in collagenase type 1A (Sigma, MO, USA) solution at 37 °C with gentle shaking. Detached endothelial cells were centrifuged at 1500 rpm for 10 min and the cell pellets were re-suspended and cultured in endothelial cell growth medium.
(EGM, Gibco, invitrogen, CA, USA) containing 20% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in addition of endothelial cell growth supplement (50 μg/ml, BD Transduction Laboratory, CA, USA). The cells were kept in a humidified atmosphere containing 5% CO₂ at 37 °C for 45 min and culture medium was then replaced and allowed to grow into confluence. Cells from passages between 1 and 3 were used for the present study. The endothelial cells were verified by positive staining to PECAM-1 (Santa Cruz, CA, USA) and negative staining to smooth muscle marker, β-actin (Dako, Denmark).

6.3.10 Measurement of intracellular ROS formation in MAECs

The intracellular ROS production in MAECs was measured by a fluorogenic probe, CM-H₂DCFDA and the fluorimetric signal was captured on an Olympus FV1000 laser scanning confocal system (Olympus, America Inc., Melville, NY, USA). Briefly, the confluent MAECs were seeded on circular cover slip and incubated in a low serum medium (EGM with 2% FBS) for 4 h. The cells were then exposed to high glucose (30 mM) for 36 h with or without co-treatment with boldine (1 μM) or tempol (100 μM). At the end of treatment, cells were washed twice in NPSS and preloaded with CM-H₂DCFDA (1 μM) for 20 min at 37 °C before the fluorescence signal was measured at 488 nm excitation and 520 nm emission.

6.3.10 Western blotting

Aortas and MAECs were homogenized in ice-cold 1X RIPA buffer containing leupeptin 1 μg/ml, aprotonin 5 μg/ml, PMSF 100 μg/ml, sodium orthovanadate 1 mM, EGTA 1 mM, EDTA 1 mM, NaF 1 mM, and β-glycerolphosphate 2 mg/ml. The lysates were centrifuged at 20,000 g for 20 min and supernatant was collected for Western blotting. Protein concentrations of the supernatant were determined by modified Lowry assay
(Bio-Rad Laboratories, Hercules, CA, USA). A 15 μg of protein loaded in each lane was separated on 7.5% or 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel and then transferred to an immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) at 100 V. The blots were blocked for non-specific binding with 2% bovine serum albumin (BSA) or 5% non-fat milk in Tris-buffered saline containing 0.1 % Tween 20 (TBS) for 1 h at room temperature with gentle shaking. After it was rinsed in TBS-T, the blots were incubated with either primary polyclonal anti-eNOS at Ser1177 (p-eNOS^{Ser1177}) (1:500, Cell Signaling Technology, MA, USA), monoclonal anti-eNOS (1:500, BD Transduction laboratory), anti-nitrotyrosine (1:1000, Milipore, MA, USA ), anti-AT_{1}R (1:1000, Abcam, Cambridge, UK), anti-BMP4 (1:500, Sigma, MO, USA). After overnight incubation at 4 °C, the membranes were washed three times and incubated with respective secondary antibodies conjugated to horseradish peroxidase for 2 h at room temperature. The membranes were developed with Amersham™ ECL plus Western blotting detection system (Amersham, Bukinghamshire, UK) and images were captured under ChemiDoc-It® Imaging system (UVP, Cambridge, UK). The densitometric analysis was performed using VisionWorks®LS analysis software and the respective protein expression levels were normalized to housekeeping protein β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

6.3.11 Statistical analysis

Results represent mean ± standard error of mean (SEM) for number (n) of mice. Concentration-response curves were fitted to a sigmoidal curve using non-linear regression with the aid of the statistical software GraphPad Prism version 4, USA. The observed responses were analyzed for statistical significance using Student’s t-test for unpaired observations and the one-way analysis of variance (ANOVA) followed by
Bonferroni’s multiple comparison test for multiple value comparison (Prism 5.0, GraphPad Software, USA). A value of P < 0.05 was taken statistically significant.

6.4 Results

6.4.1 Boldine improves endothelial function in diabetic db/db mice

Figure 1 shows the impaired endothelium-dependent relaxations to ACh (Figure 6.1A) but not endothelium-independent relaxations to SNP (Figure 6.1B) in aortic rings from db/db mice compared with those from db/m+ mice. One-week oral administration of boldine (20 mg/kg/day) or tempol (20 mg/kg/day) to db/db mice rescued the impaired ACh-induced relaxations (Figure 6.1A & Table 6.1) without affecting SNP-induced responses in aortic rings (Figure 6.1B). Such treatment did not modulate plasma lipid profile or glucose levels (Appendix H & I). In addition, treatment with either boldine or tempol restored the lost phosphorylation of eNOS at Ser1177 in db/db mouse aortas (Figure 6.1C). Likewise, 12 h in vitro treatment with boldine (1 μM) or tempol (100 μM) also rescued the impaired ACh-induced relaxations in db/db mouse aortas (Figure 6.2A & B) without affecting SNP-induced relaxations (Figure 6.2D). The impaired relaxations were also reversed by treatment with AT1R antagonist losartan (3 μM) and bone morphogenic protein-4 (BMP4) antagonist, noggin (100 ng/ml) (Figure 6.2C).
Table 6.1: The agonist sensitivity (pEC$_{50}$) and percentage of maximum response ($R_{\text{max}}$) of ACh-induced endothelium-dependent relaxations in aortic rings isolated from $db/m^+$ and $db/db$

<table>
<thead>
<tr>
<th>Group</th>
<th>pEC$_{50}$ (log M)</th>
<th>$R_{\text{max}}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$db/m^+$</td>
<td>6.60 ± 0.06</td>
<td>82.42 ± 2.03</td>
</tr>
<tr>
<td>$db/db$</td>
<td>4.35 ± 0.47 #</td>
<td>29.00 ± 6.25 #</td>
</tr>
<tr>
<td>$db/db$ + boldine</td>
<td>6.28 ± 0.12 *</td>
<td>71.00 ± 7.65 *</td>
</tr>
<tr>
<td>$db/db$ + tempol</td>
<td>6.81 ± 0.15 *</td>
<td>70.00 ± 8.66 *</td>
</tr>
</tbody>
</table>

Results are means ± SEM of 6 separate experiments. # P< 0.01 compared to $db/m^+$
* P<0.01 compared to $db/db$
Figure 6.1: The ACh-induced endothelium-dependent (A) and SNP-induced endothelium-independent relaxations (B) in aortic rings from db/db mice orally treated with vehicle (20% EtOH), or boldine (20 mg/kg/day), or tempol (20 mg/kg/day). (C) Chronic boldine treatment increased the level of eNOS phosphorylation at Ser1177 in aortas of db/db mice. Results are shown as mean ± SEM of 6 separate experiments. # P <0.05 compared to db/m+ mice; * P <0.05 compared to db/db mice.
Figure 6.2: In vitro exposure to boldine (1 μM) or tempol (100 μM) for 12 h prevents impaired ACh-induced endothelium-dependent relaxations in db/db mouse aortas (A, B); whilst, SNP-induced endothelium-independent relaxations were unaffected in all group (D). Treatment (12 h) with BMP4 antagonist noggin (100 ng/ml) and AT1R antagonist losartan (3 μM) improved endothelial function in aortas of db/db mice (C). Results are means ± SEM of 6 separate experiments. # P <0.05 compared to db/m+ mice; * P <0.05 compared to db/db mice.
6.4.2 Boldine reduces vascular oxidative stress markers in db/db mice

One-week treatment with boldine or tempol normalized the elevated ROS accumulation in *en face* endothelium (Figure 6.3A), across the vascular wall (Figure 6.3B), and superoxide anion levels (Fig. 6.3C) in aortas from *db/db* mice as reflected by DHE fluorescence dye and using lucigenin enhanced-chemiluminescence method. The Western blot results show that the elevated protein expression of BMP4 (Figure 6.4A) and nitrotyrosine (another oxidative stress index, Figure 6.4B) in *db/db* mouse aortas was reversed by chronic treatment with boldine or tempol.

6.4.3 Boldine reduces the expression of AT$_1$R in db/db mouse aortas

Immunohistochemistry staining showed the presence of AT$_1$R in both endothelial cells and smooth muscle cells in mouse aortas and chronic treatment with boldine or tempol reduced the expression of AT$_1$R in the aortas in *db/db* mice (Figure 6.5A). Such effects were confirmed by the Western blot data (Figure 6.5B).
Figure 6.3: Chronic boldine treatment reversed the elevated ROS in en face endothelium (A) and cryostat section of db/db mouse aortas (B) as indicated by changes of the DHE fluorescence intensity and inhibited the increased generation of superoxide anion in these aortas (C) as detected using lucigenin-enhanced chemiluminescence method. Results are means ± SEM of 4-6 separate experiments. # P <0.05 compared with db/m+ mice; * P <0.05 compared with db/db mice.
Figure 6.4: Western blotting showing the up-regulation for expressions of BMP4 (A) and nitrotyrosine (B) in aortas from db/db mice with and without receiving one-week treatment with boldine or tempol. Results are means ± SEM of 4 separate experiments.

# P <0.05 compared with db/m+ mice; * P <0.05 compared with db/db mice.
Figure 6.5: One-week treatment with boldine or tempol reduced the AT$_1$R expression in $db/db$ mouse aortas as detected by immunohistochemistry (A) and Western blotting (B). Arrows indicate the endothelial layer of the artery. Results are shown as mean ± SEM of 3-4 separate experiments. # P < 0.05 compared with $db/m^+$ mice; * P < 0.05 compared with $db/db$ mice.
6.4.4 Boldine protects against Ang II-induced BMP4-dependent endothelial dysfunction

Ang II (500 nM) attenuated the ACh-induced relaxations in aortas from non-diabetic C57 mice (Figure 6.6A & B) and this impairment was reversed by co-treatment with BMP4 inhibitor noggin or AT1R antagonist losartan (Figure 6.6A). Ang II elevated the expression of BMP4 in cultured mouse aortas and this effect was reversed by noggin or losartan (Figure 6.6C). Like noggin or losartan, both boldine and tempol also reversed the impairment of ACh-induced relaxations (Figure 6.6B) and up-regulation of BMP4 expression in Ang II-treated aortas from non-diabetic mice (Figure 6.6D).
Figure 6.5: Ang II-induced impairment of ACh-induced relaxations was reversed by in vitro treatment with boldine (1 µM, A), tempol (100 µM, A), noggin (100 ng/ml, A) and losartan (3 µM, A) in aortas from non-diabetic mice. These four inhibitors normalized Ang II-induced increase in BMP4 expression (C & D). Results are means ± SEM of 4-6 separate experiments. #P <0.05 compared with control; *P <0.05 compared with Ang II.
6.4.5 Boldine reverses high glucose-induced endothelial dysfunction in mouse aortas

Exposure to high glucose (36 h) attenuated ACh-induced relaxations and this effect was reversed by co-treatment with boldine (1 μM) or tempol (100 μM) (Figure 6.7A & B). Treatment with high glucose for 36 h elevated the ROS production in MAECs as indicated by changes of the DCF-DA fluorescence intensity, while ROS elevation was prevented by pre-treatment (8 h, Appendix J) and co-treatment (Figure 6.7C & D) with boldine or tempol. In addition, high glucose-induced reduction in eNOS phosphorylation in MAECs was reversed by co-treatment with boldine or tempol (Figure 7E).
Figure 6.6: *In vitro* treatment with boldine or tempol reversed high glucose-induced impairment of ACh-induced relaxations in non-diabetic mouse aortas (A & B) and normalized the elevated ROS production in high glucose (HG)-treated MAECs (C & D). (E) Boldine and tempol increased the eNOS phosphorylation in HG-treated MAECs. NG: normal glucose (5 mM glucose and 25 mM mannitol as osmotic control of HG). Results are means ± SEM of 4-6 separate experiments. # P <0.05 compared with NG; *P < 0.05 compared with HG.
6.5 Discussions

The present study provide new experimental evidence showing that *in vivo* treatment with boldine can effectively restore the impaired endothelium-dependent relaxations in aortas of *db/db* mice and reduces oxidative stress as signified by marked reduction in the expression of BMP4, nitrotyrosine and AT₁R in diabetic mouse arteries. The renin-angiotensin system (RAS) and associated oxidative stress play a crucial role in maintaining endothelial dysfunction in diabetic mice (Wong *et al.*, 2010b) while BMP4 is a novel important mediator of endothelial dysfunction in hypertension (Tian *et al.*, 2012b). To further elucidate the possible inhibitory effect of boldine on Ang II-mediated vascular dysfunction, Ang II was used to trigger ROS generation and thus reduce the bioavailability of NO in the vascular wall. As expected, *in vitro* Ang II treatment impairs endothelium-dependent relaxations accompanied by the elevated BMP4 expression in aortas from non-diabetic mice. Co-treatment with boldine reverses the harmful effects of Ang II on relaxations and BMP up-regulation. It appears that boldine reduces Ang II-induced BMP expression mainly through limiting ROS generation in the inflamed arteries as the known ROS inhibitor tempol produces the same benefits as boldine. In addition, reducing the AT₁R expression and associated ROS over-production also play a positive role in boldine-induced improvement of endothelial function in diabetic *db/db* mice.

Boldine was described before to ameliorate the development of diabetes in streptozotocin-induced rats by inhibiting oxidative stress-associated tissue damage and restoring the antioxidant enzyme activities (Jang *et al.*, 2000a). We have recently described that treatment with boldine reverses endothelial dysfunction in hypertensive rats through suppression of NADPH oxidase-mediated ROS over-production (Lau *et al.*, 2012a) and protects endothelial function in hyperglycaemia-induced oxidative stress
through inhibiting NADPH oxidase expression (Lau et al., 2013). In addition, boldine was reported to reduce the carrageenan-induced guinea pig paw oedema probably through inhibiting the biosynthesis of pro-inflammatory prostaglandins (Backhouse et al., 1994). However, the effect of boldine on endothelial dysfunction and vascular inflammatory response in type 2 diabetes remains unclear. The present study reports novel findings on the endothelial cell protective effect of boldine involving inhibition of Ang II-mediated BMP4 up-regulation and ROS overproduction induced by hyperglycemia.

The RAS and associated NADPH oxidase-derived ROS mediate endothelial dysfunction in the mouse model of type 2 diabetes through decreasing NO bioavailability in the vascular wall (Wong et al., 2010b). The present study shows that the impaired endothelium-dependent relaxations in db/db mouse aortas or in high glucose-treated mouse aortas were reversed by both in vivo and in vitro treatment with boldine or tempol. This improved relaxations correlate with the restored level of eNOS phosphorylation. Additional support comes from experiments on cultured mouse endothelial cells in which the high glucose-induced reduction in the level of p-eNOS was reversed by boldine or tempol.

Bone morphogenetic protein 4, an important matrix cytokine is present in atherosclerotic plaques and it stimulates the expression of adhesion molecules and induces endothelial dysfunction through NADPH oxidase-dependent pathway (San Martin et al., 2007). Up-regulation of BMP4 in db/db mouse aortas may involve ROS-dependent vascular inflammation (San Martin et al., 2007). Likewise, the present results demonstrate that endothelial dysfunction in diabetic mice was accompanied by augmented oxidative/nitrosative stress and BMP4 up-regulation. Treatment with boldine
rescued the impaired endothelial function in \textit{db/db} mice and inhibited both ROS over-production and BMP4 up-regulation. The present study thus reveals that the increased expression and activity of the RAS is likely to up-regulate the expression of BMP4 and the latter is associated ROS over-generation, leading to the impaired endothelial function in diabetes, while boldine protects endothelial cell function through inhibiting AT1R-BMP4-ROS axis. Similarly, boldine reversed the Ang II-induced endothelial dysfunction \textit{in vitro} and reduced the Ang II-induced BMP4 protein expression. Both actions of Ang II were also alleviated in the presence of losartan, AT1R blocker and noggin, an inhibitor of BMP4, suggesting that the impairment of endothelial dysfunction in \textit{db/db} mouse aorta may be attributed to the Ang II-induced BMP4 oxidative stress cascade. A recent study also reported that BMP4 plays a pathophysiological role in Ang II-induced cardiomyocyte hypertrophy where BMP4 expression was increased by Ang II treatment in cultured cardiac fibroblasts (Sun \textit{et al.}, 2013). However, from the present findings, participation of other BMPs such as BMP2, BMP6 and BMP7 in vascular oxidative stress and vascular inflammation in animal model of diabetes mellitus cannot be entirely ruled out.

\textbf{6.6 Conclusion}

Taken together, the present study provides novel evidence demonstrating that boldine is effective in inhibiting the AT\textsubscript{1}R-mediated cellular signaling cascade and ameliorating endothelial dysfunction in diabetic mice. Our findings further suggest a therapeutic potential of boldine-containing medicinal herbs in alleviating diabetic vasculopathy.
CHAPTER VII
CONCLUSION

Oxidative stress associated with the imbalance of excessive generations of free radicals and low levels of antioxidants have received considerable attention in recent years. Increasing evidence indicates that developments of chronic cardiovascular related diseases are strongly correlated with elevated oxidative stress. Oxidative stress is caused by overproduction of ROS such as $\text{O}_2^-$ and $\text{ONOO}^-$ and it is an important contributor to the development of endothelial dysfunctions in chronic diseases such as hypertension, diabetes mellitus and atherosclerosis. The pathophysiology of endothelial dysfunction is complex and however, is always associated with decreased NO bioavailability. The protective role of antioxidants in oxidative stress-related diseases is well documented and forms an important complimentary therapy to conventional treatments. Although many antioxidants such as vitamin E, ascorbic acid, and calcitriol have been given to animals and in clinical studies to prevent the development of cardiovascular complications, numerous “natural” antioxidants derived from plants remain unexplored scientifically.

In the present study, we further examine plant derived antioxidants as potential endothelial protective agents against the damaging effects of free radicals in oxidative stress related diseases such as hypertension and diabetes mellitus. Preliminary results showed that the methanolic extracts of *Phoebe grandis* (MPG) a local plant, in which boldine is a major compound exhibited potent antioxidant activity in several antioxidant assays including DPPH, ORAC and cell-free xanthine/xanthine oxidase superoxide generation system. In the isolated aorta of Sprague Dawley rats, MPG was further demonstrated to significantly reverse the endothelial dysfunction induced by the
oxidative stress inducer $\beta$-NADH and restoring the endothelium-dependent relaxations to ACh as in the vehicle-treated vessels. In addition, MPG inhibited the generation of vascular superoxides induced by $\beta$-NADH in the isolated rat aorta. This endothelial protective effect of MPG served as an important basis for further exploration of ‘natural’ antioxidants derived from Malaysian plants and their potential medicinal uses.

Impaired endothelium-dependent relaxation and decreased NO bioavailability are commonly reported in animal models of hypertension and diabetes mellitus (both type 1 and type 2). In the subsequent study, the endothelial protective effect of boldine was investigated in a hypertensive rat model. Endothelial dysfunction in the SHRs is associated with increased SBP and NADPH oxidase mediated oxidative stress. Increased NADPH-induced O$_2^-$ production scavenges endothelium-derived NO, leading to reduced NO levels as well as increased formation of ONOO$^-$ which further diminishes the protective role of NO in the regulation of the vasculature. Repeated treatment of the SHRs with boldine for 7 days attenuated the elevated SBP and improved the endothelium dependent relaxations to ACh in the isolated aorta from these rats. This provided further support that the endothelial protective effects of boldine in hypertensive rats is at least in part due to inhibition of NADPH-mediated superoxide production. Protein expression studies denote that the inhibitory effect of boldine on the NADPH oxidase is associated with down-regulation of the membrane-bound regulatory cytosolic protein subunit, p47$\text{phox}$. This novel finding of the cellular action of boldine provide further support to the antioxidant actions and therapeutic potential of the alkaloid in the regulation of vascular tone and the maintenance of vascular patency to preserve cardiovascular health.
Similarly, boldine exhibited potent antioxidant effects in both *in vitro* and animal models of type 1 and type 2 diabetes. As in hypertension, excessive generations of ROS interfere with NO signalling and play a critical pathological role in the development of the vascular complications associated with diabetes. In hyperglycaemia, glucose autoxidation increases production of free radicals leading to overproduction of ROS and subsequent inactivation of NO.

In both STZ-induced diabetic rat (type 1 diabetes) and *db/db* mice (type 2 diabetes), endothelial dysfunctions were markedly observed by the impaired endothelium-dependent relaxations to ACh in isolated rat or mouse aorta. Acute (30 min pre-treatment) and repeated treatment (7 days) of boldine significantly reversed the impaired endothelial function in aorta of both diabetic animals. As in hypertensive vessels, the endothelial protective actions of boldine strongly attributed to decreasing ROS formation and enhancing NO bioavailability via improving activity of eNOS in the aortic wall of the diabetic animals. Incubation of rat and mouse aortic endothelial cells grown in high glucose medium with boldine, decreased the generation of O$_2^-$ in these cells and thus protecting NO degradation. In the type 1 diabetic rats, treatment with boldine normalized the overproduction of ROS and this correlated with the downregulation of the NADPH oxidase subunits NOX2 and p47phox. Repeated treatment with boldine in the *db/db* mouse decreased protein expression of AT$_1$R and BMP4 in the vascular wall. This finding suggests that in the type 2 diabetic mice, boldine may additionally, reduced the generation of superoxide anions by inhibiting the AT$_1$R-BMP4-ROS axis and increasing eNOS phosphorylation.

Taken together, it appears that boldine may exert positive effects on the endothelium via several mechanisms including mainly by protecting NO from degradation via inhibiting
the excessive production and scavenging of superoxides, and additionally by increasing NO bioavailability by promoting eNOS phosphorylation. The present results further support the potential use of boldine as a naturally effective antioxidant with protective actions against endothelial dysfunctions associated with oxidative stress as in chronic diseases such as hypertension and diabetes mellitus.

Despite mixed outcomes from clinical trials of vitamin E and C, the good safety profile of boldine and its potent antioxidant action, should provided new impetus to conduct preliminary investigations of the effectiveness of the alkaloid either for treatment or as prophylaxis, in hypertensive and diabetic patients. Combining boldine with current treatments for hypertension and diabetes mellitus would be another area of study given that drug combinations have been shown to be successful for the treatment of hypertension. Finally, other potential studies with boldine should examine the effects of longer treatment durations, and other mechanisms including anti-inflammatory and the central actions of the aporphine alkaloid.
Appendix A: Effect of 0.1 % DMSO (vehicle) and MPG extracts on ACh endothelium-dependent relaxation. (A) 0.1 % DMSO did not alter the ACh responses in the presence of NADH (300 µM) (B) 0.5 μg/ml, 5 μg/ml and 50 μg/ml Phoebe grandis (stem bark) methanolic extract did not affect the ACh relaxation in rat aortic rings. Results are mean ± SEM (n = 5-6). * P < 0.05 compared to control.
Appendix B: Effect of chronic boldine treatment on total plasma nitrate/nitrite (NO$_x$) in WKY and SHR rats. The reduced total NO$_x$ observed in SHR was increased in boldine-treated SHR rats. Data are expressed as mean ± SEM (n= 6-7). # P < 0.01 compared to WKY, * P < 0.05 compared to SHR.
Appendix C: The protein expression of p67phox (A) and Rac1 (B) was not changed by co-treatment of boldine or tempol. Results are mean ± SEM of 3 experiments using RAECs from different rats. NG: normal glucose (5 mM glucose + 25 mM mannitol as osmotic control).
Appendix D: SNP-induced endothelium-independent relaxations were not altered by boldine treatment. Results are shown as mean ± SEM (n=6).
Appendix E: The protein expression of glutathione (GSH) antioxidant was not affected by treatment of boldine or tempol in rat aortic endothelial cells (RAECs) exposed to high glucose for 48 h. Results are mean ± SEM of 3 experiments using RAECs from different rats. NG: normal glucose (5 mM glucose + 25 mM mannitol as osmotic control).
Appendix F: The protein expression of eNOS was not affected by treatment of boldine or tempol in rat aortic endothelial cells (RAECs) exposed to high glucose for 48 h of Results are mean ± SEM of 3 experiments using RAECs from different rats. NG: normal glucose (5 mM glucose + 25 mM mannitol as osmotic control).
Appendix G: The graph showing the ratio of phenylephrine (Phe, 1 μM)-induced contraction before and after the incubation of boldine (1 μM) or prazocin (0.3 nM) for 30 min from isolated aorta of SD rat. Prazocin significantly reduced the ratio (second Phe contraction over the first Phe contraction) compared to boldine.
Appendix H: Pre- and post-treatment plasma glucose level among different groups of mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma glucose (mmol/l)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>29.83 ± 0.60</td>
<td>26.80 ± 2.28</td>
<td></td>
</tr>
<tr>
<td>Boldine</td>
<td>29.30 ± 0.76</td>
<td>27.64 ± 1.86</td>
<td></td>
</tr>
<tr>
<td>Tempol</td>
<td>29.31 ± 1.13</td>
<td>27.58 ± 1.62</td>
<td></td>
</tr>
</tbody>
</table>

$db/db$ mice were given with boldine (20mg/kg/daily, i.p.). Results are mean±SEM of 6 separate experiments.

Appendix I: Lipid profile in $db/db$ chronic treated with vehicle, boldine or tempol.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Boldine</th>
<th>Tempol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>70.33 ± 5.90</td>
<td>84.11 ± 20.01</td>
<td>74.91 ± 6.74</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>71.15 ± 11.63</td>
<td>82.56 ± 0.07</td>
<td>118.20 ± 20.80</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>47.24 ± 2.26</td>
<td>45.81 ± 9.03</td>
<td>44.44 ± 1.85</td>
</tr>
<tr>
<td>non-HDL (mg/dl)</td>
<td>23.09 ± 6.30</td>
<td>38.30 ± 12.99</td>
<td>30.46 ± 6.55</td>
</tr>
</tbody>
</table>

Effect of chronic boldine treatment (20mg/kg/daily, i.p.) on levels of total cholesterol, triglyceride, HDL and non-HDL in vehicle- or boldine-treated $db/db$ mice. Results are mean ± SEM of 6 separate experiments.
Appendix J: Pre-treatment with boldine or tempol reduced ROS production that was elevated in high glucose (HG)-treated MAECs NG: normal glucose (5 mM glucose and 25 mM mannitol as osmotic control of HG). Results are means ± SEM of 4-6 separate experiments. # P <0.05 compared with NG; * P < 0.05 compared with HG.

![CM-H2DCF-DA fluorescence (fold of NG)](image_url)


Bibliography


