

**Effects of cyanidin 3-*O*-glucoside on cardiovascular complications and
immune response in spontaneously hypertensive rats**

by

Basma Aloud

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Department of Physiology and Pathophysiology

University of Manitoba

Winnipeg

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Abstract

Several epidemiological studies have elucidated that consumption of foods rich in anthocyanins decreases the risk of cardiovascular disease (CVD). Therefore, the first objective of this study was to systematically review randomized controlled trials (RCTs) assessing the effects of purified anthocyanin mixtures and anthocyanin-rich extracts on CVD risk factors. The findings of this systematic review show that purified anthocyanin mixtures may have positive effects on certain markers of CVD; however, there is limited evidence surrounding the potential of purified anthocyanin mixtures in subjects with hypertension. Therefore, our second objective was to investigate the effects of the purified anthocyanin (cyanidin-3-*O*-glucoside, C3G) *in vitro* in adult rat cardiomyocytes and cardiac fibroblasts (CF) exposed to endothelin 1 (ET1), and *in vivo* in spontaneously hypertensive rats (SHRs) and Wistar-Kyoto rats (WKY) alone or combination (10 mg/kg/day each) with the diuretic (hydrochlorothiazide, HCT). C3G prevented ET1-induced cardiomyocyte death and hypertrophy and reduced CF activation. HCT slowed the rise of blood pressure in SHRs over time, but C3G had no effect on blood pressure. SHRs treated with C3G, HCT, and C3G+HCT had lower left ventricular mass and shorter isovolumetric relaxation time compared to control SHRs. We next focused on characterizing the immune system dysfunction and investigating whether C3G and HCT can affect T-cell dysfunction in SHRs. Splenocytes of SHRs produced lower concentrations of cytokines *ex vivo* in response to concanavalin A stimulation, compared to WKY rats. While there was no effect of C3G on cytokine production in both WKY rats and SHRs, HCT treatment significantly decreased cytokine production in SHRs.

SHRs had a lower proportion of regulatory T-cells (Tregs) compared to WKY rats with no effect of C3G or HCT. In conclusion, C3G may have the potential to mitigate cardiac abnormalities in SHRs independently of any effects on blood pressure. The observed cardioprotective effects of C3G and HCT are not mediated through Tregs. Future studies are needed to explore the potential of C3G in subjects with established hypertension and to identify the mechanisms underlying its cardioprotective effects observed in this study.

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List of abbreviations

ACE – Angiotensin-converting enzyme

ACEI - Angiotensin-converting enzyme inhibitors

ALT - Alanine aminotransferase

APC – Antigen presenting cells

AST - Aspartate aminotransferase

CCBs - calcium channel blockers

C3G - Cyanidin 3-O-glucoside

CF - Cardiac Fibroblasts

ConA – Concanavalin A

CVD – Cardiovascular disease

DASH - Dietary approaches to stop hypertension

DBP - Diastolic blood pressure

DC – Dendritic cells

DOC - Desoxycortisone

EF - Ejection fraction

ED-A fibronectin - Extra domain A fibronectin

ET1 - Endothelin 1

FMD – Flow-mediated dilation

Foxp3 -Forkhead box p3

FS - Fractional shortening

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

HDL - High density lipoprotein

HCT - Hydrochlorothiazide

HHD - Hypertensive heart disease

IFN γ - Interferon gamma

IHD – Ischemic heart disease

IVRT - Isovolumetric relaxation time

IVSd - Interventricular septal wall thickness at diastole

IVSs - Interventricular septal wall thickness at systole

IL - Interleukin

LDL – Low density lipoprotein

LVH - Left ventricular hypertrophy

LVIDs - Left ventricular internal dimensions at systole

LVIDd - Left ventricular internal dimensions at diastole

LVPWs - Left ventricular posterior wall thickness at systole

LVPWd - Left ventricular posterior wall thickness at diastole

MDA – Malondialdehyde

MI – Myocardial infarction

MS - Metabolic syndrome

RAAS - Renin-angiotensin-aldosterone system

RCTs – Randomized controlled trials

α -SMA - Alpha-smooth muscle actin

SBP - Systolic blood pressure

SERCA -Sarco/endoplasmic reticulum Ca^{2+} -ATPase

SHR - Spontaneously hypertensive rats

SHRSP – Stroke-prone spontaneously hypertensive rats

SNS – Sympathetic nervous system

SOD - Superoxide dismutase

TCR – T-cell receptors

Th – Helper T-cells

TNF α - tumor necrosis factor alpha

Treg – Regulatory T-cells

VEC - Vascular endothelial cells

V_{max} - maximal SERCA2a activity

WKY - Wistar-Kyoto rats

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CHAPTER 1

Efficacy of anthocyanins on cardiovascular disease risk factors: a systematic review of randomized controlled trial

1.1 Introduction

Cardiovascular disease (CVD) remains a major cause of morbidity and premature death worldwide.¹ The World Health Organization (WHO) estimates that CVD is responsible for 31% of all deaths, most of these were attributed to ischemic heart disease and stroke.¹ While some CVD risk factors such as family history and aging cannot be avoided, identifying and managing modifiable CVD risk factors including tobacco smoking, unhealthy diet, sedentary lifestyle, and harmful alcohol consumption can help decrease the increasing incidence of CVD.^{1,2} These factors manifest in individuals as hypertension, type 2 diabetes, obesity and dyslipidemia.¹ According to the WHO, 80% of premature heart attacks and cerebrovascular accidents are preventable; therefore, detecting and implementing risk factor amelioration strategies in individuals at high risk for CVD is crucial.^{1,2}

A high consumption of fruits and vegetables has been recommended by the American Heart Association as part of the Dietary Approaches to Stop Hypertension diet, which has been demonstrated to have not only a direct blood pressure lowering effect, but it can also decrease the risk of CVD.^{3,4} Research has indicated that these protective effects are attributed to different bioactive phytochemicals that have useful biological effects including antioxidant, antihyperlipidemic, and blood pressure lowering effects.^{4,5}

Anthocyanins are phytochemicals that belong to a family of water-soluble pigments known as flavonoids. Chemically, anthocyanins are polyhydroxylated or polymethoxylated derivatives of flavium salts.⁶ Although there are over 635 characterized

anthocyanins, six anthocyanidins: cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin, represent more than 90% of anthocyanins in the plant kingdom.⁶ Anthocyanins are found in high concentrations in many colored fruits, vegetables, and grains, suggesting that consuming significant quantities of anthocyanins through dietary sources is achievable.⁷ Therefore, there has been an increased focus in research on the biological activities of anthocyanins as potential health promoting phytochemicals.

Several epidemiological studies have elucidated that consumption of foods rich in anthocyanins decreases the risk of CVD.⁸⁻¹⁰ A meta-analysis of anthocyanin consumption in relation to CVD risk suggests that dietary anthocyanin intake reduces the risk of coronary heart disease and CVD-related mortality.¹¹ Another meta-analysis of randomized controlled trials (RCTs) that explored the efficacy of anthocyanins on cardiometabolic risk factors in healthy and diseased subjects revealed that anthocyanins significantly decreased fasting glucose, total cholesterol, and low density lipoprotein cholesterol (LDL-c) levels.¹² The effect of purified anthocyanins on lipid profiles and blood pressure in healthy subjects and individuals with elevated markers of CVD in RCTs was systematically reviewed by Wallace *et al.*, and their main findings showed that anthocyanins have favorable effects on LDL-c in hyperlipidemic individuals.¹³ Our main objective in this systematic review was to provide an update on the effect of purified anthocyanins and anthocyanin-rich extracts in subjects with increased risk for CVD by systematically reviewing RCTs to answer the following question: Do purified anthocyanins or anthocyanin-rich extracts compared to no anthocyanin treatment (placebo) significantly improve CVD risk factors such as blood pressure, (low and high-

density lipoprotein cholesterol (LDL-c and HDL-c), total cholesterol, and triglycerides), fasting glucose, glycated hemoglobin, and atherosclerosis in adult subjects with hypertension, dyslipidemia, type 2 diabetes mellitus, and atherosclerosis?.

1.2 Methods

This systematic review was reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA).¹⁴ A PRISMA checklist is presented in **Appendix 1.1**.

1.2.1 Search strategy

A systematic search strategy was developed in four databases (Medline, Embase, Web of Science, and Cochrane Library) for randomized controlled trials published in English from inception until September 2018. The bibliographies of related systematic reviews were hand-searched to locate additional eligible articles.

Search terms consisted of subject headings (MeSH, EMTREE) and keywords for the concept ‘anthocyanins’, or anthocyanin-rich dietary sources (**Table 1.1**) combined with subject headings (MeSH, EMTREE) and keywords for the following search concepts: “essential hypertension or hypertension or pre-diabetes or diabetes or glucose intolerance or dyslipidemia or LDL-c or HDL-c or total cholesterol or triglycerides or atherosclerosis” and “randomized controlled trials” (**Appendix 1.2**). A librarian was involved in developing the search strategy (J.R.).

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	II
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3-4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	No
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	10
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	8-9
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	10
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	10-11
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	No
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	No
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	No
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	No

Section/topic	#	Checklist item	Reported on page #
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	No
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	No
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	14
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	12
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	No
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	16, 18, 20 & 21
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	No
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	No
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	No
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	21-24
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	24
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	24-25
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	No

Appendix 1.1: Preferred Reporting Items for Systematic Reviews and Meta-Analyses checklist.

Anthocyanin-rich dietary sources	Database search keywords
Black currant	“Black currant”
Elderberries	“Elderberr”
Bilberries	“Bilberr”
Whortleberries	“Whortleberr”
Chokeberries	“Chokeberr”
<i>Hibiscus sabdariffa</i>	“Hibiscus”
Red grapes	“Red grape”
Caucasian Whortleberry	“Vaccinium arctostaphylos”
Cranberries	“Cranberr”
Blueberries	“Blueberr”
Black raspberries	“Black raspberr”
cornelian cherry	“Cornus mas”

Table 1.1: A list of selected anthocyanin-rich dietary sources with selected database search keywords.

01. (anthocyanin*):ti,ab,kw
02. ("black currant"):ti,ab,kw
03. (elderberry):ti,ab,kw
04. (bilberry):ti,ab,kw
05. (whortleberry):ti,ab,kw
06. (chokecherry):ti,ab,kw
07. (hibiscus):ti,ab,kw
08. ("red grape"):ti,ab,kw
09. ("vaccinium arctostaphylos"):ti,ab,kw
10. (cranberry):ti,ab,kw
11. (blueberry):ti,ab,kw
12. ("black raspberry"):ti,ab,kw
13. ("cornus mas L"):ti,ab,kw
14. MeSH descriptor: [Anthocyanins] explode all trees
15. {or #1-#14}
16. (hypertension):ti,ab,kw
17. (hypertensive):ti,ab,kw
18. MeSH descriptor: [Hypertension] explode all trees
19. (diabetes):ti,ab,kw
20. MeSH descriptor: [Diabetes Mellitus] explode all trees
21. (prediabetes):ti,ab,kw

22. ("glucose intolerance"):ti,ab,kw
23. ("glycation end products"):ti,ab,kw
24. (atherosclerosis):ti,ab,kw
25. MeSH descriptor: [Atherosclerosis] explode all trees
26. ("ischemic heart disease"):ti,ab,kw
27. MeSH descriptor: [Myocardial Ischemia] explode all trees
28. ("myocardial ischemia"):ti,ab,kw
29. ("myocardial infarction"):ti,ab,kw
30. MeSH descriptor: [Myocardial Infarction] explode all trees
31. (dyslipidemia):ti,ab,kw
32. MeSH descriptor: [Dyslipidemias] explode all trees
33. (triglyceride*):ti,ab,kw
34. MeSH descriptor: [Triglycerides] explode all trees
35. (Cholesterol):ti,ab,kw
36. MeSH descriptor: [Cholesterol] explode all trees
37. ("high density lipoprotein"):ti,ab,kw
38. MeSH descriptor: [Lipoproteins, HDL] explode all trees
39. ("low density lipoprotein"):ti,ab,kw
40. MeSH descriptor: [Lipoproteins, LDL] explode all trees
41. {or #16-#40}
42. #15 and #41

Appendix 1.2: Search strategy (Cochrane Library).

1.2.2 Study selection

Studies were included in this review based on the following eligibility criteria: clinical RCTs studying the efficacy of purified anthocyanins or anthocyanin-rich extracts compared with no intervention or placebo control groups, involved adult participants with established CVD risk factors (hypertension, dyslipidemia, type 2 diabetes and/or atherosclerosis) as the primary outcome measures, and the dose of anthocyanin intervention was specified. No limitations were applied to sample size, study duration, study setting or geography. Studies that were excluded fit the following criteria: trials that used anthocyanin-rich foods (not purified anthocyanin or anthocyanin-rich extracts) as the intervention, studies that were published in non-English languages, and studies that included healthy subjects (those with outcomes in the normal range).

Eligibility assessment of articles was carried out by screening titles and abstracts by two reviewers (B.M.A. and T.N.). Studies which did not meet the inclusion criteria were excluded. Ineligible studies were excluded from the review. Potentially relevant articles were selected for full-text revision and their eligibility was assessed by the reviewers. Differences between reviewers were resolved by discussion until consensus was reached.

1.2.3 Data extraction

The following data were extracted from selected studies: study design, source of anthocyanins, anthocyanin dosage versus control, number of participants, age, gender; outcomes (systolic and diastolic blood pressures, LDL-c, HDL-c, total cholesterol, and

triglycerides); and study duration. Data were checked for completeness and accuracy after extraction. **Table 1.2** represents the characteristics of the studies included in the review.

Reference	Study design	Intervention	anthocyanin dose mg/day	Control	Number of participants	Gender	Age (years)	Duration	CVD-related parameters
Hassellund <i>et al.</i> , 2012 ¹⁵	Crossover	Purified anthocyanins	640	Placebo capsules	31	Men	35-51	4 weeks	Mild hypertension (> 140/90mmHg)
Li <i>et al.</i> ,2015 ¹⁶	Parallel	Purified anthocyanins	320	Placebo capsules	58	Women and men	56-67	24 weeks	Dyslipidemia with type 2 diabetes
Yang <i>et al.</i> , 2017 ¹⁷	Parallel	Purified anthocyanins	320	Placebo capsules	160	Women and men	40-75	12 weeks	Prediabetes or early untreated diabetes
Zhu <i>et al.</i> , 2014 ¹⁸	Parallel	Purified anthocyanins	320	Placebo capsules	122	Women and men	55	24 weeks	Hypercholesterolemia
Zhu <i>et al.</i> , 2012 ¹⁹	Parallel	Purified anthocyanins	320	Placebo capsule	150	Women and men	40-65	24 weeks	Hypercholesterolemia
Qui <i>et al.</i> , 2009 ²⁰	Parallel	Purified anthocyanins	320	Placebo capsules	120	Women and men	40-65	12 weeks	Dyslipidemia
Zhu <i>et al.</i> ,2011 ²¹	Parallel	Purified Anthocyanins	320	Placebo capsules	150	Women and men	40-65	12 weeks	Hypercholesterolemia
Hassellund <i>et al.</i> , 2013 ²²	Crossover	Purified anthocyanins	640	Placebo capsules	31	Men	35-51	4 weeks	Mild hypertension

Table 1.2: Characteristics of studies included in the systematic review.

1.3 Results

1.3.1 Search results

The literature search of selected databases (Medline, Embase, Web of Science, and Cochrane Library) retrieved a total of 524 articles. One additional article which was not indexed in selected databases was identified by hand searching. After deduplication, 287 articles were excluded. Titles and abstracts of 238 articles were assessed for relevance according to the inclusion criteria for this review. 59 trials relevant trials were identified and further examined as full texts. No studies that investigated the effects of anthocyanin-rich extracts met the inclusion criteria. Therefore, at the end, 8 studies that investigated the effectiveness of purified anthocyanins on CVD risk factors were fully reviewed and included in the review (**Figure 1.1**).

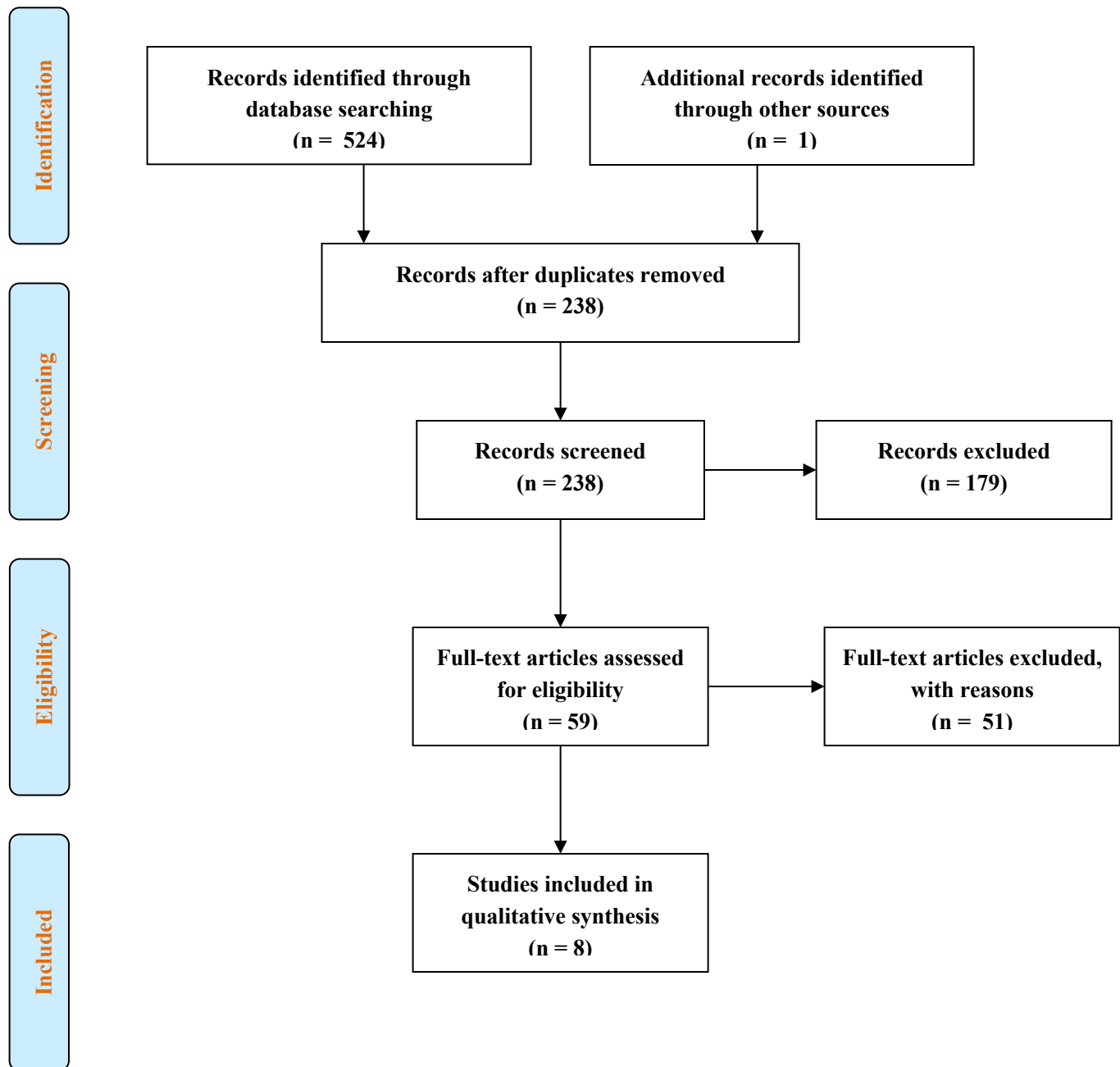


Figure 1.1: Preferred Reporting Items for Systematic Reviews flow diagram of the study selection process.

1.3.2 Effects of purified anthocyanins on blood pressure

One study assessed the effects of purified anthocyanin supplementation on blood pressure (**Table 1.3**). The results of this study show that anthocyanin intervention (320 mg twice daily) for 4 weeks has no effect on blood pressure in subjects with mild hypertension in a placebo-controlled randomized double-blind crossover study, compared with placebo intervention.¹⁵

Reference	Anthocyanin dose mg/day	Stage of hypertension	Systolic BP (mmHg)			Diastolic BP (mm Hg)		
			Placebo	Intervention	<i>P</i>	Placebo	Intervention	<i>P</i>
Hassellund <i>et al.</i> , 2012 ¹⁵	640	Stage 1 (> 140/90mm Hg)	133 ± 2	135 ± 2	0.25	82 ± 1	83 ± 1	0.32

Table 1.3: Effects of purified anthocyanins on blood pressure.

1.3.3 Effects of purified anthocyanins on lipid profiles

Seven studies assessed the effects of purified anthocyanins on the levels of LDL-c, HDL-c, total cholesterol, and triglycerides.¹⁶⁻²² The results of six studies showed a significant decrease (3-11%) in LDL-c levels by purified anthocyanins (320 mg/day) compared with placebo intervention.¹⁶⁻²¹ In one study, there was no effect of purified anthocyanin intervention (640 mg/day) on LDL-c levels in prehypertensive subjects (**Table 1.4**).²²

With the exception of one study that reported no influence of anthocyanin supplementation on HDL-c levels in subjects with prediabetes or early untreated diabetes,¹⁷ all other studies reported a significant increase (5-29%) in HDL-c levels by purified anthocyanins in subjects with hyperlipidemia and hypertensive subjects with intermediate levels of HDL-c, compared with placebo intervention.^{16, 18-22}

Of the seven studies that reported the effect of purified anthocyanins on lipid profiles, only one study reported significant decreases in total cholesterol and triglyceride levels (2.2 and 20%, respectively) by purified anthocyanins in diabetic patients with dyslipidemia, compared with a placebo control group.¹⁶

Reference	Anthocyanin dose (mg/day)	CVD-related parameters	LDL-c (mmol/L)			HDL (mmol/L)			Total cholesterol (mmol/L)			Triglycerides (mmol/L)		
			Control	Intervention	<i>P</i>	Control	Intervention	<i>P</i>	Control	Intervention	<i>P</i>	Control	Intervention	<i>P</i>
Li <i>et al.</i> , 2015 ¹⁶	320	Dyslipidemia	3.21 ± 0.48	2.92 ± 0.54	0.030	0.95 ± 0.07	1.23 ± 0.12	0.012	4.99 ± 0.86	4.88 ± 0.94	0.041	1.96 ± 0.45	1.57 ± 0.72	<0.01
Yang <i>et al.</i> , 2017 ¹⁷	320	Prediabetes or early untreated diabetes	3.20 ± 0.79	3.1 ± 0.69	0.040	1.3 ± 0.32	1.29 ± 0.40	0.694	6.04 ± 1.26	5.99 ± 1.24	0.358	1.87 ± 1.55	1.81 ± 1.26	0.820
Zhu <i>et al.</i> , 2012 ¹⁹	320	Hypercholesterolemia	3.30 ± 0.52	3.01 ± 0.41	0.030	1.23 ± 0.20	1.37 ± 0.22	0.036	6.25 ± 0.83	6.18 ± 0.82	0.556	2.34 ± 1.35	2.35 ± 1.37	0.462
Qui <i>et al.</i> , 2009 ²⁰	320	Dyslipidemia	4.07 ± 0.94	3.62 ± 0.92	<0.001	1.21 ± 0.26	1.32 ± 0.23	<0.001	5.75 ± 1.03	5.70 ± 0.88	0.435	2.26 ± 1.03	2.14 ± 0.97	0.576
Zhu <i>et al.</i> , 2011 ²¹	320	Hypercholesterolemia	3.28 ± 0.47	3.01 ± 0.41	0.045	1.22 ± 0.25	1.37 ± 0.22	0.028	6.24 ± 0.86	6.19 ± .82	0.385	5.32 ± 3.18	5.38 ± 3.14	0.469
Hassellund <i>et al.</i> , 2.13 ²²	640	Prehypertension	3.09 ± 0.16	3.19 ± 0.15	0.341	1.18 ± 0.09	1.24 ± 0.08	0.043	4.88 ± 0.16	4.96 ± 0.17	0.432	1.37 ± 0.18	1.18 ± 0.13	0.127

Table 1.4: Effects of purified anthocyanins on lipid profiles. Abbreviations: CVD, cardiovascular disease; LDL-c, low density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol.

1.3.4 Effects of purified anthocyanins on fasting glucose and insulin levels, insulin sensitivity, and glycated hemoglobin levels in prediabetic and diabetic subjects

Two studies reported the impact of anthocyanins on glycemic control (**Table 1.5**). The findings of the first study show that while purified anthocyanin supplementation (160 mg twice daily) for 24 weeks had no effects on plasma insulin and glycated hemoglobin levels in diabetic patients compared with placebo supplementation, it lowered fasting plasma glucose levels by 8.5% and improved insulin sensitivity as indicated by a 13% decrease in the homeostasis model assessment for insulin resistance index.¹⁶ In the second study, purified anthocyanin supplementation (320 mg/day) for 12 weeks in subjects with prediabetes or early untreated diabetes resulted in a 5% decrease in glycated hemoglobin levels with no effects on fasting glucose and insulin levels.¹⁷

Reference	Anthocyanin dose (mg/day)	Study duration	End points	Placebo		Intervention		P
				Baseline	End of intervention	Baseline	End of intervention	
Li <i>et al.</i> , 2015 ¹⁶	320	24 weeks	Fasting glucose, mmol/L	7.3 ± 1.7	7.1 ± 1.5	7.1 ± 2.2	6.5 ± 1.8	0.042
			Plasma insulin, mU/L	11.6 ± 4.13	11.7 ± 3.76	11.9 ± 4.30	11.1 ± 3.98	0.14
			Glycated hemoglobin, %	6.6 ± 1.5	6.5 ± 1.4	6.5 ± 1.7	6.2 ± 1.9	0.06
			HOMA-IR	3.76 ± 0.53	3.69 ± 0.64	3.74 ± 0.55	3.21 ± 0.76	0.35
Yang <i>et al.</i> , 2017 ¹⁷	320	12 weeks	Fasting glucose, mmol/L	6.11 ± 0.61	6.26 ± 0.71	6.15 ± 0.87	6.19 ± 0.68	0.444
			Plasma insulin, mU/L	11.99 ± 5.90	11.89 ± 6.90	11.55 ± 6.23	11.14 ± 6.47	0.600
			Glycated hemoglobin, %	5.96 ± 0.61	5.80 ± 0.61	6.13 ± 0.59	5.84 ± 0.49	0.024

Table 1.5: Effects of purified anthocyanin supplementation on fasting plasma glucose, plasma insulin, glycated hemoglobin, and insulin resistance in diabetic patients. HOMA-IR, homeostasis model assessment for insulin resistance index.

1.3.5 Effects of purified anthocyanins on markers of atherosclerosis

One study reported the impact of purified anthocyanins on flow mediated dilation (FMD) of the brachial artery, as a marker of early atherosclerosis. In this study, 12-week purified anthocyanin supplementation resulted in a 28.4% increase in FMD in hypercholesterolemic individuals, and this effect was mediated by activation of the NO-cGMP pathway.²¹

1.4 Discussion

In this systematic review of RCTs we evaluated whether purified anthocyanins and anthocyanin-rich extracts have the potential to improve outcomes in individuals at high risk for CVD. Only eight RCTs that evaluated the effects of purified anthocyanins on outcomes related to blood pressure, lipid profiles, diabetes, and atherosclerosis were identified. Therefore, further research is required to provide sufficient scientific evidence supporting the use of purified anthocyanins to help reduce the risk of CVD.

The reason for including RCTs evaluating the CVD protective effects of only purified anthocyanins and anthocyanin-rich extracts was because including RCTs that evaluated anthocyanin-containing foods (with considerable concentrations of other bioactive phytochemicals) would make the interpretation of anthocyanin-related biological effects difficult. Of note, based on our selection criteria, no studies using anthocyanin-rich extracts were identified. All the RCTs included in our systematic review utilized Medox anthocyanin capsules that contain high concentrations (80 mg/capsule) of 17 purified anthocyanins (mainly cyanidin 3-*O*- β -glucoside and delphinidin-3-*O*- β -

glucoside) in relation to other phytochemicals.²² Therefore, the reported effects in the RCTs included in this systematic review are mainly contributed to anthocyanins.

One RCT studied the antihypertensive effects of purified anthocyanin intervention at a dose of 640 mg/day. The findings of this study show that purified anthocyanins do not have blood pressure lowering effects in subjects with mildly elevated blood pressure.¹⁵ A potential explanation for the lack of antihypertensive effect of purified anthocyanin intervention in subjects with mild hypertension is because the antihypertensive effects of a particular intervention are more pronounced in patients with higher baseline blood pressure.¹⁵ However, including subjects with higher blood pressure levels in an anthocyanin interventional trial without taking their conventional blood pressure lowering medications is ethically unacceptable.¹⁵

Seven RCTs reported the effect of purified anthocyanins on lipid profiles in subjects with high lipid levels.¹⁶⁻²² Although only one study reported a significant decrease in triglyceride levels with purified anthocyanin intervention,¹⁶ six studies showed that anthocyanins could significantly decrease LDL-c and increase HDL-c concentrations.¹⁶⁻²¹ A meta-analysis of 17 RCTs has been performed to estimate the effects of anthocyanin supplementation on lipid profiles in both healthy subjects and subjects with elevated markers for CVD. The findings of this meta-analysis showed that anthocyanin supplementation significantly decreased triglyceride and LDL-c levels, while it increased HDL-c levels.²³ A growing body of evidence suggests that regular consumption of anthocyanin-rich dietary sources has cardiovascular protective effects in

dyslipidemic individuals through different biological activities including antioxidant and anti-inflammatory effects.¹² Anthocyanins were reported to enhance the activity of HDL-associated esterase/lactonase paraoxonase 1, an esterase that protects lipoproteins from oxidative damage; and improve cholesterol efflux in hypercholesterolemic individuals.²⁴ Purified anthocyanin supplementation was also reported to cause significant decreases in high sensitivity C-reactive protein, vascular adhesion molecule-1, and interleukin-1 β in hypercholesterolemic subjects.¹² Several mechanisms have been suggested to explain the anti-hyperlipidemic effects of anthocyanins including decreasing cholesterol synthesis by down-regulating the gene for the rate-limiting enzyme in cholesterol biosynthesis (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase), decreasing LDL-c concentration by inhibiting cholesteryl ester transfer protein, and facilitating fecal cholesterol excretion.²³

The effects of purified anthocyanins on glycemic control in pre-diabetic and diabetic subjects were investigated in two RCTs.^{16, 17} The findings of these studies show that purified anthocyanin supplementation for 12 or 24 weeks can significantly improve insulin sensitivity, fasting plasma glucose and glycated hemoglobin levels at a dose of 320 mg/day. The results of a meta-analysis of 32 RCTs showed favorable effects of anthocyanins on glycemic control in healthy subjects and subjects with cardiometabolic disorders.¹² The findings of this meta-analysis also show that purified anthocyanins or anthocyanin-rich-extracts were more effective than non-purified anthocyanin-containing dietary sources. Of note, anthocyanin-containing interventions used in the RCTs included in this meta-analysis contained different concentrations of anthocyanins from different sources including purified anthocyanins, anthocyanin-rich fruit juice, and freeze-dried

berry fruits. Therefore, the variability in results between purified anthocyanins and non-purified anthocyanin-containing dietary sources could be related to the presence of other constituents in non-purified anthocyanin-rich dietary sources that may interfere with biological activities of anthocyanins.¹²

This systematic review includes a number of limitations. First, the use of language restriction by including only English-language studies in the review introduces a language bias and does not take into account results published in other languages that may alter the conclusions of the review. Second, the assessment of risk of bias of individual studies included in the review was not performed. Third, a meta-analysis to evaluate the significance of the impact of purified anthocyanins on lipid profile was not performed, although could be considered for a subset of the results included here. RCTs included in this systematic review also have several drawbacks of their own including variabilities in the durations of the studies and ages of participants included in the trials. In addition, dose response effects of purified anthocyanins were not evaluated in any of the RCTs included in this systematic review.

1.5 Conclusions

The present systematic review of RCTs adds to the contemporary scientific evidence suggesting that purified anthocyanin consumption may have the potential to improve lipid profiles and glycemic regulation in subjects with increased risk for CVD. However, further well-designed RCTs of adequate duration and appropriate sample size are needed to confirm the current findings. Only one RCT compared the effect of purified

anthocyanins to placebo in subjects with mildly elevated blood pressure. Therefore, the current scientific evidence is not sufficient to allow us to draw definitive conclusions about the efficacy of purified anthocyanins in reducing blood pressure in patients with hypertension. More studies with optimal number of subjects and sufficient duration are required to evaluate the antihypertensive effects of purified anthocyanins alone or in combination with established antihypertensive medications in subjects in which SBP and DBP are the main outcomes.

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CHAPTER 2

Review of literature

2.1 General introduction

Cardiovascular disease (CVD) is a collective term for a number of related medical conditions that affect the heart and blood vessels. It includes ischemic heart disease (IHD) such as angina and myocardial infarction, hypertensive heart disease, peripheral arterial disease, rheumatic heart disease, cerebrovascular disease, congenital heart disease and other conditions. Worldwide CVD is associated with high mortality rates, accounting for 31% of all deaths every year, most of these deaths are attributed to IHD and cerebrovascular accidents.^{1, 2} It is estimated that approximately 75% of cardiovascular events can be prevented by reducing CVD risk factors which can help decrease the expanding burden of CVD.¹

The increased incidence of major CVD risk factors such as diabetes, dyslipidemia, hypertension, smoking, and obesity in industrialized as well as developing countries increases the incidence of vascular damage, which in severe stages can translate into heart failure with a life expectancy similar to aggressive cancers.³ Although aging is an established risk factor for the pathogenesis of CVD, autopsy evidence indicates that the progression of CVD can be alleviated by risk factor amelioration.² Hypertension or high blood pressure is among the most prevalent and strongest risk factors for CVD and increased mortality.⁴ Globally, more than 1 in 5 adults are diagnosed with hypertension and it is responsible for almost 50% of mortality attributed to cerebrovascular event and heart disease. Furthermore, hypertension continues to be a leading cause of premature death worldwide accounting for 9.4 million deaths every year.⁵ In Canada, about 25% of the adult population are living with hypertension.⁶ Of adults with hypertension, 80% are

being treated with antihypertensive medications; however, hypertension remains uncontrolled in 34% of hypertensive patients that are receiving antihypertensive treatment.⁷

A systolic blood pressure above 140 mm Hg and/or a diastolic blood pressure above 90 mm Hg is considered diagnostic of hypertension and represents the threshold value for initiating antihypertensive treatment to prevent the development of hypertension-related complications.⁸ Despite the major health, social, and financial impact of hypertension, it remains undiagnosed and/or ineffectively treated.⁸ Several factors have been implicated in the development of hypertension, including (1) family history, (2) ethnicity, (3) obesity, (4) high sodium intake, (5) high alcohol intake, (6) low potassium intake, (7) low calcium intake, and (8) sedentary life style.⁸⁻¹⁰

Regulation of blood pressure is a complex integrated response involving a variety of organ systems including the central nervous system, cardiovascular system, kidneys, and adrenal glands. Uncontrolled hypertension can damage these organs, and it is a major contributor to the development of congestive heart failure, end-stage renal disease, and stroke.⁸ Hypertensive heart disease is one of the major complications of untreated hypertension. The cardiac consequences of high blood pressure include abnormalities in the structure and function of the myocardium, including left ventricular hypertrophy, systolic and diastolic dysfunction, and in severe cases, overt heart failure.⁸ It has been reported that the lifetime risk for developing heart failure is 1 in 9 men and 1 in 6 women among patients with hypertension and without an established myocardial infarction,

highlighting the risk conferred by hypertension alone.¹¹ Although the exact molecular mechanisms by which hypertension predisposes patients to heart failure have not been fully elucidated, it is generally believed that chronic blood pressure elevation leads to the development of left ventricular hypertrophy, a compensatory response by which the heart walls increase in size in an attempt to counter the increased ventricular systolic wall stress.¹⁰ At the cellular level, the development of cardiac hypertrophy involves an increase in cardiomyocyte size, enhanced protein synthesis, and sarcomeric reorganization.¹² Prolonged cardiac hypertrophy becomes maladaptive and it is associated with a rapid decline in the contractile performance of the myocardium (systolic and diastolic dysfunction), eventually leading to heart failure.⁸

While the development of hypertension has been attributed to aberrations in vasculature, central nervous system, and kidneys, accumulating evidence suggests that immune system dysregulation also contributes. Cells of the innate and adaptive immune systems have been reported to play important roles in the initiation and maintenance of hypertension in different animal models. For example, studies have shown that mice lacking adaptive immune cells are resistant to blood pressure elevation in response to angiotensin II and high salt.¹³ Hypertension is characterized by enhanced expression of adhesion molecules on blood vessels, the heart, and the kidneys; therefore, enhancing extravasation and accumulation of immune cells such as macrophages and T lymphocytes in these organs. These infiltrating cells secrete and stimulate other immune cells to secrete pro-hypertensive cytokines such as IL-6, TNF- α , and IL-17.¹³ Therefore, assessment of immune cell function in hypertension is crucial in the discovery and

mechanistic understanding of novel therapeutic strategies to prevent and treat hypertension.

The established antihypertensive drug classes available for treating hypertension/heart failure include diuretics, β -adrenergic receptor blockers, calcium channel blockers, angiotensin converting enzyme inhibitors, angiotensin receptor blockers, and aldosterone antagonists.¹⁴ However, many of these medications have off-target effects that may adversely impact the quality of life. Therefore, new effective and safer therapeutic agents for hypertension and hypertensive heart disease need to be developed. One important strategy may be the use of functional foods and nutraceuticals which have specific medical benefits. Cyanidin 3-*O*-glucoside (C3G), one of the most widely investigated nutraceuticals, is an anthocyanin that is particularly abundant in many colored fruits and vegetables providing them the red, blue, and purple colors.¹⁵ C3G has been reported to have a wide range of biological activities including antidiabetic,^{5, 16} antihyperlipidemic,¹⁷ antioxidant,¹⁸ and anti-inflammatory activities.¹⁹ C3G has also been reported to reverse high-fat diet-induced cardiovascular complications.²⁰

2.2 Hypertension

Hypertension, or systemic arterial hypertension, is defined as a chronic increase in systolic blood pressure (SBP) of ≥ 140 mm Hg and/or diastolic blood pressure (DBP) of ≥ 90 mm Hg measured in a relaxed sitting posture.²¹ Hypertension can also be diagnosed in patients who have high blood pressure more often in the clinical sittings, also known as white coat syndrome, by ambulatory blood pressure monitoring for up to 24 hours. In

this case, the cut-off value to diagnose hypertension is 140/90 mm Hg for more than 25% of the measurements in a day.²¹ However, the 2017 Guidelines for Prevention, Detection, Evaluation, and Management of High Blood Pressure formulated by the American College of Cardiology and the American Heart Association have lowered the cut-off values for blood pressure classification in adults as follows: normal (<120 mm Hg SBP and <80 mm Hg DBP), elevated (120-129 mm Hg SBP and <80 mm Hg DBP), stage 1 hypertension (130-139 mm Hg SBP or 80-89 mm Hg DBP), and stage 2 hypertension ($\geq$ 140 mm Hg SBP or $\geq$ 90 mm Hg DBP).²²

Observational trials have reported an increased risk of CVD as SBP and DBP increase.²³ In a meta-analysis of 61 prospective trials every 20/10 mm Hg increase in blood pressure was correlated with a doubling of the risk of mortality from heart disease and cerebrovascular accident.^{22, 24} It is important to recognize that the lifetime risk of developing hypertension is three times higher in the prehypertensive population as compared with normotensive subjects.²⁵ Therefore, it is essential to detect and manage elevated blood pressure at early stages to reduce the risk of cardiovascular comorbidities and premature death.

Hypertension is classified as either idiopathic/essential (primary) or non-essential (secondary) hypertension.^{26, 27} Essential hypertension affects more than 90% of hypertensive patients and is a heterogenous disorder of genetic origin influenced by different environmental factors.²⁶ In contrast, non-essential hypertension is usually caused by a specific secondary condition such renal or endocrine disorders.²⁷ Several

interrelated contributing factors have been reported to be involved in the pathogenesis of essential hypertension, including genetic predisposition, enhanced sympathetic nervous system activity, increased peripheral vascular resistance, endothelial dysfunction, and imbalance in the renin-angiotensin-aldosterone system.²⁶ Non-essential hypertension is the direct result of a specific disorder such as renal parenchymal disease (chronic kidney disease); renovascular disease including fibromuscular dysplasia and atherosclerotic renal artery stenosis; as well as endocrine abnormalities such as primary aldosteronism, pheochromocytoma, high cortisol levels, and thyroid or parathyroid abnormalities.²⁷

2.2.1 Regulation of blood pressure

Blood pressure regulation is defined as the control of blood supply to a particular organ to match its metabolic needs.²⁸ The main cardiovascular factors that determine blood pressure are cardiac output and total peripheral resistance, which are mainly influenced by neurohormonal mechanisms and intravascular blood volume.²⁹

Control of blood pressure involves different complex mechanisms including neural mechanisms, renal-endocrine mechanisms, and endothelium-dependent mechanisms.^{28, 29} Imbalance or dysregulation of blood pressure control mechanisms can lead to chronic blood pressure elevation, that will eventually lead to target organ damage including overt heart failure, end-stage renal disease, and stroke.^{8, 29} Blood pressure control mechanisms can be classified into local and global regulation mechanisms. Local mechanisms regulate blood pressure by controlling blood supply to body tissues acutely through vasoconstriction and vasodilation, and chronically by modifying the number and

diameter of blood vessels supplying body organs.²⁸ Besides local blood pressure control mechanisms, global control mechanisms regulate blood flow by modifying cardiac output and control blood pressure mainly by regulation of the sympathetic nervous system. The renal-endocrine system is another crucial chronic control mechanism that regulates blood pressure by regulating sodium and fluid homeostasis.²⁸

2.2.1.1 *Sympathetic nervous system*

An increase in the activity of sympathetic nervous system (SNS) is associated with higher cardiac output, tachycardia, higher norepinephrine levels, and peripheral vasoconstriction in hypertensive young adults.²⁸ SNS is also more active in hypertensive patients with prediabetes, sleep apnea, heart disease, and kidney disease.^{28, 29} Furthermore, studies have revealed that the occurrence and maintenance of essential hypertension are related to an imbalance in the autonomic nervous system with increased stimulation of SNS and decreased parasympathetic nervous system stimulation.²⁹

Baroreceptors, pressure changes sensing receptors, are located in different places in the arterial system, but their main locations are the carotid sinus and the aortic arch. Acute rise in blood pressure causes the carotid artery to stretch and stimulate baroreceptors to send impulses to the central nervous system to decrease the activity of the SNS.^{28, 29} SNS also plays an important role as a chronic blood pressure regulator by stimulating renin release in the juxtaglomerular apparatus in the kidney by activating the sympathetic renal nerve.²⁸ Enhanced sympathetic renal nerve activity with associated salt

retention and sustained hypertension have been reported in obesity-induced hypertension models.³⁰

2.2.1.2 *Renin-angiotensin-aldosterone system*

The renin-angiotensin-aldosterone system (RAAS) is a hormonal system that plays a fundamental role in blood pressure regulation by controlling vasoconstriction and sodium and fluid homeostasis, and it is an important mediator of essential hypertension.²⁹ The most important function of the RAAS is to maintain pressure-fluid homeostasis in the renal system by preserving adequate renal perfusion in cases of extracellular fluid depletion as a consequence of increased sodium and fluid excretion. To maintain pressure-volume balance when the volume of the extracellular fluid expands, the activity of the RAAS is suppressed to enhance sodium and water excretion.²⁹

Renin, also referred to as angiotensinogenase, is an aspartic protease enzyme that is synthesized in the juxtaglomerular apparatus in the kidneys. Upon stimulation with different stimuli including stimulation of the renal sympathetic nerve, and vasodilation, renin is released and activates the hydrolysis of angiotensinogen (a protein that is synthesized and released to the systemic circulation by the liver) to angiotensin I.²⁹ Angiotensin I is a mild vasoconstrictor and it is not strong enough to induce major circulatory changes.²⁸ Angiotensin converting enzyme (ACE) is a key mediator in the RAAS that regulates blood pressure by converting angiotensin I to the potent vasoconstrictor angiotensin II, which plays a central role in the pathogenesis of essential hypertension. Angiotensin II mediates its actions through binding to and activating the

transmembrane G protein coupled receptors (AT1 and AT2). It activates renal sodium reabsorption, induces endothelial dysfunction, and has proinflammatory properties; therefore, angiotensin II is implicated in the pathogenesis of hypertension-related microvascular (nephropathy and retinopathy) and macrovascular damages (heart attacks and cerebrovascular accidents).²⁹ In addition to ACE, ACE2 has a crucial role in blood pressure regulation by mediating the conversion of angiotensin II to angiotensin (1-7). Angiotensin (1-7) stimulates vasodilation, enhances sodium and fluid excretion, and has been reported to have cardiovascular protective activities.²⁹

Aldosterone is a mineralocorticoid hormone produced primarily by the adrenal cortex in the adrenal gland that regulates blood pressure by enhancing renal sodium reabsorption via activating renal epithelial sodium channels in the collecting tubules.^{28, 29} Aldosterone contributes to the development of hypertension by enhancing vascular extracellular matrix accumulation, endothelial dysfunction, and oxidative stress.²⁹

2.2.1.3 *Endothelium*

The endothelium plays a crucial role in the control of blood pressure by regulating blood vessel tone through nitric oxide production (also referred to as endothelium derived relaxing factor). In response to blood flow-induced shear stress, nitric oxide is synthesized by endothelial cells from L-arginine by the action of nitric oxide synthase. Nitric oxide induces vasodilatation by stimulating guanylyl cyclase to produce cyclic guanosine monophosphate which mediates vascular smooth muscle relaxation. Studies have demonstrated that hypertensive patients have lower levels of nitric oxide compared

with their normotensive counterparts.²⁹ Furthermore, a decrease in nitric oxide levels has been reported to potentiate the vasoconstrictor actions of angiotensin II.²⁸

In addition to nitric oxide, endothelial cells produce the potent vasoconstrictor polypeptide, endothelin 1 (ET1). By binding to ETA receptors located in the vascular smooth muscle cells, ET1 induces systemic vasoconstriction resulting in an increase in arterial blood pressure. ET1 can also elevate blood pressure by enhancing SNS.²⁸ Even though results from different studies have not consistently shown increases in ET1 levels in hypertension, studies have reported that hypertensive patients have higher sensitivity to the vascular effects of ET1.²⁹

Endothelial dysfunction strongly contributes to the development of hypertension. In addition to pressure-related vascular damage, studies have demonstrated that oxidative stress contributes to the development of endothelial dysfunction in hypertension.²⁹ Increased superoxide anions levels as a result of decreased superoxide dismutase activity reduces the bioavailability of nitric oxide by binding to it and forming the powerful oxidant peroxynitrite. Several factors including angiotensin II, ET1, catecholamines, and cigarette smoking have been reported to induce oxidative stress and play a fundamental role in the development of endothelial dysfunction by activating the superoxide anions generating enzyme, nicotinamide adenine dinucleotide phosphate oxidase.²⁹

2.2.2 Genetic factors in the pathogenesis of essential hypertension

As previously mentioned, only a small percentage of hypertensive patients (<10%) have identified secondary causes for their raised blood pressure, including chronic kidney

disease and primary aldosteronism.²⁷ The majority of hypertensive patients (>90) are diagnosed with essential hypertension, which means that hypertension cannot be related to a single specific etiologic factor.³¹ However, different pathophysiologic factors have been reported to play a crucial role in the maintenance of essential hypertension, including genetic and environmental factors, abnormalities in the blood pressure regulating mechanisms, endothelial dysfunction, and insulin resistance.³¹

Several lines of evidence indicate that genetic factors have a major effect on blood pressure. Studies have shown similarities in blood pressure in monozygotic as compared to dizygotic twins, and within families. Of note, the reported similarity in blood pressure levels is not likely to be related to shared environmental factors because studies revealed higher similarity in blood pressure amongst biological siblings than adopted siblings.²⁶ Furthermore, having one or both parents with elevated blood pressure doubles the risk of developing hypertension.³⁰ Even though particular genes have been implicated in development and maintenance of hypertension, several genetic factors are linked to the pathogenesis of hypertension. Liddle syndrome is an example of monogenetic disorders that can lead to hypertension due to mutations in the β or γ subunits in the renal epithelial sodium channels, causing activation of the channel and enhanced sodium and water retention.²⁶

2.2.3 Hypertensive heart disease

Several prospective cohort studies have revealed that high blood pressure increases the risk of premature mortality and cardiovascular complications.³² Hypertensive subjects

have more than twice the risk for developing ischemic heart disease, and more than triple the risk for developing congestive heart failure and cerebrovascular accident.³³ Several forms of heart disease including left ventricular hypertrophy, coronary heart disease, heart failure and sudden cardiac death have been etiologically linked to hypertension.³²

2.2.3.1 *Left ventricular hypertrophy*

Left ventricular hypertrophy (LVH) is defined as enlargement and thickening of the left ventricle of the myocardium. Based on echocardiographic measurements, LVH is diagnosed when left ventricular weight indexed to body surface area is $> 131 \text{ g} \cdot \text{m}^{-2}$ for men and $> 100 \text{ g} \cdot \text{m}^{-2}$ for women. The risk of developing LVH in normotensive subjects has been estimated to be 1.3%-1.6%, whereas the risk increases in subjects with mild and severe hypertension to 2.7%-5.6% and 75.6-82.6%, respectively.³³ LVH is associated with higher mortality rates for ischemic heart disease, heart failure, and cerebrovascular accident. The Framingham heart study demonstrated that hypertensive subjects with LVH have a poor prognosis irrespective of blood pressure levels.^{32, 33} LVH is also linked to a higher risk of cerebrovascular accident, myocardial infarction, and peripheral arterial disease.²⁶

Ventricular enlargement can be classified into concentric or eccentric LVH. While in concentric LVH the walls of the left ventricle thicken relative to the internal cavity, eccentric LVH mainly involves the enlargement of the intraventricular septum. Concentric LVH is commonly seen in patients with mildly to severely elevated blood pressure and is associated with normal or decreased cardiac output.³³

Several etiological factors have been implicated in the pathogenesis of LVH in hypertension including pressure overload with an increase in the total peripheral resistance, as well as neurogenic and hormonal factors.³³

A) Left ventricular pressure overload

The main determinants of pressure overload of the left ventricle in the context of hypertension are aortic stenosis and increased arterial blood pressure. Both increased intraventricular pressure and aortic stenosis result in cardiomyocyte hypertrophy and enhanced peri-myocytic extracellular matrix deposition.³³

B) Renin-angiotensin-aldosterone-system (RAAS)

Different hypertrophic factors including the components of the RAAS such as angiotensin-II and aldosterone have an important role in the development of hypertensive heart disease by inducing cardiac hypertrophy.³⁷ Experimental data suggest that the stimulation of the RAAS by suprarenal aortic constriction or intravenous administration of angiotensin-II or aldosterone can directly induce cardiac hypertrophy and fibrosis. The involvement of the RAAS in the development of LVH in hypertension is evidenced by the efficacy of the angiotensin converting enzyme inhibitors and angiotensin-II receptor blockers in preventing/regressing hypertensive LVH.³³

C) Aldosterone

Besides its blood pressure elevating effects such as enhancing sodium retention, and stimulating the SNS, aldosterone has been reported to induce cardiac fibrosis and potentiate the fibrotic effects of angiotensin-II in hypertension. Therefore, aldosterone

has a critical role in the pathogenesis of hypertension-associated LVH. It has also been suggested that effects of aldosterone with regards to the pathogenesis of hypertensive heart disease and heart failure are independent of renin and angiotensin-II because the administration of the aldosterone receptor antagonist (Aldactone) in combination with other standard heart failure medications markedly improved the outcomes in individuals with severe heart failure.³³

D) Sympathetic nervous system

It has been postulated that activation of the SNS in hypertension may contribute to the development of LVH. The role of SNS in stimulating LVH is evidenced by the presence of LVH and heart failure in subjects with pheochromocytoma, a catecholamine-secreting tumor of the medulla of the adrenal gland.³³

E) High sodium intake

Another important factor that can affect left ventricular mass in hypertension is high dietary sodium consumption. In spontaneously hypertensive rats the development of LVH was potentiated by a high-sodium diet. Furthermore, high-sodium intake caused an increase in the myocardial weight in Wistar Kyoto Rats independently of blood pressure.^{33, 34} Salt sensitivity has been identified as a critical determinant of the influence of dietary sodium on LVH since hypertensive patients with salt sensitivity are more likely to develop LVH compared with salt resistant hypertensive patients.³⁵

2.2.3.2 *Coronary heart disease*

Prospective studies have demonstrated that high-normal blood pressure and hypertension increase the risk of coronary heart disease. Different hypertension-associated factors accelerate the development of coronary artery disease including LVH, enhanced atherosclerosis in the coronary vessels, and defective endothelium dependent relaxation.^{32, 33} LVH increases the risk of ischemic heart disease by increasing the resistance of coronary blood vessels, decreasing capillaries density, and inducing myocyte hypertrophy. Coronary heart disease is an independent risk factor for cardiac arrhythmias, myocardial infarction, and sudden cardiac death.³³

2.2.3.3 *Heart failure*

Hypertension is a common risk factor for the development of cardiac structural aberrations that will eventually lead to cardiac functional abnormalities and overt heart failure. If left untreated, hypertension can lead to eccentric LVH which is usually associated with abnormalities in the extracellular matrix and cardiac fibrosis, an important determinant of diastolic dysfunction.³⁶ It has been reported that enhanced cardiac collagen deposition in hypertensive patients with LVH and clinical heart failure is associated with increased levels of tissue inhibitors of matrix metalloproteinase-1 compared with hypertensive subjects diagnosed with LVH with no clinical evidence of heart failure.³⁷ Furthermore, a study of 85 hypertensive patients indicated that serum levels of matrix metalloproteinase-2 may be more accurate than brain natriuretic peptide levels in diagnosing heart failure with preserved ejection fraction in hypertensive

subjects.³⁸ Collectively these findings indicate that enhanced cardiac fibrosis plays an important role in the transition from LVH to overt heart failure in hypertension.³⁶

2.2.3.4 *Arrhythmias and sudden cardiac arrest*

Besides being a blood pressure-independent risk factor for ischemic heart disease and heart failure, LVH is a strong risk factor for sudden cardiac death. LVH can increase the propensity for ventricular arrhythmias and premature ventricular contractions.³⁹ Atrial fibrillation is also prevalent in hypertensive patients with LVH and can lead to a significant decrease in stroke volume and heart failure. Moreover, hypertensive patients with atrial fibrillation are at increased risk for cerebrovascular accident and thromboembolism.³³

2.2.4 Prevention of hypertension and hypertensive heart disease

Averting hypertension and hypertension-induced cardiac damage continues to be a major public health hurdle. The high incidence of CVD in hypertensive patients emphasizes the importance of controlling blood pressure in hypertensive patients as well as in prehypertensive subjects. Blood pressure reduction in individuals with high normal blood pressure levels is critical to decrease the prevalence of hypertension with age and reduce the risk of CVD.²⁹ It has been estimated that even moderate blood pressure reduction can be effective in decreasing hypertension-related morbidity and mortality.^{10, 29} For example, a 5 mm Hg decrease in SBP would decrease mortality caused by cerebrovascular accident, ischemic heart disease, and all-cause mortality by 14, 9, and 7%, respectively.⁴⁰

2.2.4.1 *Lifestyle modifications*

Different non-pharmacological approaches have the potential to prevent and treat hypertension and decrease the risk of hypertension-related CVD. The most effective prevention measures are weight reduction; decreased dietary sodium intake; increased potassium intake; increased physical activity; decreased alcohol intake; and following dietary patterns based on dietary approaches to stop hypertension (DASH) diet, which is rich in fruits and vegetables and low-fat dairy products, and reduced in saturated fat and cholesterol.^{29, 41}

2.2.4.2 *Pharmacological management*

The use of antihypertensive medications at low doses has been reported to be effective in decreasing blood pressure and preventing the development of hypertension in prehypertensive subjects.²⁹ The results of the PREVER-Prevention Trial showed that treatment with chlorthalidone (a thiazide like diuretic) in combination with amiloride (a potassium-sparing diuretic) at a low-dose reduced blood pressure, prevented hypertension, and decreased left ventricular mass.⁴² It should be noted that the use of pharmacological interventions to prevent hypertension and CVD should only be considered in individuals who are at increased risk of developing hypertension even though they have strictly followed established lifestyle modification approaches.^{29, 42}

2.2.5 *Antihypertensive medications*

Blood pressure lowering drugs are one of the highly prescribed medications. Usually a combination of two or more antihypertensive drug classes is needed to achieve the

therapeutic goals within evidence-based clinical guidelines.⁴³ The established antihypertensive drug classes available for treating hypertension include diuretics, angiotensin-converting enzyme inhibitors (ACEI), angiotensin II receptor blockers, calcium channel blockers (CCBs), and β -adrenergic receptor blockers.¹⁴ Depending on the patient's blood pressure, the first-line blood pressure lowering treatment could include a single medication, or a combination of different antihypertensives. First-line blood pressure lowering medications include ACEI, angiotensin II receptor blockers, thiazide diuretics, and calcium channel blockers. The selection of a particular antihypertensive treatment should be based on different factors including the efficacy and tolerability of the medication, and ethnic origin of the patient.²⁹

2.2.5.1 *Diuretics*

The use of diuretics in the treatment of hypertension has been reported to be effective in controlling blood pressure as well as preventing cerebrovascular accident and hypertensive heart disease. There are three different classes of diuretic used to manage hypertension: A) thiazide-type and thiazide-like diuretics, B) loop diuretics, and C) potassium sparing diuretics.⁴⁴

A) Thiazide-type and thiazide-like diuretics

Thiazide-type (such as hydrochlorothiazide) and thiazide-like diuretics (such as chlorthalidone and indapamide) stimulate renal sodium excretion by inhibiting the renal sodium and chloride symporter.²⁹ Thiazide-type diuretics can be used as a monotherapy or added to β -blocker, ACEI, or angiotensin II receptor blockers to achieve optimal

control of blood pressure.⁴⁴ The use of Thiazide-type and thiazide-like diuretics necessitates monitoring serum electrolyte levels as their most common side effect is electrolyte disturbances such as hypokalemia and hyponatremia. To decrease the risk of hypokalemia thiazide-type and thiazide-like diuretics are commonly prescribed with potassium supplements or potassium-sparing antihypertensives such as ACEI, angiotensin II receptor blockers and potassium-sparing diuretics.²⁹

B) Loop diuretics

Loop diuretics such as furosemide, bumetanide, and torsemide inhibit the reabsorption of sodium, potassium, and calcium in the ascending limb of loop of Henle. Unlike thiazide-type diuretics which can lose their efficacy in cases with reduced renal function, furosemide is effective and can be safely used at high doses in patients with renal disease. The use of loop diuretics may also increase the risk of hypokalemia which can be reduced by adding potassium supplements or potassium-sparing diuretics to the treatment regimen.⁴⁴

C) Potassium-sparing diuretics

The addition of potassium-sparing diuretics to thiazide-type and thiazide-like diuretics has been reported in randomized controlled trials to prevent ventricular ectopy and sudden death.⁴⁵ Potassium-sparing diuretics including spironolactone and eplerenone are superior to potassium supplements in conserving serum and intracellular potassium levels. Spironolactone decreases blood pressure by inhibiting the mineralocorticoid receptors in the kidney and sodium-potassium exchange in the collecting duct. When

added to thiazide-type diuretics spironolactone is effective in patients with resistant hypertension. In addition to its blood pressure lowering effects, spironolactone has been shown to decrease proteinuria in patients with renal disease and revert LVH in patients with primary aldosteronism.⁴⁵

2.2.5.2 *Angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers*

ACEI and angiotensin II receptor blockers are commonly prescribed as first-line antihypertensives that decrease blood pressure by inhibiting the components of the RAAS. They have been reported to decrease the risk of CVD and improve the outcomes in patients with heart failure.^{29, 46} Side effects associated with ACEI include cough, hyperkalemia, a decrease in renal function, and angioedema.²⁹ Angiotensin II receptor blockers can also cause hyperkalemia and a decline in the renal function, but they do not commonly elicit cough and angioedema. The use of ACEI and angiotensin II receptor blockers is contraindicated in pregnant women with hypertension due to the high risk of renal teratogenicity. They are also contraindicated in lactating women because these medications are secreted in breast milk and can elicit adverse effects in breast-fed babies.²⁹

2.2.5.3 *Calcium-channel blockers*

Dihydropyridine CCBs such as amlodipine and nifedipine block the L-type calcium channels in the vascular smooth muscles, thereby decreasing systemic vascular resistance and blood pressure. They can be combined with all first-line antihypertensives with added

antihypertensive and cardioprotective effects. A common side effect of dihydropyridine CCBs is peripheral edema due to peripheral arterial vasodilatation.²⁸ Non-dihydropyridine CCBs (for example, verapamil) can also block cardiac calcium channels and decrease cardiac contractility, heart rate, and cardiac output. These cardiac effects of non-dihydropyridine CCBs result in decreased peripheral blood pressure.^{29, 43}

2.2.5.4 *β-Adrenergic receptor blockers*

β-Blockers antagonize the biological effects of epinephrine and norepinephrine and decrease the sympathetic nervous system activity by blocking β- adrenergic receptors. Decreased sympathetic nervous system activity results in decreased cardiac contractility and heart rate, thereby decreasing blood pressure. Prolonged use of β-blockers will also decrease the systematic vascular resistance and elicit a further decrease in blood pressure. β-blockers are classified into two groups: non-selective and selective β-blockers. Non-selective β-blockers (for example, propranolol and carvedilol) block both the cardiac (β₁) and bronchial (β₂) receptors; therefore, they may enhance bronchoconstriction in asthmatic patients. Selective β-blockers (for example, acebutolol and atenolol) selectively block the cardiac β-receptors; therefore, they decrease the risk of bronchoconstriction in patients with asthma. β-blockers are mainly indicated in heart failure patients with decreased left ventricular ejection fraction and myocardial infarction.^{29, 45}

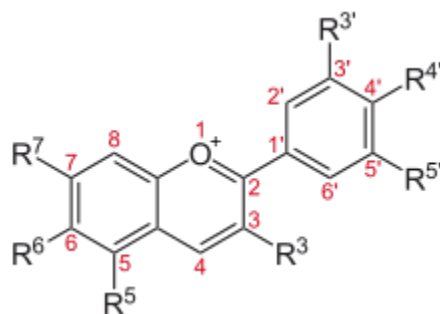
2.3 Anthocyanins

Anthocyanins are the most abundant and the largest class of polyphenolic water-soluble pigments that present ubiquitously in the plant kingdom, providing the red, blue, and

purple colors of many fruits and vegetables.¹⁵ Anthocyanins are present in significant concentrations in different cereal species, vegetables (such as eggplants, red cabbage, and blue onion), but are particularly abundant in berry fruits such as blueberries, cranberries, and raspberries. Of note, various factors can affect the concentration and quality of anthocyanins in fruits and vegetables including: species, growing conditions, and storage.⁴⁷

2.3.1 Chemistry of anthocyanins

Chemically, anthocyanins are polyhydroxylated and polymethoxylated glycosylated derivatives of flavium salts that belong to the flavonoid family of polyphenols. The most common anthocyanins are cyanidin, pelargonidin, malvidin, delphinidin, peonidin, and petunidin. The chemical structure of these anthocyanins differs based on the number of hydroxyl and methyl groups in the molecule; the type, number, and site of attachment of sugars attached to anthocyanin molecules; and the type and number of acids attached to the sugar moiety (**Figure 2.1**). The most common sugar molecules attached to anthocyanins are glucose, arabinose, galactose, rhamnose, and rutinose.⁴⁸ Stability of anthocyanins is affected by pH, temperature, and light. They are more stable at acidic conditions and degrade rapidly at higher pH levels.⁴⁹



R3, R5, and R7 = OH

R6 = H

Cyanidin: R3' = -OH , R5' = -H

Pelargonidin: R3' = -H , R5' = -H

Malvidin: R3' = -OCH3, R5' = OCH3

Delphinidin: R3' = -OH, R5' = -OH

Peonidin: R3' = -OCH3, R5' = -H

Petunidin: R3' = -OH , R5' = -OCH3

Figure 2.1: Chemical structures of the most common anthocyanins.

2.3.2 Biological activities of anthocyanins

Anthocyanins have been reported to have a wide range of biological activities including antioxidant,⁵⁰ anti-inflammatory,⁵¹ anti-carcinogenic,⁵² anti-hyperlipidemic,¹⁶ and cardioprotective activities.⁵³ Therefore, a high dietary intake of anthocyanins is highly recommended for its potential beneficial effects in various human diseases. Epidemiological studies suggest that the daily consumption of diets rich in anthocyanins is associated with a decrease in the risk of CVD;⁵³ however, it is unknown whether these cardiovascular benefits can be achieved via anthocyanin extracts or individual phytochemicals alone.

2.3.3 Cyanidin-3-O-glucoside

Cyanidin-3-O-glucoside (C3G), one of the most abundant anthocyanins in human diet, is the best known and most investigated anthocyanin. Purified C3G has been reported to have anti-oxidant,⁵⁴ anti-hyperglycemic,¹⁶ and anti-hyperlipidemic effects.¹⁷

2.3.3.1 *Anti-oxidant effects*

Enhanced oxidative stress and accumulation of reactive oxygen species play a critical role in the pathogenesis of CVD.⁵⁴ C3G has been reported to possess anti-oxidant properties in different experimental models. C3G was reported to inhibit glycated low-density lipoprotein-induced activation of NADPH oxidase and superoxide production in vascular endothelial cells (VEC) *in vitro*.¹⁸ In another study C3G prevented angiotensin II-induced oxidative stress and inflammation in VEC by upregulating superoxide dismutase and inhibiting the nuclear-factor kappa B signaling pathway.⁵³ C3G has also

been reported to inhibit cell death and oxidative stress by recovering glutathione levels and inhibiting activities of glutathione oxidase and glutathione S-transferase.⁵⁶

2.3.3.2 *Anti-diabetic effects*

Two *in vivo* studies on diabetic animals revealed that C3G has anti-diabetic effects. For example, C3G was reported to ameliorate hyperglycemia and lipid peroxidation in the streptozotocin-induced diabetic rats,⁵ and reduce fasting glucose levels and increase insulin sensitivity in high-fat diet-fed and db/db mice.¹⁶

2.3.3.3 *Other effects of C3G*

C3G was reported to protect apolipoprotein E-deficient mice from hypercholesterolemia-induced endothelial dysfunction and atherosclerosis in by maintaining the activity of endothelial nitric oxide synthase and levels of nitric oxide.¹⁷ C3G was also reported to ameliorated diet-induced cardiovascular and hepatic abnormalities in rats.¹⁸

Despite the reported beneficial effects of C3G in different models of CVD,^{5, 16-18, 20, 54-56} no studies have reported the effect of purified C3G on the development of hypertension, and its adverse effects on heart structure and function. Furthermore, based on the findings of our systematic review reported in Chapter 1, there is limited evidence surrounding the potential of purified anthocyanins in subjects with hypertension. Therefore, in the present study we first investigated the effects of purified C3G alone or in combination with the diuretic (hydrochlorothiazide, HCT) on blood pressure and cardiac structure and function in the spontaneously hypertensive rat model (SHR), a genetic model of essential hypertension. Different studies have reported that the

development of hypertension in SHR is strongly related to immune system dysfunction.⁵⁷ Our second objective, therefore, was to characterize the immune system dysfunction and investigate whether C3G and HCT can affect T-cell dysfunction in SHR.

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CHAPTER 3

Effects of 3-*O*-glucoside on maladaptive cardiac hypertrophy and diastolic heart dysfunction in 20-week-old spontaneously hypertensive rats

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B. M. Aloud, P. Raj, J. McCallum, C. Kirby, X. L. Louis, F. Jahan, L. Yu, B. Hiebert, T. A. Duhamel, J. T. Wigle, H. Blewett and T. Netticadan, Cyanidin 3-*O*-glucoside prevents the development of maladaptive cardiac hypertrophy and diastolic heart dysfunction in 20-week-old spontaneously hypertensive rats, *Food & Function*, 2018, **9**, 3466-3480. With permission from the Royal Society of Chemistry.

3.1 Rationale and hypothesis

Worldwide, hypertension continues to be a leading cause of premature mortality accounting for approximately 9.4 million deaths every year.^{1, 2} Despite the major health, social, and financial impacts of hypertension, it often remains undiagnosed and inefficiently treated.³ Hypertensive heart disease (HHD) is one of the major complications of untreated hypertension. Chronic blood pressure elevation leads to the development of left ventricular hypertrophy, a compensatory response by which the heart walls increase in size in an attempt to counter increased ventricular systolic wall stress.⁴ Prolonged cardiac hypertrophy becomes maladaptive and it is associated with a rapid decline in the contractile performance, eventually leading to heart failure.³ Despite the moderate success of established antihypertensive therapies,⁵ the high incidence of hypertension-related cardiovascular complications remains a major public-health challenge. Therefore, it is critical to explore novel therapeutic strategies to treat hypertension and prevent the development of heart failure. One important approach may be the use of food derived compounds which have specific therapeutic/preventative potential in hypertension and HHD.⁶

Cyanidin3-*O*-glucoside (C3G), one of the most abundant anthocyanins in dark fruits, vegetables, and grains, has been reported to ameliorate hyperglycemia and lipid peroxidation in streptozotocin-induced diabetic rats,⁷ and reduce fasting glucose levels and increase insulin sensitivity in high-fat diet-fed and db/db mice.⁸ Furthermore, C3G was proven to prevent hypercholesterolemia-induced endothelial dysfunction and inhibit atherosclerosis in apolipoprotein E-deficient mice by preserving endothelial nitric oxide

synthase (eNOS) activity and nitric oxide (NO) bioavailability.⁹ C3G has also been reported to reverse diet-induced cardiovascular, liver, and metabolic aberrations in rats.¹⁰ Another study showed a decrease in cecal ligation and puncture-induced mortality and mitigation of acute lung injury in C3G-treated rats.¹¹ C3G was also reported to ameliorate light-induced retinal damage in rabbits.¹² However, no study to date has investigated the effects of C3G alone or in combination with an established blood pressure lowering agent on the development of hypertension, and its adverse effects on heart. Therefore, we started our experimental approach by testing the effects of C3G on endothelin-1 (ET1)-induced cardiomyocyte cell death and hypertrophy, as well as on the proliferation of cardiac fibroblasts (CF) and fibroblast-to-myofibroblast phenoconversion. We next aimed to validate these *in vitro* results *in vivo* by testing the hypothesis that C3G alone or in combination with hydrochlorothiazide (HCT), a thiazide diuretic used frequently in the treatment of hypertension, would slow the development of hypertension, and hypertension-induced impairment of cardiac structure and function in the spontaneously hypertensive rat (SHR) model. The rationale for combining C3G and HCT treatments is that most patients with hypertension who are in stage 2 or 3 hypertension need a combination BP lowering medications to sufficiently control hypertension.¹³

We hypothesize that treatment with C3G for 15 weeks will ameliorate the development of hypertension and hypertension-induced cardiac complications in the SHR model.

3.2 Methods

3.2.1 Materials

Chemicals used in adult rat cardiomyocytes isolation, ET1, HCT, ascorbic acid, alpha-smooth muscle actin (α -SMA) mouse monoclonal antibody, lactate dehydrogenase, calcium ionophore, and cyclopiazonic acid were obtained from Sigma-Aldrich (Missouri, USA)). Dulbecco's Modified Eagle's Medium (DMEM)/F12, Spinner Minimum Essential Medium (SMEM), penicillin, and streptomycin were from Gibco (California, USA)). Pyruvate kinase was obtained from Roche Diagnostics (Mannheim, Germany). Collagenase II was from Worthington Biochemical Corporation (Minnesota, USA). Extra domain A (ED-A) fibronectin mouse monoclonal antibody was from Millipore (Massachusetts, UAS). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) rabbit polyclonal antibody was from Abcam (Massachusetts, USA).

3.2.2 Animal ethics

Experimental procedures used in this study were approved by the University of Manitoba Office of Research Ethics and Compliance and Animal Care Committee and were performed in accordance with guidelines by the Canadian Council on Animal Care and Use of Experimental Animals.

3.2.3 High-purity cyanidin 3-O-glucoside chloride preparation and quality assessment

Cyanidin-3-*O*-glucoside chloride (C3G) was extracted and purified to >98% from ProC3G powder, a commercially available, highly concentrated black rice extract

containing ~30% C3G (w/w) (Chromadex, Irvine, CA). In brief, C3G (and other anthocyanins) were extracted from ProC3G powder, using weakly acidified methanol (0.01% HCl), in a sonic bath at 37°C, for 1 hour. The resulting methanolic extract was vacuum filtered (Whatman No.4) and reduced to near dryness via rotary evaporation (Heidolph, Schwabach, Germany). The resulting solid was then reconstituted in Milli-Q water (EMD Millipore, Billerica, MA), prior to chromatographic purification via a Büchi X50 flash system (Büchi, Geneva, Switzerland), with separation of anthocyanins attained using 100 gram C18 RediSep RF Gold™ flash columns (15mm x 230mm; column volume 87.7mL), with a 20-40 µm particle size (Teledyne Isco, Lincoln, NE), as per our previously published method.¹⁴ Absorbance at 520 nm was monitored and the C3G peak was isolated from other anthocyanins via robotic fraction collection.

Each C3G fraction was purity checked via UPLC-MS/MS (Waters Canada, Mississauga, ON), and suitably pure fractions were subsequently pooled and dried using rotary evaporation. ¹H NMR spectra in CD₃OD and 5% TFA-D (Cambridge Isotope Laboratories, Andover, MA) were collected using a Bruker Avance III 600 NMR (Bruker Biospin Ltd., Milton, ON), as per previously published methods¹⁴ and were used to assess the purity of C3G batches and to monitor for possible crystallized water or solvent contaminants.

Additional purity checks relying on empirically calculated molar extinction coefficients (ϵ) were used to detect possible crystallized salt (ionic) contaminants, which would be otherwise undetectable via LC-MS or NMR techniques. Absorbance at 530nm

versus concentration (moles/L) was plotted for C3G-chloride dilution series, prepared from individual batches of C3G in 1% HCl methanol, where starting masses were precisely measured using a XP26 microbalance (Metler Toledo, Mississauga, ON), accurate to microgram quantities.

Plotting absorbance versus molar concentration, ϵ can be determined as the slope of the line, according to the Beer-Lambert law:

$$A = \epsilon cl,$$

where A = absorbance; c = concentration (moles/L) using a molecular weight of 484.84 g/mol for cyandin-3-*O*-glucoside chloride; l = spectrophotometer path length of 1 cm; and ϵ is the molar extinction coefficient.

C3G batches were deemed free from possible crystalized salt contaminants, when empirically determined ϵ were found to lie in the range of literature values of 30200 and 34300 $M^{-1}cm^{-1}$, as reported by Swain, and Siegelman and Hendricks.^{15, 16}

Individual batches of C3G that met stringent purity (98+%) requirements were mixed together, homogenized, and subsequently aliquoted into individual 50 or 100mg dosed vials, and stored at -20°C until required for gavage. Due to the number of animals used and the duration of the feeding trial, a total of 14 grams of highly purified C3G chloride was prepared for this study.

3.2.4 Adult rat cardiomyocyte isolation and culture

Ventricular myocytes were isolated from twelve-week-old male Sprague-Dawley rats (200-250 g) following the procedure established by us previously.¹⁷ In brief, hearts were washed with calcium-free buffer (mmol/L: NaCl 90, KCl 10, KH₂PO₄ 1.2, MgSO₄ · 7H₂O 5.0, NaHCO₃ 15, taurine 30, glucose 20, pH 7.4) for approximately 5 minutes and then transferred to a Langendorff apparatus and perfused for 30 minutes with calcium-free buffer containing collagenase II (0.05%) and bovine serum albumin (0.2%). After perfusion, hearts were removed from the Langendorff apparatus and ventricles were cut into small pieces and transferred to recirculated collagenase buffer (at 37°C) for further digestion. Cardiomyocytes were resuspended in medium-199 supplemented with 5% fetal bovine serum, 5% horse serum, and penicillin/streptomycin (100 IU/mL). Finally, cells were transferred to culture dishes precoated with laminin (10 µg/mL) and incubated at 37°C and 5% CO₂ for 2 hours. The culture medium was then replaced with serum-free medium-199 supplemented with 5 mmol/L taurine, 2 mmol/L l-carnitine, 1 mmol/L creatine, 1 µmol/L insulin, and 100 IU/mL penicillin/streptomycin. Cells were incubated overnight at 37°C and 5% CO₂ prior to further experimental procedures.

3.2.5 Assessment of cardiomyocyte viability

Four different concentrations of C3G were tested to determine their potential in preventing ET1-induced cardiomyocyte death: 7.5, 15, 30, and 60 µM. Cardiomyocytes were separated into different groups: (untreated) control group, cardiomyocytes stimulated with ET1 (10⁻⁷ M), and cardiomyocytes pretreated with 4 different concentrations of C3G (7.5, 15, 30, and 60 µM) for 45 minutes and then co-incubated with ET1 (10⁻⁷ M) for 24 hours. The dose of ET1 was taken from previous studies carried

out on cardiomyocytes.¹⁸ Following treatments, images of cardiomyocytes were taken using a phase contrast microscope (Olympus, Ontario, Canada). Cardiomyocyte cell viability was measured by calculating the ratio of rod-shaped cells to round-shaped cells using ImageJ software (National Institutes of Health, Maryland, USA).^{19, 20}

3.2.6 Measurement of Cardiomyocyte cell size

Based on data obtained from the cell viability assessment experiments, 60 μ M C3G was the dose selected for further analyses. Hypertrophic growth of cardiomyocytes was assessed by measuring surface area of individual cardiomyocytes, as previously described.¹⁹ After incubation with treatments for 24 hours, images of cardiomyocytes were taken using a phase contrast microscope (Olympus, Ontario, Canada).

3.2.7 Isolation and culture of cardiac fibroblasts from adult rats

CFs were isolated from adult male Sprague-Dawley rats (150–200 g). Isolated hearts were perfused with (DMEM)/F12 using the Langendorff perfusion system followed by SMEM. The hearts were then digested for 20 minutes at room temperature with SMEM containing 0.1% w/v collagenase II and minced for 15 minutes in diluted collagenase solution (0.05% w/v). Minced tissue was placed into a 50 mL centrifuge tube and suspended in DMEM-F12 growth medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin and 1 μ M ascorbic acid and large tissue pieces were allowed to settle to the bottom of the centrifuge tube. Supernatant was transferred to another 50 mL centrifuge tube and centrifuged at 2000 rpm for 5 minutes. The supernatant was removed, and cell pellets were re-suspended in growth medium and plated on to 6-

well plates. Cells were incubated for 3-4 hours at 37°C and 5% CO₂, then washed 2-3 times with phosphate buffered saline (PBS) and maintained in fresh growth medium overnight. The following day, cells were washed 2 more times with PBS and fresh medium was added. Cells were allowed to grow at 37°C and 5% CO₂ for another 24 hours before further experimental procedures. As ET1 has been reported to stimulate CF proliferation and differentiation into myofibroblasts^{21,22} CF were stimulated with ET1 for 48 hours. To assess the potential of C3G in modulating proliferation and differentiation of CF into myofibroblasts, cells were separated into 4 groups: untreated (control group), cells stimulated with ET1 (10⁻⁷ M), cells treated with 60 µM C3G, and cells co-incubated with ET1 (10⁻⁷M) and 60 µM C3G for 48 hours.

3.2.8 Western blot analysis

Following incubation with indicated treatments, cells lysates were prepared in RIPA lysis buffer. ED-A fibronectin and α -SMA were detected by western blot analysis. Protein samples (10 µg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a 0.45 µM nitrocellulose membrane using an electrophoretic transfer tank. Membranes were then blocked in 1x tris-buffered saline containing 5% skimmed milk for 1 hour at room temperature with constant shaking. Membranes were probed with primary antibodies for ED-A fibronectin (1:2000) and α -SMA (1:5000) and incubated for 1 hour at room temperature. Appropriate secondary antibodies were used at 1:5000 dilution and incubated for 1 hour at room temperature with constant shaking. Membranes were probed with GAPDH antibody

(1:2000) as a loading control to account for loading differences between the samples. Protein bands were detected using Western Blotting Luminol Reagent and images were developed on CL-Xposure blue X-ray films using Flour S Max Multi Imager (Biorad, Hercules, CA). Protein expression was quantified by taking adjusted volume of bands measured via densitometry (GS-800 Calibrated Densitometer, Bio-Rad, USA) using QuantityOne software (Bio-Rad, USA). Adjusted volumes of bands were normalized by the respective loading controls.

3.2.9 Animal Model and study design

Experimental procedures used in this study were approved by the University of Manitoba Office of Research Ethics and Compliance and Animal Care Committee, and were performed in accordance with guidelines by the Canadian Council on Animal Care and Use of Experimental Animals. Five-week-old male SHRs and their age-matched controls, Wistar-Kyoto rats (WKY), obtained from Charles River Inc., Quebec, Canada, were used in this study. WKY and SHR rats were treated daily by oral gavage for a period of 15 weeks with vehicle (deionized water), C3G (10 mg/kg/day) and/or HCT (10 mg/kg/day) in a 2 x 2 x 2 factorial design. C3G and HCT were dissolved in deionized water. A total of 8 groups (8 rats per group) were examined. (1) WKY treated with vehicle, (2) WKY treated with C3G, (3) WKY treated with HCT, (4) WKY treated with C3G+HCT; (5) SHR treated with vehicle, (6) SHR treated with C3G, (7) SHR treated with HCT, and (8) SHR treated with C3G+HCT.

3.2.10 Blood and tissue collection

At the end of the study period, all rats were weighed and anesthetized with ketamine/xylene injection. Non-fasting (random) blood samples were collected with and without heparin and centrifuged at 2500 rpm to obtain plasma and serum which were stored at -80°C for further assessments. Heart tissues were excised, cleaned, and weighed. The atria and the right ventricle were removed, and the left ventricle was weighed. The separated heart tissues were flash frozen in liquid nitrogen and stored at -80°C for further analyses. Tibia length was measured and heart weight and left ventricular weights (both normalized to tibia length) were calculated. Thoracic aortas were excised, loosely connected fibrous tissue cleaned off, cut into rings 5 mm in length, and embedded in optimal cutting temperature (OCT) compound in an upright position, and stored at -80°C for further analyses.

3.2.11 Blood Pressure Measurements

Blood pressure measurements were conducted on all groups of animals at 1, 8, and 15 weeks after the commencement of the treatment using a CODA multichannel, noninvasive blood pressure system (Kent Scientific, Torrington, CT, USA) as previously described.²³

3.2.12 Examination of cardiac structure and function *in vivo*

Cardiac structure and function were assessed in all groups of animals using transthoracic echocardiography at 15 weeks after the start of the treatments as described previously.²⁴ Rats were weighed and anaesthetized with isoflurane and echocardiograms were obtained

by two-dimensionally (2D) guided M-Mode and Pulsed-Wave Doppler imaging modalities with a Sonos 5500 ultrasound system (Agilent Technologies, Massachusetts, USA) equipped with a 12-MHz (s12) transducer. 2D M-mode measurements included percentage of left ventricular fractional shortening (FS), left ventricular ejection fraction (EF), cardiac output (CO), left ventricular mass, interventricular septal wall (IVS) thickness, left ventricular posterior wall (LVPW) thickness at systole and diastole, and left ventricular internal dimensions (LVID) at systole and diastole. Doppler measurements included isovolumetric relaxation time (IVRT), peak early diastolic filling velocity (E wave), and peak late diastolic filling velocity (A wave).

3.2.13 Biochemical assessments

Lipid peroxidation levels were estimated in blood and heart tissues by measuring the amount of the lipid peroxidation product, malondialdehyde (MDA) using a thiobarbituric acid reactive substance (TBARS) assay kit (Cell Biolabs, California, USA). Activities of the major antioxidant enzymes superoxide dismutase (SOD) and catalase in heart tissue were measured using Cayman Chemicals assay kits (Michigan, USA). Levels of hydroxylproline (an indicator of collagen content) in the heart tissue were measured using the hydroxyproline assay kit (Sigma-Aldrich, Ontario, Canada) as described previously.²⁵ The concentrations of inflammatory cytokines, tumor necrosis factor alpha (TNF α), Interleukin 6 (IL-6), and Interferon gamma (IFN γ) in the heart tissue were determined using the MSD multiplex assay kit (Meso Scale Discovery, Maryland, USA). Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, urea, glucose, cholesterol, high-density lipoprotein (HDL), and triglycerides were measured

using a Cobas c111 clinical chemistry analyzer (Roche Diagnostics, Quebec, Canada).

All assays were conducted according to the manufacturer's instructions.

3.2.14 Histological evaluation of the aorta

Aortic samples embedded in OCT were cut into 8 μm sections (three sections per slide) using a cryostat (Leica Biosystems, Nussloch, Germany). Slides were kept at -80°C until stained. To assess histological changes of the aortic wall, sections were stained with hematoxylin and eosin (H & E) according to the manufacturer's instructions (Sigma-Aldrich (Missouri, USA)). Three aortic sections for each rat were randomly selected and stained. The stained sections were examined with light microscopy (Axioskop 2 mot plus microscope, Carl Zeiss, Oberkochen, Germany). Images were captured using 4 X and 20 X objectives with a digital microscope camera (AxioCamStemi 2000 camera). Image-pro analytical software (Maryland, USA) was used to measure wall thickness, lumen diameter, and wall to lumen ratio. Three images of each stained aortic sections were analyzed. Aortic structure data analysis was carried out in a blinded manner.

3.2.15 Assessment of sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA)

Activity

The kinetic properties of Ca^{2+} -dependent SERCA ATP hydrolysis (i.e. SERCA activity) were examined using a spectrophotometric assay²⁶ modified by Duhamel et al.²⁷ for use on a plate reader (SPECTRAmax, Molecular Devices, California, USA). Left ventricular tissue homogenates were mixed with 4.25 mL of buffer cocktail containing ATPase buffer, lactate dehydrogenase and pyruvate kinase. To allow calcium to permeate cell

membranes, 8.8 μL calcium ionophore was added to the assay buffer. Calcium-dependent activity was then measured by varying the amount of calcium (10-25 μL 10mM CaCl_2) loaded in an Eppendorf tube that contained 250 μL buffer cocktail and left ventricle protein. To determine basal ATPase activity, 2 μL of the SERCA inhibitor cyclopiazonic acid (CPA) was used in one sample, and this basal activity was subtracted from total Ca^{2+} -stimulated ATPase activity, which enabled the calculation of Ca^{2+} -stimulated SERCA activity. After plotting a graph of enzyme activity versus Ca^{2+} concentration, maximal enzyme activity (V_{max}), Ca_{50} and the Hill slope were calculated using GraphPad Prism software. Ca_{50} was defined as the concentration of Ca^{2+} that elicits 50% of V_{max} when graphed using a sigmoidal fit of the data. The Hill slope was defined as the slope of the relationship between Ca^{2+} and SERCA activity, obtained by using the portion of the SERCA activity curve that corresponds to 10-90% V_{max} .²⁸ Additionally, we determined the relative amounts of passive Ca^{2+} leak across the SR membrane by comparing the ratio between V_{max} in the presence and absence ($V_{\text{max}(-)}$) of 1 μM Ca^{2+} -ionophore (ionophore ratio). Maximal SERCA activity (V_{max}) and $V_{\text{max}(-)}$ were both determined at pCa 6.13.

3.2.16 Statistical analyses

Sample size of 8 rats per group was determined using a standard deviation of 5.7 to pick up a systolic blood pressure difference of 8 mmHg at a power of 80% and significance level of 0.05%.²⁹ Two-way ANOVA was utilized to analyze the effects of ET1 (factor 1) and C3G (factor 2) and their interaction in the *in vitro* data. For the *in vivo* experiments, a three-way ANOVA was utilized to analyze the effect of C3G (factor 1), HCT (factor 2), genotype (factor 3), and their interaction effects for all outcome variables measured at

one time point. For BP, a repeated measures three-way ANOVA was utilized to analyze the effect of C3G (factor 1), HCT (factor 2), and genotype (factor 3) on BP over time. Mixed models were applied to all repeated measure outcome variables. Time was included in the model as both a fixed and random effect. Type 3 fixed effects and corresponding p-values were calculated for C3G, HCT, genotype, time point, and interaction effects. Least square means were calculated for each treatment group, genotype, and time period combination and expressed as mean $\pm$ SEM. Post-hoc testing was performed comparing each treatment, genotype, and time period combination. Multiple comparisons for post-hoc testing were accounted for by controlling the False Discovery Rate (FDR) with an FDR adjusted p-value of 0.05 considered statistically significant (Benjamini & Hochberg). For the *in vitro* studies, we used a one-way Analysis of Variance (ANOVA) to determine if different doses of C3G protected against ET1-induced cell death. All statistical analysis was performed using SAS statistical software (version 9.4; SAS Institute Inc. Cary, NC, USA).

3.3 Results

3.3.1 Effect of ET1 and C3G on Cardiomyocyte viability

Cardiomyocytes exposed to ET1 for 24 hours had a lower ratio of rod-shaped to round-shaped cells (indicating reduced cell viability) as compared with the un-stimulated control cardiomyocytes. Pretreatment with C3G (30 and 60 μ M) for 45 minutes significantly protected against ET1-induced cell death as compared with untreated cardiomyocytes stimulated with ET1. Pretreatment of cardiomyocytes with lower concentrations of C3G

(7.5 and 15 μ M) did not protect against ET1-induced cell death (**Figure 3.1A**). The most effective dose of C3G (60 μ M) was used for all subsequent *in vitro* experiments.

3.3.2 Effect of C3G on ET1-induced Cardiomyocyte hypertrophy

Cardiomyocyte surface area was significantly higher in the ET1-exposed group as compared with un-stimulated control cardiomyocytes. Pre-treatment with C3G (60 μ M) for 45 minutes prevented ET1-induced cardiomyocyte hypertrophy as compared with untreated cardiomyocytes stimulated with ET1. Exposing cardiomyocytes to C3G (60 μ M) alone for 24 hours had no significant effect on cardiomyocyte size as compared with un-stimulated control cardiomyocytes (**Figure 3.1B**).

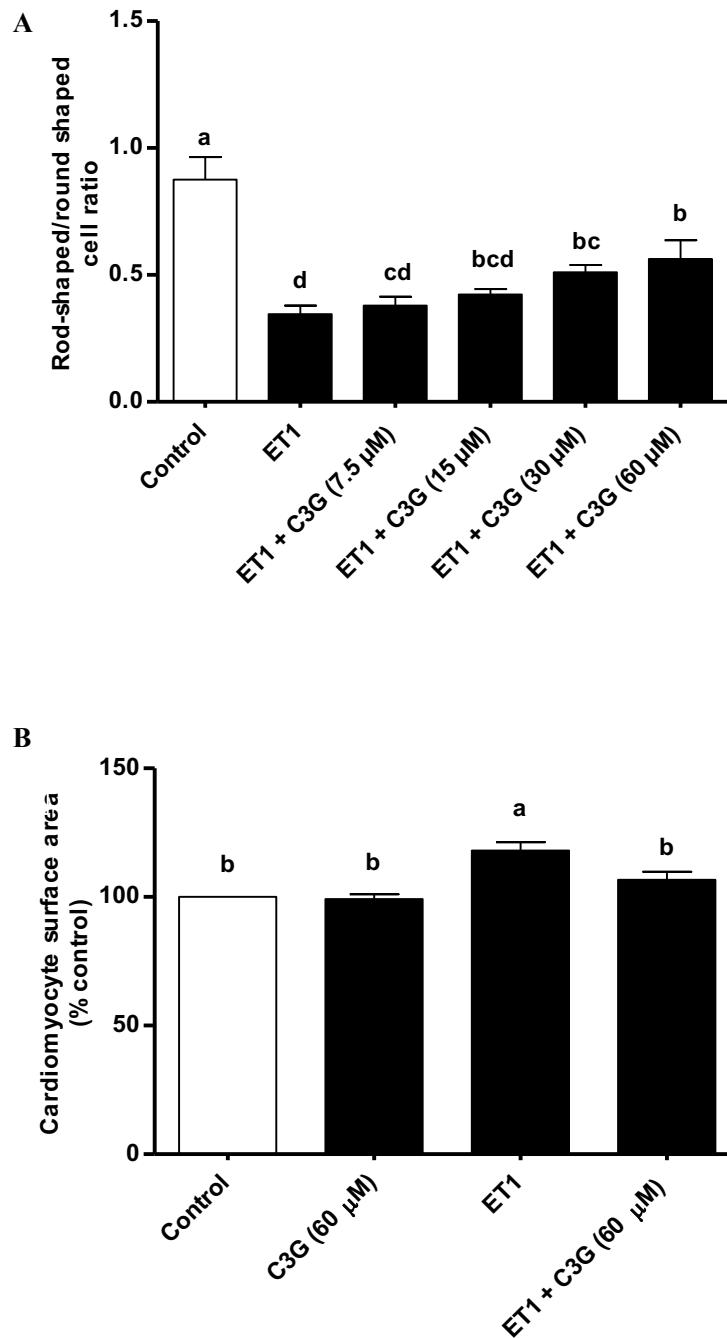


Figure 3.1: Morphological changes in adult rat cardiomyocytes treated with C3G and/or ET1 (10^{-7} M). (A) Effect of ET1 and different concentrations of C3G (7.5, 15, 30, and

60 μ M) on cardiomyocyte viability. Values are means $\pm$ SEMs (n = 100 cells from 5 different rats). (B) Effect of C3G (60 μ M) on ET1-induced cardiomyocyte hypertrophy. Values are means $\pm$ SEMs (n = 100 cells from 4 different rats). Labeled means with different letters are significantly different from each other, $P \leq 0.05$. C3G, Cyanidin 3-*O*-glucoside; ET1, endothelin-1.

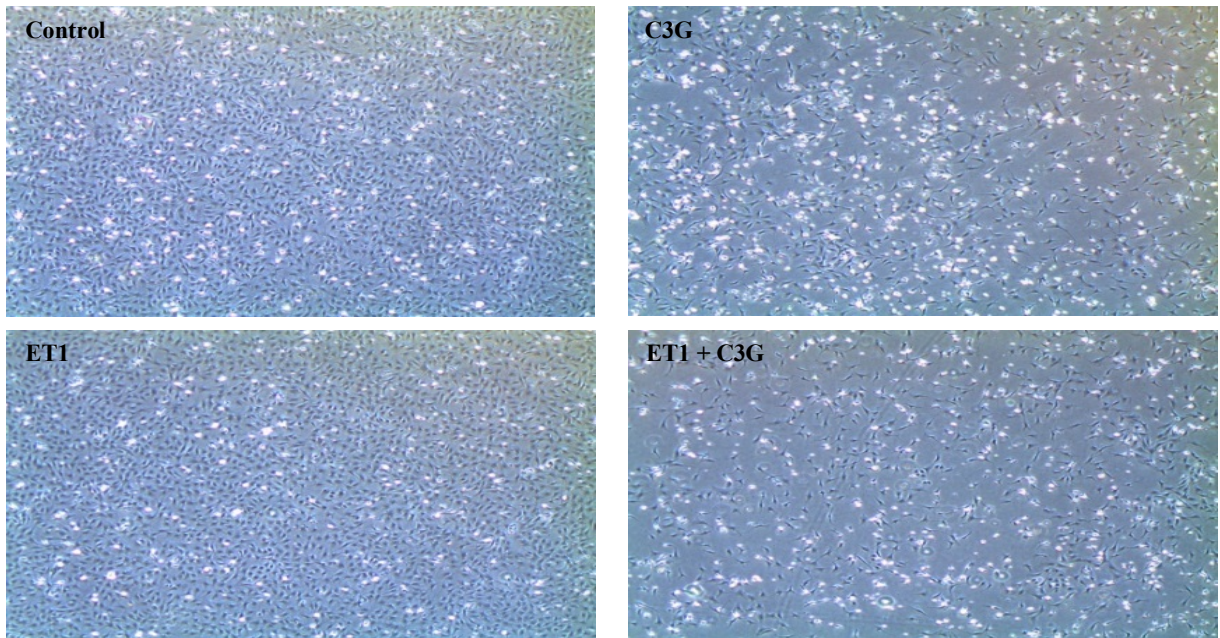
3.3.3 Effect of ET1 and C3G on cardiac fibroblast cell number

Exposing CF to ET1 for 48 hours did not change cell number as compared with unstimulated control cells. CF treated with C3G (60 μ M) alone or with a combination of C3G and ET1 for 48 hours had a significantly lower number of cells as compared with control cells or cells stimulated with ET1 (**Figure 3.2A**).

3.3.4 Effect of ET1 and C3G on the expression of myofibroblast markers

The expression levels of ED-A fibronectin and α -SMA in CF exposed to ET1 for 48 hours were not significantly different from untreated control CF. ED-A fibronectin and α -SMA protein levels were significantly lower in the C3G treated group as compared with untreated control CF. These findings show that the C3G effect is not simply a prevention of ET1 effect. There was no significant difference in the protein levels of ED-A fibronectin and α -SMA between control CF and CF exposed to C3G+ET1 (**Figure 3.2B**).

A



B

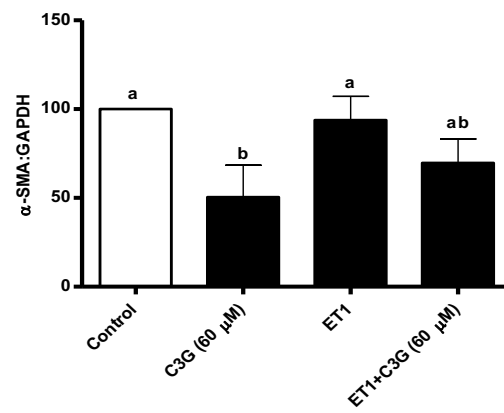
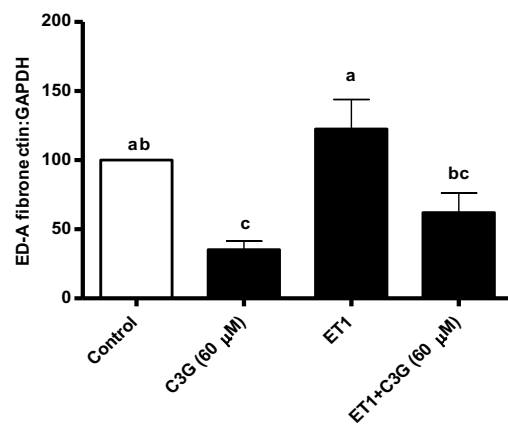
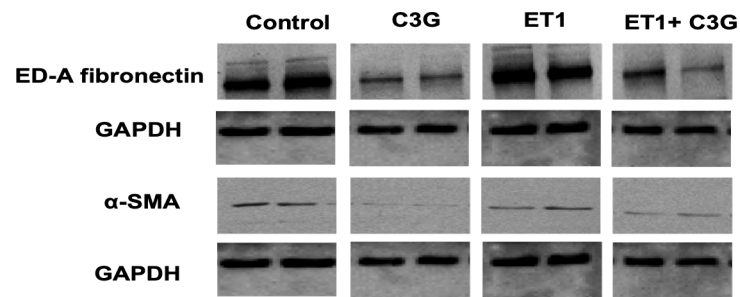


Figure 3.2: Effect of C3G (60 μ M) or ET1 (10⁻⁷ M) on fibroblast growth and activation. (A) Effect of ET1 and C3G on cardiac fibroblast cell number. (B) Western blot analysis and histographic representation of ED-A fibronectin and α -SMA expression in cardiac fibroblasts exposed to ET1 and C3G. Values are means $\pm$ SEMs (n =4 rats). Labeled means with different letters are significantly different from each other, $P \leq 0.05$. C3G, Cyanidin 3-*O*-glucoside; ET1, endothelin-1; ED-A fibronectin, extra domain A fibronectin; α -SMA, Alpha-smooth muscle actin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

3.3.5 Body weights

There was no significant effect of C3G or HCT on final body weights in WKY rats or SHRs. There was no significant interaction between C3G and HCT treatments on final body weights in WKY rats or SHRs (**Figure 3.3A**).

3.3.6 Left ventricle weight to body weight and left ventricle weight to tibia length ratios

SHRs treated with C3G, HCT, and C3G+HCT had significantly lower left ventricle weight to body weight ratio, as well as left ventricle weight to tibia length ratio compared to control SHRs (**Figures 3.3B and 3.3C**).

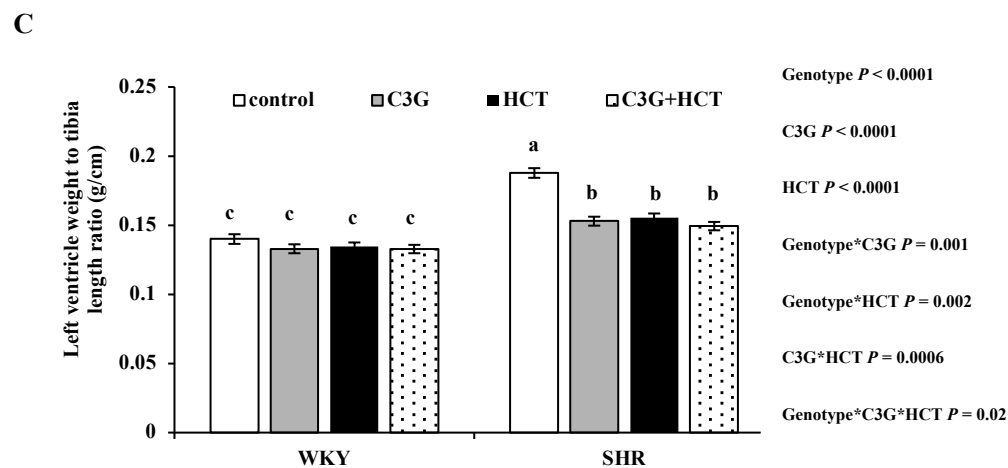
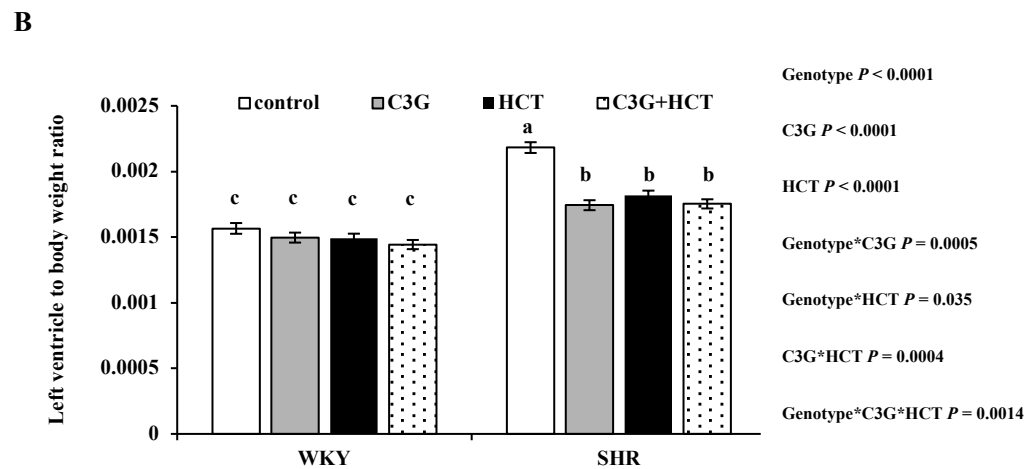
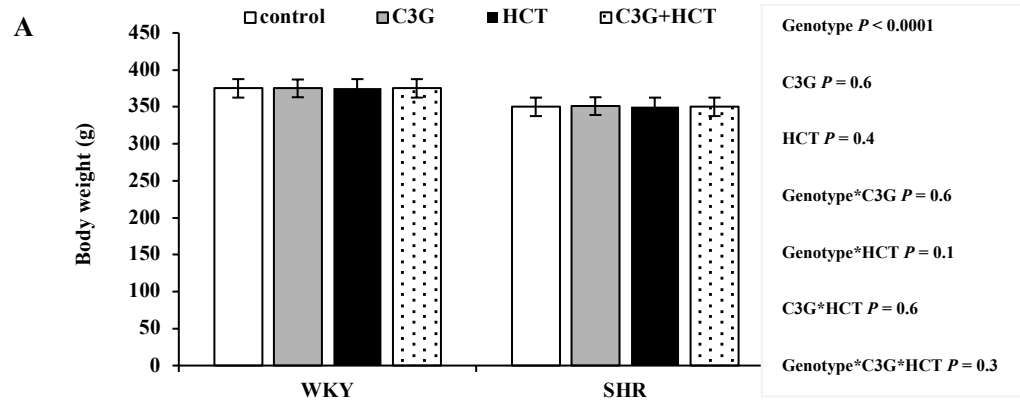


Figure 3.3: Effect of C3G (10 mg/kg/day), HCT (10 mg/kg/day), and C3G+HCT (10 mg/kg/day each) on body weights and left ventricle hypertrophy indices of WKY rats and SHRs. (A) Final body weights. (B) Left ventricle weight to body weight ratio. (C) Left ventricle weight to tibia length ratio. Values are means $\pm$ SEMs (n = 8 rats). Labeled means with different letters are significantly different from each other, $P \leq 0.05$. C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHRs, spontaneously hypertensive rats.

3.3.7 Systolic blood pressure

HCT treatment slowed the rise of SBP in the SHR overtime (week 1: SHRs control = 161 ± 6.3 mmHg, SHRs HCT = 129 ± 6.3 mmHg; Week 15: SHRs control = 201 ± 7.3 mmHg, SHRs HCT = 168 ± 7.3 mmHg), but C3G had no effect in these animals (week 1: SHRs control = 161 ± 6.3 mmHg, SHRs C3G = 126 ± 6.3 mmHg; Week 15: SHRs control = 201 ± 7.3 mmHg, SHRs C3G = 186 ± 7.3 mmHg). The combination of C3G and HCT had a greater effect than either treatment alone ($P = 0.006$) (**Figure 3.4A**).

3.3.8 Diastolic blood pressure

HCT treatment slowed the rise of DBP in SHRs overtime (week 1: SHRs control = 130 ± 6.2 mmHg, SHRs HCT = 100 ± 6.2 mmHg; Week 15: SHRs control = 146 ± 5.4 mmHg, SHRs HCT = 117 ± 5.4 mmHg) but C3G had no effect (week 1: SHRs control = 130 ± 6.2 mmHg, SHRs C3G = 102 ± 6.1 mmHg; Week 15: SHRs control = 146 ± 5.4 mmHg, SHRs C3G = 140 ± 5.4 mmHg) (**Figure 3.4B**).

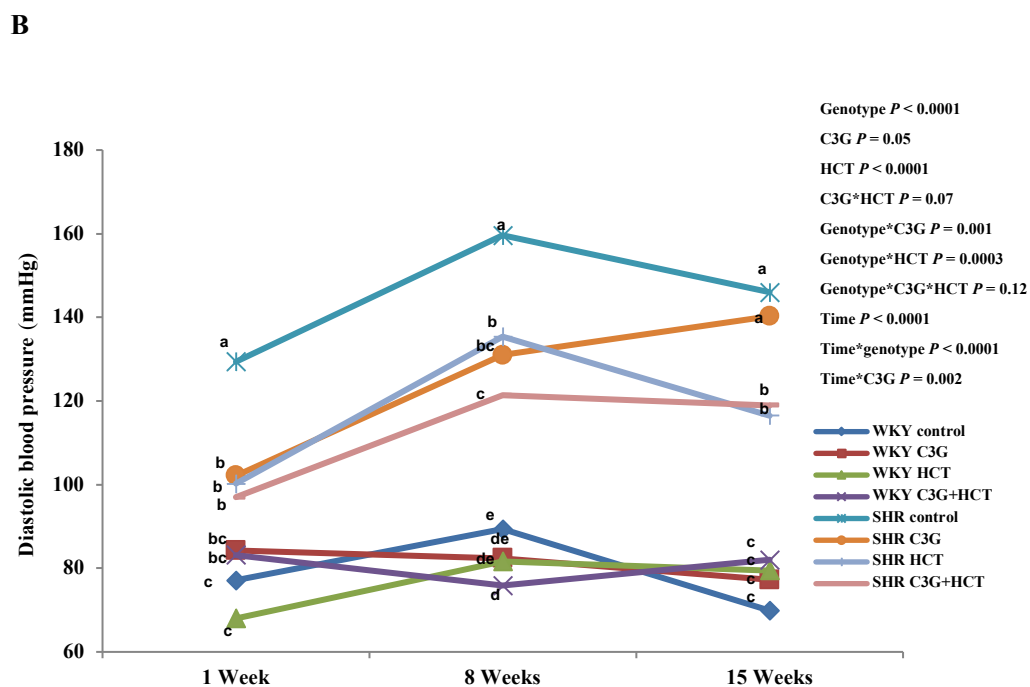
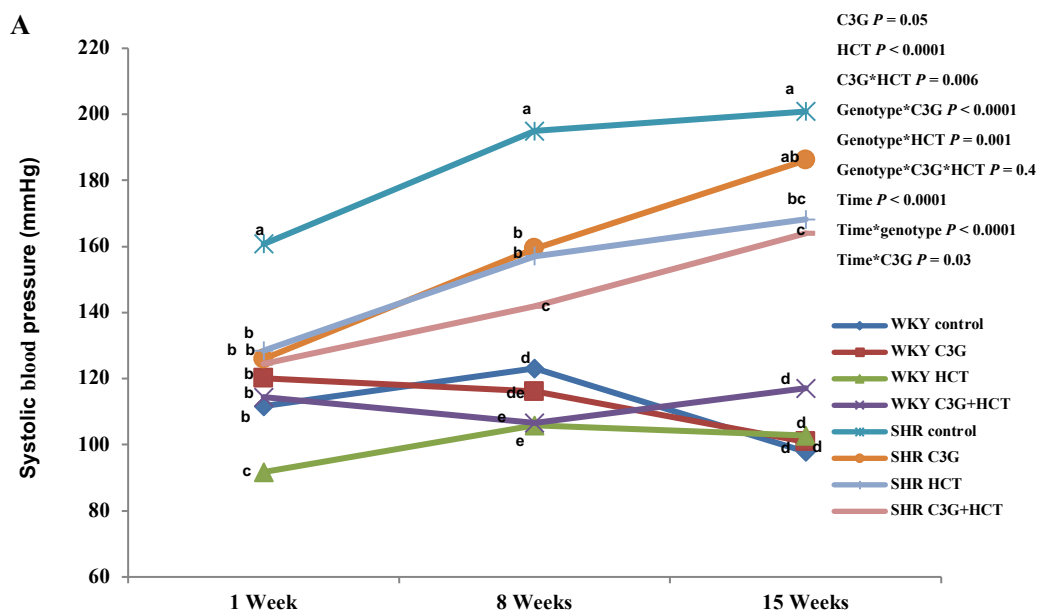


Figure 3.4: Effect of C3G (10 mg/kg/day), HCT (10 mg/kg/day), and C3G+HCT (10 mg/kg/day each) on blood pressure in WKY rats and SHRs at 1, 8, and 15 weeks. (A) Systolic blood pressure. (B) Diastolic blood pressure. Values are means $\pm$ SEMs (n = 8 rats). Labeled means within the same time point with different letters are significantly different from each other, $P \leq 0.05$. C3G 0.05. C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

3.3.9 Cardiac structure

3.3.9.1 *Interventricular septal wall thickness at systole (IVSs)*

Treatment with C3G or HCT resulted in a significant decrease in IVSs in SHR rats but not in WKY rats. The combination treatment of C3G and HCT was not different than either treatment alone (**Table 3.1**).

3.3.9.2 *Interventricular septal wall thickness at diastole (IVSd)*

Treatment with C3G or HCT resulted in a significant decrease in IVSd in SHR rats but not in WKY rats. The combination treatment of C3G and HCT had greater effect than either treatment alone (**Table 3.1**).

3.3.9.3 *Left ventricular internal dimensions at systole (LVIDs)*

SHRs had significantly higher LVIDs compared to WKY rats. There was no significant effect of C3G or HCT treatment in both WKY rats and SHRs (**Table 3.1**).

3.3.9.4 *Left ventricular internal dimensions at diastole (LVIDd)*

There was no significant effect of genotype on LVIDd. There was no significant effect of C3G or HCT treatment on LVIDd in WKY rats or SHRs (**Table 3.1**).

3.3.9.5 *Left ventricular posterior wall thickness at systole (LVPWs)*

There was no significant effect of C3G on LVPWs. HCT treatment lowered LVPWs significantly in SHRs (**Table 3.1**).

3.3.9.6 *Left ventricular posterior wall thickness at diastole (LVPWd)*

When used alone, both C3G and HCT decreased LVPWd significantly in SHR. The combination of C3G and HCT had a greater effect on LVPWd than either treatment alone, and the lowering effect was greater in SHR rats (**Table 3.1**).

	WKY				SHRs				<i>P</i> values						
	Control	C3G	HCT	C3G+HCT	Control	C3G	HCT	C3G+HCT	Genotype	C3G	HCT	Genotype*C3G	Genotype*HCT	C3G*HCT	Genotype*C3G*HCT
IVSs, mm	2.5 +/- 0.1 ^c	2.5 +/- 0.1 ^c	2.6 +/- 0.1 ^{b,c}	2.7 +/- 0.1 ^{b,c}	3.2 +/- 0.08 ^a	2.8 +/- 0.08 ^b	2.8 +/- 0.08 ^b	2.7 +/- 0.09 ^{b,c}	<0.0001	0.08	0.15	0.03	0.001	0.08	0.12
IVSd, mm	1.6 +/- 0.08 ^c	1.6 +/- 0.08 ^c	1.5 +/- 0.08 ^c	1.6 +/- 0.09 ^{b,c}	2.2 +/- 0.07 ^a	1.8 +/- 0.07 ^b	1.8 +/- 0.07 ^b	1.8 +/- 0.07 ^b	<0.0001	0.08	0.02	0.02	0.20	0.01	0.39
LVIDs, mm	4.0 +/- 0.2	4.1 +/- 0.2	3.9 +/- 0.2	3.8 +/- 0.2	4.4 +/- 0.3	4.5 +/- 0.2	4.2 +/- 0.2	4.0 +/- 0.2	0.04	0.68	0.06	0.85	0.53	0.36	0.67
LVIDd, mm	6.8 +/- 0.2	6.9 +/- 0.2	7.3 +/- 0.2	6.9 +/- 0.2	6.9 +/- 0.2	7.2 +/- 0.2	7.2 +/- 0.2	6.7 +/- 0.2	0.95	0.23	0.62	0.69	0.07	0.01	0.44
LVPWs, mm	2.7 +/- 0.1 ^c	2.7 +/- 0.1 ^c	2.8 +/- 0.1 ^c	2.6 +/- 0.1 ^c	3.1 +/- 0.1 ^a	2.9 +/- 0.1 ^{a,b}	2.7 +/- 0.1 ^{b,c}	2.7 +/- 0.1 ^{b,c}	0.01	0.13	0.04	0.95	0.03	0.73	0.34
LVPWd, mm	2.0 +/- 0.1 ^b	2.0 +/- 0.1 ^b	1.9 +/- 0.1 ^b	1.9 +/- 0.1 ^b	2.4 +/- 0.1 ^a	1.8 +/- 0.1 ^b	1.8 +/- 0.1 ^b	1.9 +/- 0.1 ^b	0.96	0.02	0.01	0.04	0.38	0.01	0.01

Table 3.1: Effect of C3G (10 mg/kg/day), HCT (10 mg/kg/day), and C3G+HCT (10 mg/kg/day each) on cardiac structure.

Values are means $\pm$ SEMs (n = 8 rats). Labeled means in a row with different letters are significantly different from each other, $P \leq 0.05$. C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; IVSs, Interventricular septal wall thickness at systole; IVSd, Interventricular septal wall thickness at diastole; LVIDs, left ventricular internal dimensions at systole; LVIDd, left ventricular internal dimensions at diastole; LVPWs, left ventricular posterior wall thickness at systole; LVPWd; left ventricular posterior wall thickness at diastole.

3.3.10 Cardiac function

3.3.10.1 *Fractional shortening (FS)*

There was no significant effect of C3G on FS in WKY rats or SHR. Treatment with HCT caused a significant increase in FS in WKY rats and SHR (Figure 3.5A).

3.3.10.2 *Ejection fraction (EF)*

SHR had significantly lower EF compared to WKY rats. There was no significant effect of C3G or HCT or C3G+HCT on EF in WKY rats or SHR (Figure 3.5B).

3.3.10.3 *Isovolumetric relaxation time (IVRT)*

SHR treated with C3G had significantly shorter ($P = 0.045$) IVRT (the diastolic function parameter) compared to control SHR (25.1 ± 0.5 and 21.1 ± 0.5 ms, respectively). Likewise, SHR treated with HCT had significantly shorter IVRT compared with control SHR (25.1 ± 0.5 and 20.4 ± 0.5 ms, respectively). The combination treatment of C3G and HCT had less of an effect on IVRT than either treatment alone, and this effect was lesser in SHR compared to WKY rats (Figure 3.5C).

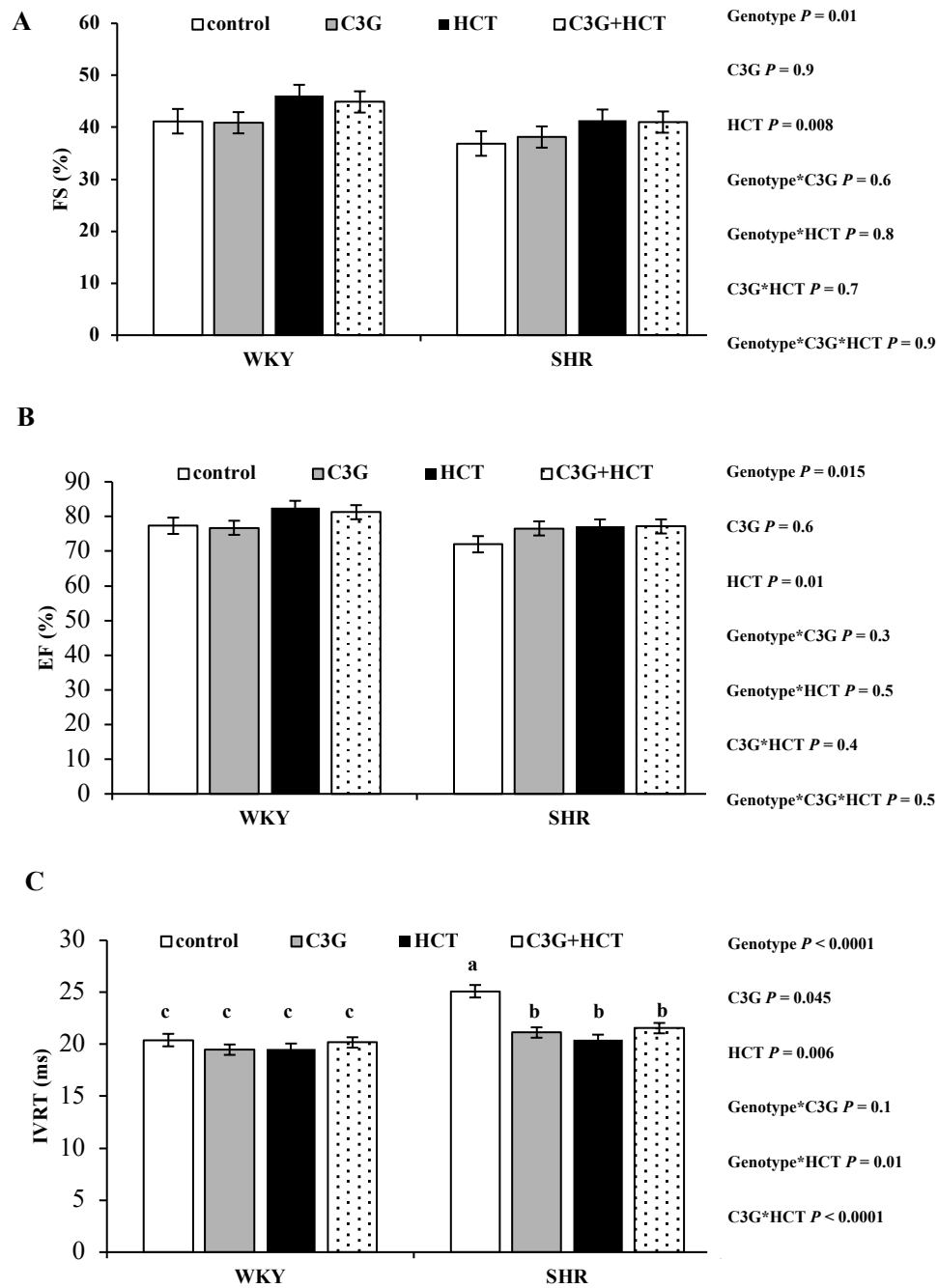


Figure 3.5: Effect of C3G (10 mg/kg/day), HCT (10 mg/kg/day), and C3G+HCT (10 mg/kg/day each) on cardiac function in WKY rats and SHRs at 15 weeks. (A) Fractional shortening. (B) Ejection fraction. (C) Isovolumetric relaxation time. Values are means $\pm$

SEMs (n = 8 rats). Labeled means with different letters are significantly different from each other, $P \leq 0.05$. C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHRs, spontaneously hypertensive rats; FS, fractional shortening; EF, ejection fraction; IVRT, isovolumetric relaxation time.

3.3.11 Oxidative stress

SHRs showed significantly higher plasma MDA levels in comparison to WKY rats. Treatments were ineffective in both WKY rats and SHRs (**Table 3.2**) in reducing these levels. There was no significant effect of C3G or HCT treatment on MDA levels in the heart tissue (**Table 3.2**).

3.3.12 Antioxidant enzymes

C3G treatment caused a significant increase in SOD activity only in WKY rats. There was no significant effect of HCT on SOD activity. There was no significant effect of C3G or HCT on catalase activity (**Table 3.2**).

3.3.13 Inflammatory and cardiac fibrosis markers

There was no significant effect of C3G or HCT on $\text{TNF}\alpha$, IL-6, and $\text{IFN}\gamma$ (**Table 3.2**). Collagen levels in the heart tissue were significantly higher in SHRs compared to WKY rats. There was no significant effect of C3G or HCT on collagen levels in the heart tissue in WKY rats or SHRs (**Table 3.2**).

	WKY				SHRs				<i>P</i> values						
	Control	C3G	HCT	C3G+HCT	Control	C3G	HCT	C3G+HCT	Genotype	C3G	HCT	Genotype*C3G	Genotype*HCT	C3G*HCT	Genotype*C3G*H
MDA in plasma, nmol/ml	15.1 +/- 2.2 ^{cd}	10.6 +/- 2.6 ^d	12.0 +/- 2.6 ^d	19.1 +/- 2.6 ^{bc}	25.0 +/- 1.9 ^a	24.6 +/- 1.9 ^a	21.1 +/- 1.9 ^{ab}	23.0 +/- 1.9 ^{ab}	<0.0001	0.42	0.91	0.81	0.05	0.01	0.07
MDA in heart (nmol/ml)	1.1 +/- 2.0	0.9 +/- 2.2	1.0 +/- 2.2	0.9 +/- 2.2	0.8 +/- 2.0	1.1 +/- 2.0	1.1 +/- 2.0	1.3 +/- 2.0	0.29	0.48	0.51	0.04	0.15	0.57	0.66
SOD activity, Unit/mg protein	0.7 +/- 0.1 ^b	1.0 +/- 0.1 ^a	0.7 +/- 0.1 ^b	0.9 +/- 0.1 ^{ab}	0.9 +/- 0.1 ^{ab}	0.7 +/- 0.1 ^b	0.8 +/- 0.1 ^{ab}	0.7 +/- 0.1 ^b	0.20	0.45	0.59	0.01	0.75	0.86	0.52
Catalase activity, Unit/mg protein	39.3 +/- 3.4	38.0 +/- 3.7	40.1 +/- 3.7	37.0 +/- 3.7	35.2 +/- 2.7	39.1 +/- 2.7	41.0 +/- 2.7	42.1 +/- 3.0	0.77	0.97	0.26	0.21	0.31	0.50	0.98
TNFα, pg/mg protein	0.7 +/- 0.1	0.7 +/- 0.1	0.7 +/- 0.2	0.6 +/- 0.2	1.0 +/- 0.1	0.6 +/- 0.1	0.7 +/- 0.1	0.7 +/- 0.1	0.27	0.18	0.53	0.47	0.84	0.40	0.19
IL-6, pg/mg protein	7.8 +/- 0.9	8.6 +/- 0.9	7.8 +/- 1.0	8.7 +/- 1.0	10.0 +/- 0.9	8.1 +/- 0.9	7.8 +/- 1.0	7.6 +/- 0.9	0.81	0.88	0.39	0.17	0.29	0.53	0.53
IFNγ, pg/mg protein	0.8 +/- 0.1	1.0 +/- 0.1	0.8 +/- 0.2	0.8 +/- 0.2	1.1 +/- 0.1	0.6 +/- 0.1	0.7 +/- 0.2	0.6 +/- 0.1	0.29	0.32	0.16	0.06	0.78	0.75	0.26
Collagen, pg/mg protein	0.012 +/- 0.004	0.016 +/- 0.004	0.016 +/- 0.004	0.017 +/- 0.004	0.022 +/- 0.004	0.023 +/- 0.004	0.024 +/- 0.004	0.022 +/- 0.004	0.01	0.66	0.58	0.60	0.66	0.60	0.96

Table 3.2: Effect of C3G (10 mg/kg/day), HCT (10 mg/kg/day), and C3G+HCT (10 mg/kg/day each) on oxidative stress, antioxidant enzymes activities, inflammatory markers, and collagen levels of WKY rats and SHRs at week 15. Values are means $\pm$ SEMs (n= 5 - 8 rats). Labeled means in a row with different letters are significantly different from each other, $P \leq 0.05$. C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; MDA, malondialdehyde; SOD, superoxide dismutase; $\text{TNF}\alpha$, tumor necrosis factor alpha; IL-6, Interleukin 6; $\text{IFN}\gamma$, Interferon gamma.

3.3.14 Plasma biochemistry

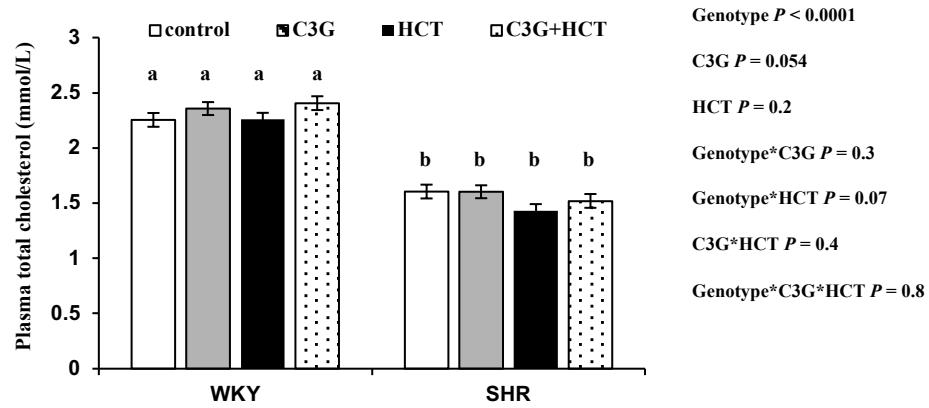
3.3.14.1 *Total cholesterol, HDL, and triglycerides*

Plasma concentrations of total cholesterol, HDL, and triglycerides were significantly higher in WKY rats compared to SHR. There was no significant effect of C3G or HCT treatment on plasma total cholesterol, HDL, and triglycerides (**Figures 3.6A, B, & C**).

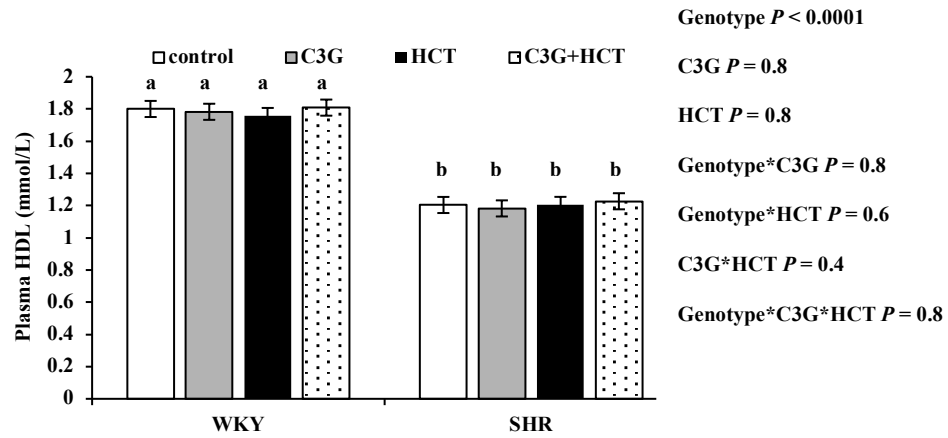
3.3.14.2 *Plasma glucose*

SHRs treated with HCT alone had higher random plasma glucose levels compared with the SHR control, C3G, and C3G+HCT (**Figure 3.6D**).

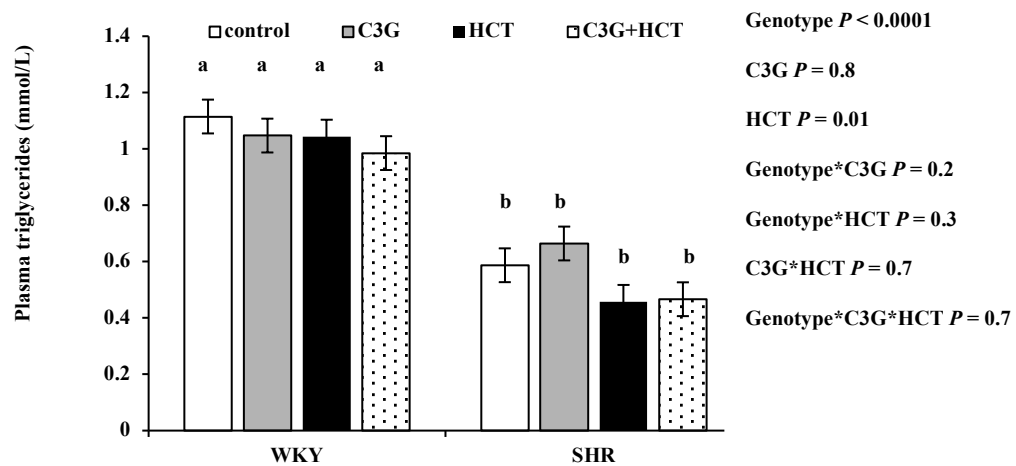
A



B



C



D

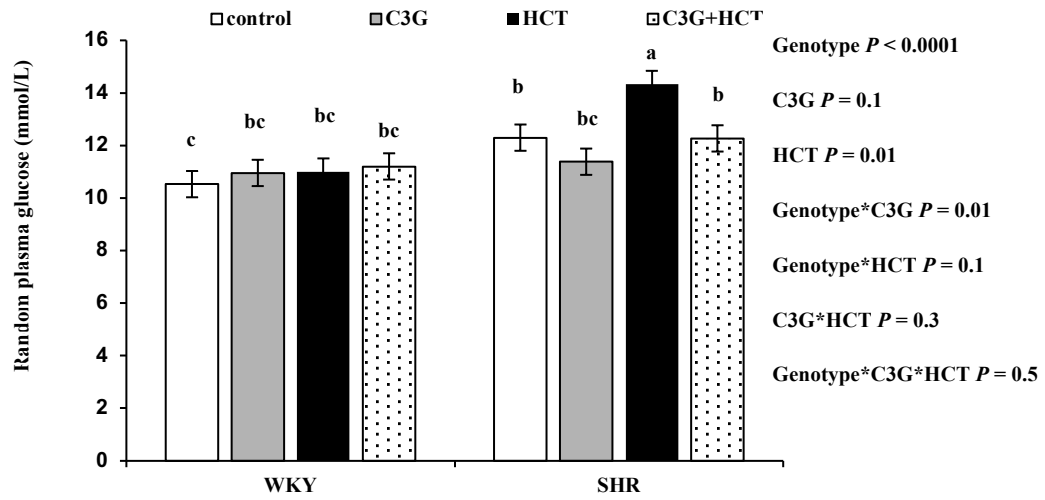


Figure 3.6: Effect of C3G (10 mg/kg/day), HCT (10 mg/kg/day), and C3G+HCT (10 mg/kg/day each) on plasma total cholesterol, HDL, triglycerides, and glucose in WKY rats and SHRs. (A) Total cholesterol. (B) High density lipoprotein. (C) Triglycerides. (D) Random glucose. Values are means $\pm$ SEMs ($n = 8$ rats). Labeled means with different letters are significantly different from each other, $P \leq 0.05$. C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHRs, spontaneously hypertensive rats; HDL, high density lipoprotein.

3.3.15 Hepatic function markers

There was no significant effect of C3G on ALT levels. HCT treatment resulted in lower ALT levels compared to control treatment in WKY rats and SHR. There was no significant effect of C3G or HCT on AST levels (**Figures 3.7A & B**).

3.3.16 Renal function markers

C3G treatment resulted in a significant increase in urea levels in SHRs compared to control treatment and lower urea levels compared to control treatment in WKY rats. The combination effect was greater in SHR rats. Plasma creatinine levels were significantly lower in SHRs treated with C3G, HCT, and C3G+HCT compared to SHR controls (**Figures 3.7C & D**).

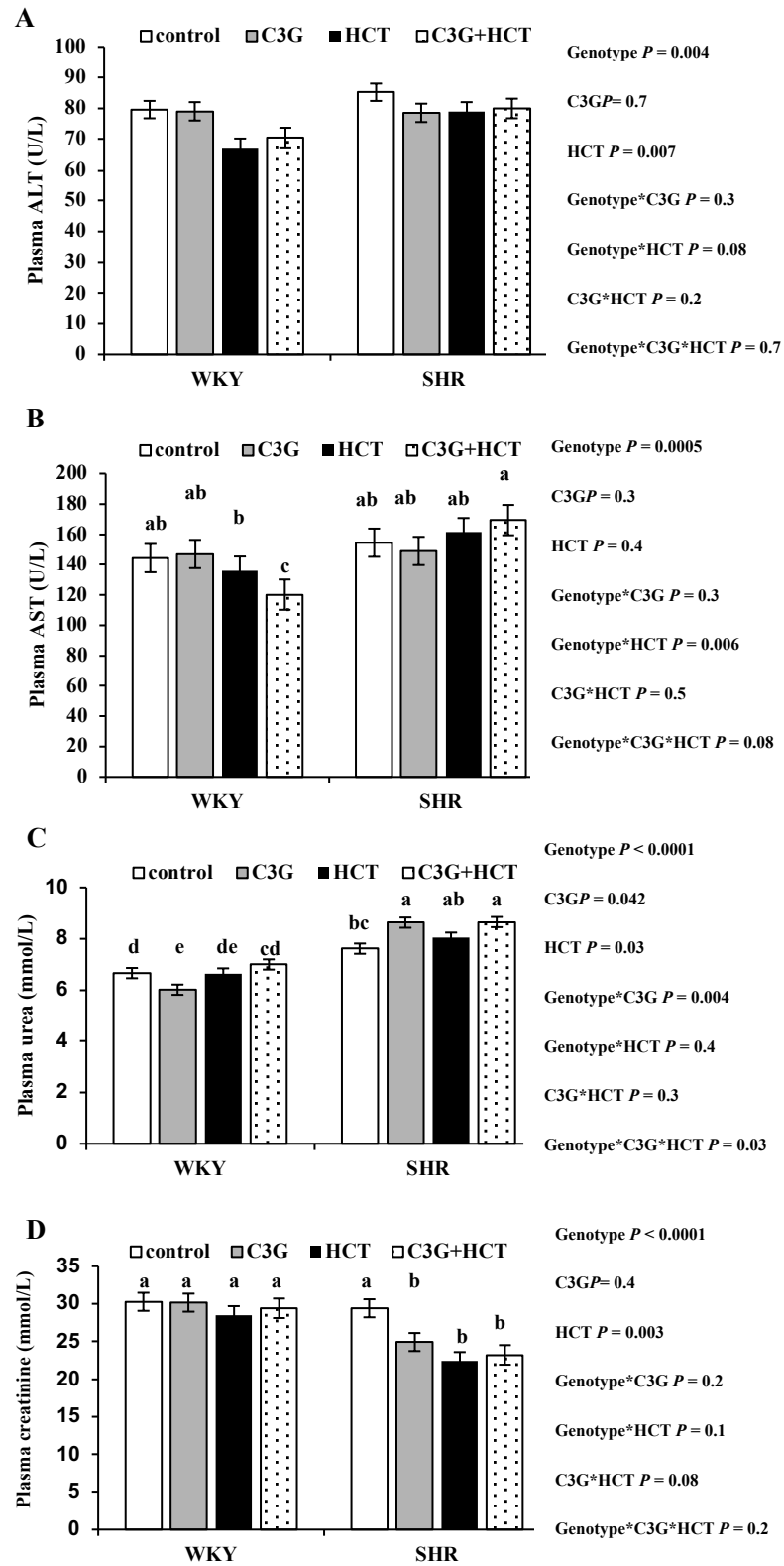
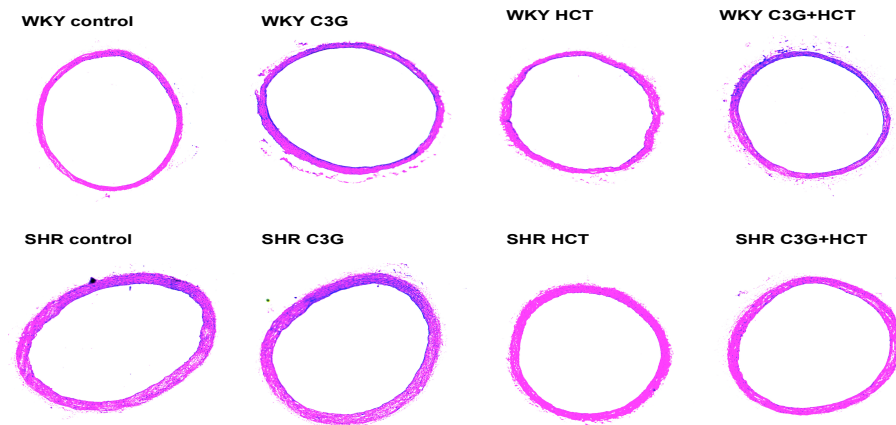


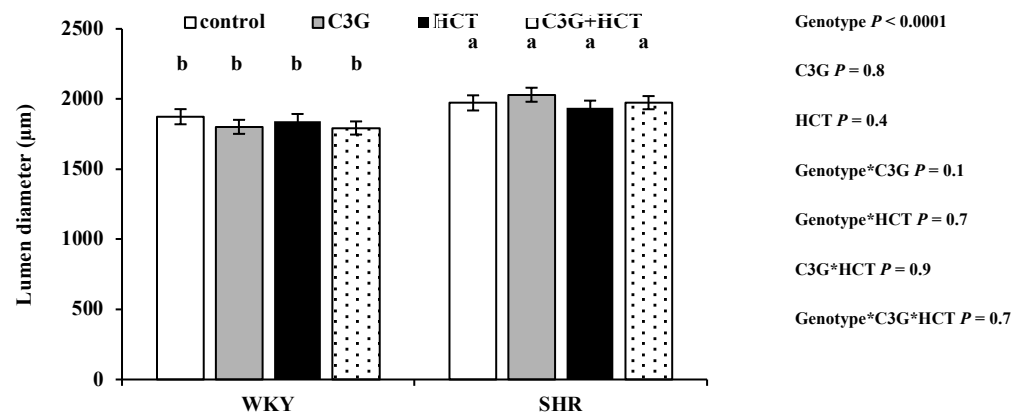
Figure 3.7: Effect of C3G (10 mg/kg/day), HCT (10 mg/kg/day), and C3G+HCT (10 mg/kg/day each) on hepatic and renal toxicity markers in WKY rats and **SHRs**. (A) ALT. (B) AST. (C) Urea. (D) Creatinine. Values are means $\pm$ SEMs (n = 8 rats). Labeled means with different letters are significantly different from each other, $P \leq 0.05$. C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHRs, spontaneously hypertensive rats; ALT, aminotransferase (ALT); AST, aspartate aminotransferase.

3.3.17 Aortic structure

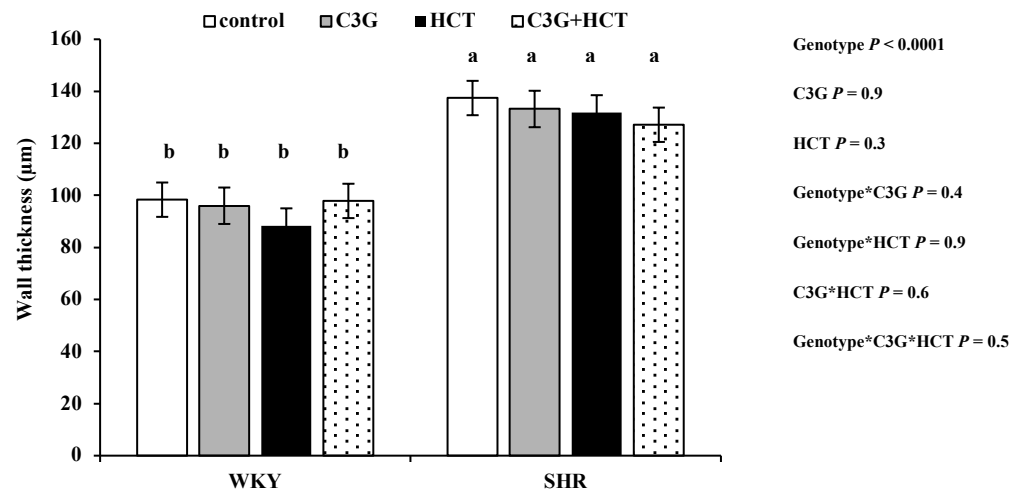
Aortic structure parameters were significantly higher in SHR rats compared to WKY rats. There was no effect of C3G or HCT on aortic structure in WKY rats or SHR rats (**Figures 3.8A, B, & C**).



A



B



C

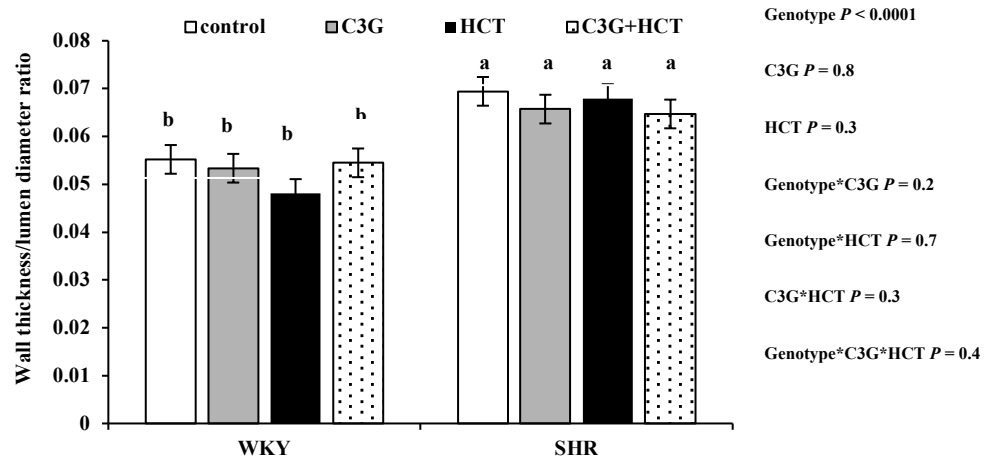


Figure 3.8: Representative photomicrographs and histomorphometric data showing effect of different treatments on aortic structure of WKY rats and SHRs. (A) Lumen diameter. (B) Wall thickness. (C) Wall thickness/lumen diameter ratio. Values are means $\pm$ standard error for $n=8$ rats. Labeled means with different letters are significantly different from each other, $P \leq 0.05$. C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHRs, spontaneously hypertensive rats.

3.3.18 Calcium-dependent SERCA2a activity

The C3G groups had lower $V_{\max}$, while HCT groups had higher $V_{\max}$ compared to controls. Ionophore ratios were lower in the HCT treated groups. Ionophore ratios in the SHR C3G group were not different from SHR controls, but WKY C3G group was higher than WKY controls. SHRs treated with a combination of C3G and HCT had higher Ca_{50} compared to other SHRs groups (Table 3.3).

	WKY				SHRs				<i>P</i> values						
	Control	C3G	HCT	C3G+HCT	Control	C3G	HCT	C3G+HCT	Genotype	C3G	HCT	Genotype*C3G	Genotype*HCT	C3G*HCT	Genotype*C3G*HCT
$V_{\max}$	55.0 +/- 4.7	51.0 +/- 4.7	70.0 +/- 4.7	64.0 +/- 4.7	68.5 +/- 4.7	61.0 +/- 4.7	77.0 +/- 4.7	58.0 +/- 4.7	0.09	0.01	0.02	0.21	0.10	0.33	0.45
$V_{\max(-)}$	46.0 +/- 3.8 ^{cd}	40.5 +/- 3.8 ^d	64.0 +/- 3.8 ^a	54.0 +/- 3.8 ^{bc}	55.0 +/- 3.8 ^{ab}	53.0 +/- 3.8 ^{b,c}	62.0 +/- 3.8 ^{ab}	53.0 +/- 3.8 ^{bc}	0.09	0.02	0.001	0.75	0.03	0.29	0.88
Ionophore ratios	1.2 +/- 0.04 ^{ab}	1.3 +/- 0.04 ^a	1.1 +/- 0.04 ^b	1.2 +/- 0.04 ^{ab}	1.2 +/- 0.04 ^{ab}	1.2 +/- 0.04 ^{ab}	1.2 +/- 0.04 ^{ab}	1.1 +/- 0.04 ^b	0.79	0.57	0.03	0.001	0.27	0.56	0.37
Hillslope	1.9 +/- 0.2	2.0 +/- 0.2	-1.8 +/- 0.2	-2.0 +/- 0.2	-2.0 +/- 0.2	-1.6 +/- 0.2	-2.3 +/- 0.2	-2.1 +/- 0.2	0.55	0.59	0.24	0.12	0.13	0.35	0.88
Ca ₅₀	132.0 +/- 14.5 ^{ab}	146.0 +/- 14.5 ^{ab}	108.0 +/- 14.5 ^b	118.0 +/- 14.5 ^{a,b,c}	109.0 +/- 14.5 ^{bc}	107.0 +/- 14.5 ^{bc}	77.0 +/- 14.5 ^c	159.0 +/- 14.5 ^a	0.21	0.01	0.43	0.16	0.09	0.06	0.04

Table 3.3: Effect of C3G (10 mg/kg/day), HCT (10 mg/kg/day), and C3G+HCT (10 mg/kg/day each) on calcium-dependent SERCA2a activity in WKY rats and SHRs at 15 weeks. Values are means $\pm$ SEMs (n = 6 - 8 rats). Labeled means in a row with different letters are significantly different from each other, $P \leq 0.05$. C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; $V_{\max}$, maximal SERCA2a activity in the presence of Ca²⁺-ionophore; $V_{\max(-)}$ maximal SERCA2a activity in the absence of Ca²⁺-ionophore; Ca₅₀, calcium concentration producing half-maximal activation.

3.4 Discussion

Cardiomyocytes and CF represent the main cell types that contribute to cardiac structural remodeling in HHD.⁴ While cardiomyocytes either undergo hypertrophy or apoptosis, depending on the nature of the causative stimuli, CF contribute to cardiac fibrosis and impaired cardiac function through proliferation and fibroblast-to-myofibroblast phenoconversion.⁴ To date, no study has examined the effects of pure C3G on adult cardiomyocytes and CF exposed to stress. The presence of high levels of ET1 has been implicated in the pathogenesis of different conditions including hypertension and heart failure.³⁰ Therefore, in the present study we first examined whether C3G has direct cardioprotective effects *in vitro* on cardiomyocytes and CF exposed to high levels of ET1.

Our *in vitro* data demonstrated that C3G protected cardiomyocytes from ET1-induced cell death in a concentration-dependent manner with the highest doses (30 & 60 μ M) resulting in a statistically significant protective effect. However, based on the morphological features of cardiomyocytes, the highest dose of C3G (60 μ M) was selected for subsequent *in vitro* experiments as it had the most protective effect against ET1-induced cell death, with no observed adverse effects on control cardiomyocytes. C3G also suppressed the development of ET1-induced cardiomyocyte hypertrophy, an important early step in cardiac structural remodeling that hastens cell death and the progression towards heart failure.^{4, 31} Therefore, the beneficial effects of C3G on cell viability in cardiomyocytes exposed to ET1 observed in this study could be mediated, at least in part, by its anti-hypertrophic effects.

Cardiac injury and stress in response to different pathological stimuli such as pressure overload secondary to hypertension can trigger resident CF to proliferate and differentiate into hypersecretory myofibroblasts.³² Both the proliferation of resident CF, and phenoconversion into myofibroblasts contribute to the excessive production and deposition of extracellular matrix structural proteins with consequent cardiac fibrosis, leading to mechanical stiffness of the myocardium, and ultimately heart failure.³² Therefore, it is important to explore novel therapeutic agents with potential to prevent cardiac fibrosis. In the current study, we examined whether C3G has direct effects on proliferation and differentiation of CF. In addition to its hypertrophic effects in cardiomyocytes, ET1 is known to trigger cardiac fibroblast proliferation and enhance the secretion of extracellular matrix proteins by neonatal CF.^{33, 34} Our results showed that stimulating adult CF with ET1 did not induce their proliferation and phenoconversion. This observed difference between our finding and those reported earlier may be due to variances in the response to ET-1 by neonatal versus adult CF. Nevertheless, in the current study, we found that C3G inhibited basal, un-stimulated CF activation and phenoconversion into cardiac myofibroblasts, as evidenced by decreased expression levels of the two key myofibroblast markers, α -SMA and ED-A fibronectin and a decrease in cell number. These findings suggest that C3G is protective against cardiac fibrosis by limiting the hyperactivity of resident CF.

These promising *in vitro* findings led us to hypothesize that C3G will be cardioprotective *in vivo* in animal models of heart disease. Therefore, in the present study we examined, for the first time, whether C3G alone or in combination with a standard

blood pressure lowering medication, HCT, can ameliorate the development of hypertension and cardiac structural and functional remodeling in the SHR model, the most widely studied animal model of hypertension and cardiovascular disease.³⁵ The nature and progression of hypertension in SHRs from compensated hypertrophy to cardiac dysfunction is similar to the clinical course and prognosis in patients with essential hypertension.³⁶ Hypertension develops in the SHR model around 5-6 weeks of age with systolic blood pressure around 180-200 mm Hg.³⁵ After a long period of established hypertension and compensated cardiac hypertrophy, SHRs develop heart failure, which becomes evident between 18-24 months of age.³⁶ Thus, the SHR model is an appropriate model to study hypertension and its associated cardiovascular complications.

Cyanidin-containing compounds are the most abundant and widely distributed anthocyanins in dark-colored fruits, vegetables and grains, accounting for almost 45% of the total anthocyanin consumption attainable through diet.³⁷ The dose of purified C3G used in the current study (10 mg/kg/day) was based on the success of a previous study that reported the efficacy of C3G in ameliorating hyperglycemia and reducing aortic lipid peroxidation in diabetic rats.³⁸ The rationale for using HCT as a positive treatment control in the current study was based on data from previous studies demonstrating that 10 mg/kg/day HCT is effective in lowering blood pressure in SHRs.^{39, 40}

To evaluate the effects of C3G alone or in combination with HCT on renal function, plasma concentrations of creatinine and urea were measured. Urea levels were

significantly higher in SHR_s compared to WKY rats. Of note, urea concentrations were significantly higher in SHR_s treated with C3G alone or in combination with HCT compared with control SHR_s. Although urea may not be the most accurate biomarker to detect renal damage at early stages,⁴¹ elevated urea levels in SHR_s treated with C3G alone or in combination with HCT may indicate the potential for C3G induced-nephrotoxicity that needs to be considered for dose adjustment in future studies with C3G. Accordingly, the use of a lower dose of C3G (5 or 7.5 mg/kg/day) may be considered for future studies.

Diuretic-based antihypertensive treatment regimens are highly effective in lowering blood pressure and have been shown to prevent stroke and cardiac disease in randomized clinical trials.⁴² Thiazide diuretics; for example, HCT used in this study, are highly effective anti-hypertensives when used as monotherapy and in conjunction with other antihypertensive medications such as ACEI or β -blockers. However, increased serum cholesterol levels, impaired glucose tolerance, and in more severe cases, increased incidence of type 2 diabetes, are major adverse effects that may develop in hypertensive patients treated with thiazide diuretics.^{42, 43} Consistent with findings from previous studies,⁴⁴ total plasma cholesterol, triglycerides, and HDL cholesterol concentrations in our study were lower in SHR_s compared to WKY rats. There was no effect of different treatments on levels of total plasma cholesterol, and HDL cholesterol in WKY rats or SHR_s. HCT treatment caused a significant decrease in plasma triglycerides in WKY rats and SHR_s. Plasma glucose levels were significantly higher in SHR_s compared to WKY rats. It should be noted that treatment with either C3G or HCT alone or in combination did not affect plasma glucose levels in WKY rats; however, SHR_s responded differently

to HCT treatment as evidenced by higher plasma glucose levels in SHRs treated with HCT compared control SHRs. Nonetheless, SHRs treated with a combination of C3G and HCT had lower plasma glucose levels compared with SHRs treated with HCT alone, suggesting that C3G exerts blood glucose lowering effects that may have contributed to the alleviation of HCT-induced hyperglycemia. These findings are consistent with previous reports indicating that C3G has insulin sensitizing and antidiabetic effects.⁴⁵

As predicted, our findings demonstrated that SHRs had significantly higher systolic and diastolic blood pressures over time compared to WKY rats. Given that diuretic-based antihypertensive treatment regimens are highly effective in lowering blood pressure and preventing stroke and heart disease in randomized clinical trials,⁴² it was interesting to compare the effects of HCT to C3G treatment alone and in combination over time. As the disease progressed at 20 weeks of age, C3G was not effective in lowering blood pressure in SHRs, and only HCT alone or in combination with C3G was able to sustain its blood pressure lowering effect, with significant additive effects on SBP observed from combination treatment. The lack of effect of C3G in lowering blood pressure was also consistent with its ineffectiveness in alleviating vascular hypertrophy (increased wall to lumen ratio) in 20-week SHR; surprisingly, HCT treatment was also ineffective, despite significantly lowering blood pressure in 20-week SHR, suggesting that, in addition to high blood pressure, in hypertension vascular structural remodeling is defined by several different factors such as oxidative stress, vascular inflammation, and the activation of renin-angiotensin-aldosterone system.⁴⁶

At 20 weeks of age, SHR presented with established cardiac hypertrophy, systolic and diastolic dysfunction. Both C3G and HCT mitigated the development of cardiac structural and diastolic functional impairment in SHR. The ability of C3G to attenuate cardiac hypertrophy and diastolic dysfunction despite its lack of a sustained antihypertensive effect suggests that these cardioprotective effects of C3G are independent of blood pressure. The findings of our study are consistent with those of a recent study reporting that 8 mg/kg/day C3G limited cardiovascular remodeling in rats with metabolic syndrome (MS).⁴⁷ However, unlike SHR, treatment with C3G lowered hypertension in rats with MS.⁴⁷ The differential impact of C3G observed could be attributed to the severity of hypertension; hypertension was relatively mild in rats with 8-week MS (~156 SBP) vs. 20-week SHR (~201 SBP). Our current findings show variance from our previously published data showing no improvement in cardiac structure and function in rats subjected to myocardial infarction (MI). It must be noted that the MI model is a more severe model of heart disease in comparison to the SHR, and this could be a possible reason for the differential effects of C3G in both models.⁴⁸

To identify mechanisms underlying the cardioprotective effects of C3G and HCT in SHR, we investigated the status of inflammation, oxidative stress and fibrosis, which are three major contributors of cardiac structural and functional remodeling, and the pathophysiology of hypertension and HHD.⁴⁹ Conversely, elevated BP has been demonstrated to induce oxidative stress and inflammation in animal models of hypertension. Evidence suggests that, these inter-related factors, if not blocked, result in target organ injury and dysfunction.⁴⁹ Consistent with findings from previous studies,⁵⁰

we found that plasma levels of MDA, a marker for oxidative stress and lipid peroxidation, were higher in SHRs compared to WKY rats. Treatment with either C3G or HCT alone or in combination did not prevent oxidative stress in SHRs. There was no significant difference in MDA levels as well as levels of TNF α , IL-6, and IFN γ in the heart tissue between WKY rats and SHRs. We also showed that there was no effect of genotype or treatment on the activity of the antioxidant enzymes, SOD and catalase in the heart tissue of WKY rats or SHRs. Altogether, these findings suggest that oxidative stress and inflammation were not affected in twenty-week-SHR hearts, thus ruling out these important contributors to pathogenesis of hypertension-related cardiac complications at this time point in SHR. In the current study, we also showed that SHRs had higher a collagen content in the heart compared to WKY rats. However, both C3G and HCT alone or in combination failed to prevent increased collagen deposition in SHRs indicating that these interventions had no effect on cardiac fibrosis.

We next examined if the C3G mediated improvement in diastolic cardiac function associated with mechanisms related to improved calcium handling in SHR. SERCA2a is a critical ATPase pump responsible for sarcoplasmic reticulum calcium uptake, which in turn governs diastolic heart function. Our results indicate that SERCA2a activity was not affected in 20-week SHR thus, ruling out SERCA2a inactivation as being a contributor to diastolic heart dysfunction at this time point in SHR. Although SERCA2a is the major determinant of cardiac relaxation, sodium-calcium exchanger and troponin I are also other important contributors.^{51, 52} It is therefore possible that C3G may positively impact these mechanisms and thereby improve diastolic heart function in SHRs.

From nutritional standpoint, the effective C3G dose needed for the health-promoting effects reported in this study maybe attainable through daily consumption of C3G-rich dietary sources such as black elderberries, plums, and blackberries. For instance, the intake of 170 g of blackberries, delivers 400-500 mg of C3G. Whilst the individual intake estimates for dietary sources abundant in anthocyanins differs largely between individuals, daily doses of more than 1 g C3G per individual are easily achievable through minor changes to our dietary routines.⁵³ Nevertheless, it must be noted that other components in these berries such as the sugar content may potentially contribute to adverse effects.

3.5 Conclusions

In conclusion, our findings show that treatment with C3G was effective in lowering blood pressure in 13-week old SHR, but it was unable to alleviate severe hypertension in 20-week old SHR. This study also shows that C3G prevented cardiac hypertrophy and diastolic dysfunction in 20-week old SHR. These findings suggest that C3G may have direct cardioprotective effects independent of blood pressure. Our results also show that combination therapy of C3G and HCT alleviated hyperglycemia associated with HCT treatment. Our results suggest that dietary intake of C3G at an early stage of hypertension alone or in combination with established antihypertensive medications may be useful in limiting the development of hypertension and its cardiac complications in hypertensive patients. Proof-of-principle and dose finding studies in patients would be needed to confirm this hypothesis.

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CHAPTER 4

Examining the effects of cyanidin 3-O- glucoside on the immune system in spontaneously hypertensive rats.

4.1 General introduction

The concept that the immune system contributes significantly to the pathogenesis of systemic hypertension has been postulated by earlier studies.¹ In 1964, White and Grollman showed that immune suppression prevents the development of hypertension in rats with partial renal infarction. They concluded that the development of hypertension in these rats may be the result of autoimmune processes that affect the renal tissue.² A few years later, Okuda and Grollman showed that transfer of lymph node cells from rats with renal infarction increased blood pressure in healthy rats.³ These observations presented additional evidence for the hypothesis that immunologic mechanisms have an important role in the development of different forms of hypertension. After these early findings by Grollman and colleges, different studies have reported a relationship between the immune system disturbances or dysfunction and hypertension in humans as well as different animal models of hypertension.¹ An overview of the immune system and the role of immune mechanisms in the development of hypertension is the basis of the following literature review.

4.2 Literature review

4.2.1 The immune system

The immune system is made up of a network of special cells, proteins, and tissues that work together to defend different body organs against antigens including microorganisms (such as bacteria, viruses, fungi, and parasites), cancer cells, and toxins. The immune system can be classified into two subsystems: the innate and the adaptive immune

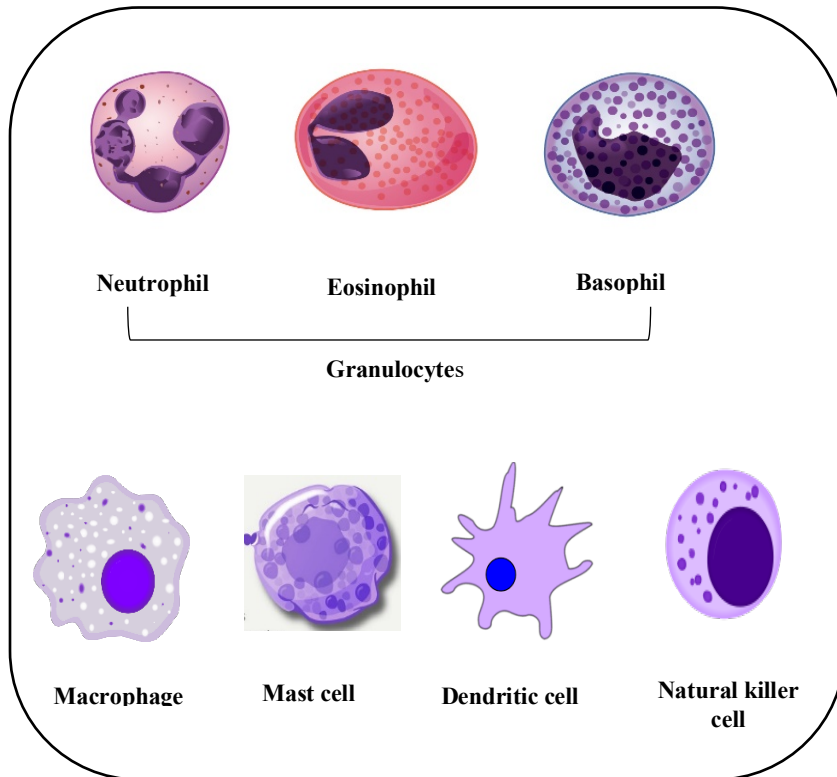
systems. The innate immune system is the first line of defense against invading pathogens. It represents generic (antigen non-specific) immune defense mechanisms that work immediately or within hours after an antigen enters the body. However, it does not have immunologic memory. On the contrary, adaptive immune system creates immunologic memory after the first exposure to pathogens; therefore, adaptive immune response is antigen-dependent (antigen-specific) that involves a lag time between pathogen invasion and maximal immunologic response upon first exposure to a specific antigen. The adaptive immune system then adapts to respond faster and more efficiently upon future exposure. In fact, innate and adaptive immune systems are not two separate systems that work independently. They complement each other with abnormalities in either one of these systems leaving the host more susceptible to diseases.⁴

4.2.2 Innate immune system

Innate defense mechanisms involve the skin and mucous membranes that provide external physical barriers to pathogen entry. In addition to these barriers, other first innate defense mechanisms include chemicals such as enzymes, acids, and mucous; cilia; tears; and temperature. If these mechanisms fail to prevent the entry of infectious pathogens, secondary innate immune mechanisms are activated, these include inflammatory cells and the complement system (a system of plasma proteins that complements the function of antibodies and phagocytic cells by identifying and coating pathogens).⁴ Innate immunity involves the mobilization of immune cells to the site of infection by releasing cytokines (a group of small proteins that promote interaction and signaling between cells).

Cytokine release activates the production of antibodies and other proteins that stimulate the complement system.⁵ Different defense cells have critical roles in innate immune response (**Figure 4.1**) including phagocytes (these cells are sub-grouped into neutrophils that are mainly found in blood and macrophages that are found in tissues), mast cells, basophils, eosinophils, dendritic cells and natural killer cells. While both macrophages and neutrophils phagocytose (coat and digest) pathogens, neutrophils assist in innate immune response by releasing enzymes that directly kill pathogens. In addition to their phagocytic role, macrophages have a long-life span and assist in antigen presentation to T lymphocytes.⁵

Innate immunity



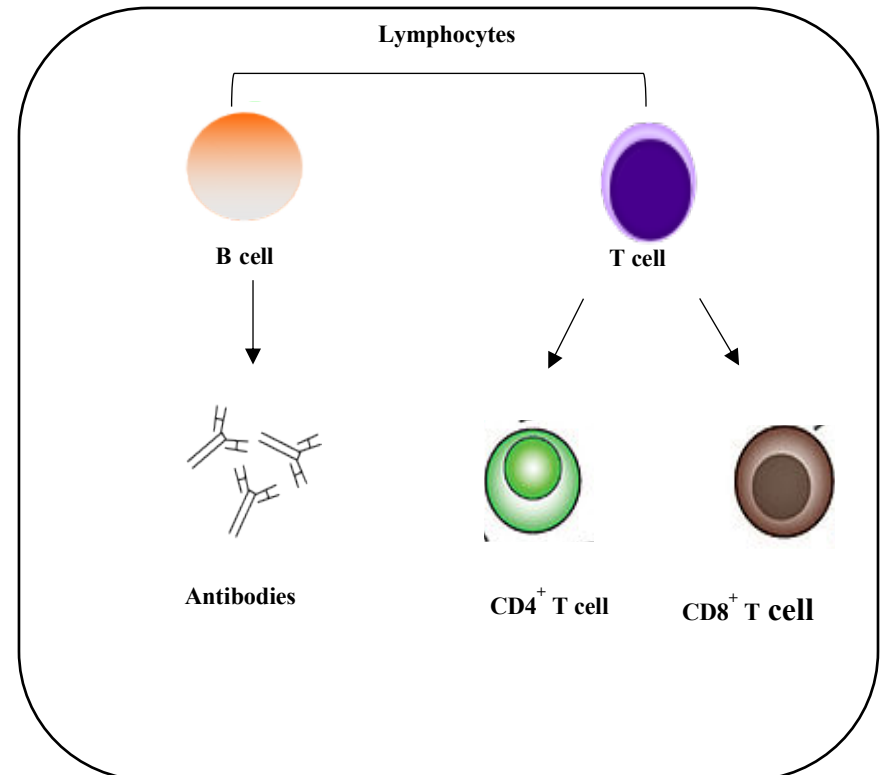
Non-specific immunity

Cytokines

Phagocytosis

Cytotoxicity

Adaptive immunity



Antigen-specific immunity

Cytokines

Antibodies

Cytotoxicity

Figure 4.1: Representative illustration for innate and adaptive immune systems.

4.2.3 Adaptive immune system

If innate immune responses are inefficient in destroying the invading pathogens, the innate defense mechanisms stimulate highly specific, long-lasting adaptive immune responses.⁴ A fundamental characteristic of the adaptive immune responses is its ability to respond specifically to antigens that induced them. Furthermore, the adaptive immune system can distinguish between self and non-self-antigens, which helps in eliminating particular pathogens or pathogen-infected cells and developing immunological memory for that particular pathogen. Occasionally, a fatal immune response may develop if the adaptive immune system fails to distinguish between self and foreign antigens.⁴ Adaptive immune defense responses are performed by leucocytes (white blood cells) called lymphocytes. There are two categories of these responses: antibody-mediated responses which are carried out by B-lymphocytes, and cell-mediated responses which are carried out by T-lymphocytes (**Figure 4.1**).⁴

4.2.4 Antibody-mediated (humoral) immunity

Humoral immune responses are mediated through antibodies produced by B-cells. B-cells mature in the bone marrow and they develop B-cell receptors on the cell surface. These receptors can recognize and bind to specific antigens to stimulate antibody production pathways. The interaction between B-cell receptors and antigens summons helper T-cells, a subset of T-lymphocytes, that play a critical role in antibody production by secreting cytokines that assist B-cells in maturing and multiplying into antibody-producing plasma cells. Produced antibodies attach to foreign antigens on surface of invading pathogens,

marking them for other immune cells and chemicals for pathogen destruction and elimination.⁶

4.2.5 Cell-mediated immunity

This class of adaptive immune responses does not involve antibodies, instead it involves the activation of macrophages and natural killer cells to eliminate intracellular pathogens, and cytotoxic T-cells to induce apoptosis of damaged or abnormal cell. In other situations, T-cells respond to invading pathogens by releasing different cytokines to activate phagocytes to destroy phagocytosed pathogens.⁴

4.2.5.1 *Biology of T cell-mediated immunity*

T-cell-mediated immune response includes developing antigen-specific T-cells to fight pathogens. Antigen specificity of T-cells involves the identification via T-cell receptors of specific antigenic peptides presented by major histocompatibility (MHC) molecules on surface of antigen presenting cells (APC).⁷ Each T-cell has a particular T-cell receptor (TCR) expressed on its surface during maturation in thymus. After maturation, T-cells become naïve T-cells, which unlike activated T-cells, have not encountered their cognate antigens and have not been activated. Naïve T-cells migrate via blood and the lymphatic system and occupy secondary lymphatic organs (spleen, lymph nodes and small masses of lymphatic tissues such as Peyer's patches, tonsils, and bronchial and gastrointestinal lymphoid tissues). Antigenic materials are processed by APC known as dendritic cells (DC) and presented to naïve T-cells in the context of MHC molecules in secondary lymphoid organs. DC take up antigens in peripheral tissues and circulate to

secondary lymphoid tissues directed by inflammatory signals and cytokines. Stimulated T-cells rapidly increase in number and differentiate into effector (cytotoxic CD8⁺ or helper CD4⁺ T-cells) or memory T-cells and circulate to tissues where antigens are present to exert their effect. After the pathogenic antigen is defeated, most effector T-cells will die off, but some cells will remain and form memory T-cells. Unlike naïve or effector cells, memory T-cells can live for several years and are easily activated to perform an immediate immune response if the same antigen is encountered again.^{4, 7}

4.2.5.2 *Structure of the T lymphocyte network*

A) Lymphatic tissues

These include primary and secondary lymphatic tissues. The primary lymphatic tissues include the bone marrow and the thymus where hematopoiesis and clonal selection of T-cells occur. The secondary lymphatic tissues include the spleen, lymph nodes, and lymphatic tissues connected with mucous membranes such as Peyer's patches and tonsils. Naïve T-cells present in high densities in specific structures in secondary lymphatic organs including the paracortex of the lymph nodes and the periarteriolar lymphatic sheet of the spleen. They occupy the spleen for only few hours and the lymph nodes for nearly one day before they migrate and enter the bloodstream through the splenic veins or through efferent lymphatic vessels, respectively.^{4, 7}

B) T-cell subsets

I) Naïve T-cells

In the blood, naïve T-cells express phenotypic markers including L-selectin (CD62L),

and CC chemokines receptor 7. These proteins help with the movement, adhesion, and extravasation of naïve cells across high endothelial venules in peripheral lymph nodes and mucosal lymphatic organs. Homeostasis and survival of naïve T-cells are maintained by low-affinity interactions of TCR with self-antigens and the existence of interleukin-7 (IL-7).⁷

II) CD4⁺ (helper) T-cells

T-helper cells express CD4 glycoprotein on their surface. They differentiate into different subpopulations including: Th1, Th2, Th9, Th17, Th22, and regulatory T-cells (Treg), and can be defined by the cytokine profile released by the cells. CD4⁺ cells are differentiated from CD4⁺ naïve cells under the influence of different cytokines; for example, Th1 cells are differentiated under the effect of the pro-inflammatory cytokines (IL-12 and IFN- γ); Th2 cells are differentiated under the effect of IL-4 and IL-10; Treg cells are differentiated under the effect of IL-2 and TGF- β . Every CD4⁺ cell subset produces a unique cytokine profile that mediates either a pro- or anti-inflammatory response. For instance, Th1 cells produce mostly IFN- γ , but also IL-2 and TNF- α . They play a major role in pro-inflammatory cell-mediated immune responses, B-cell secretion of immunoglobulin G (IgG), and induce the immune response to some protozoa. Th2 cells produce IL-4, IL-5, and IL-13. They have a crucial role in the B-cell secretion of IgG, IgA, and IgE. Treg cells produce IL-10 (a cytokine that has an immunosuppressive role and maintains the expression levels of the transcription factor (FOXP3) required for the suppressive role of Treg cells), and TGF beta; Th17 cells produce IL-17 that has a fundamental role in the

immune response during inflammation.⁷

III) CD8⁺ (cytotoxic) T-cells

Cytotoxic T-cells originate from stimulated CD8⁺ Naïve T-cells. They proliferate in the presence of IL-2, and their number can increase up to thousand-folds when exposed to foreign antigen for the first time (primary immune response). The accelerated proliferation and the efficacy of single CD8⁺ cytotoxic T-cells in killing cancer cells or infected cells mark them as very powerful antigen-specific effector T-cells. The elimination of target cells by cytotoxic T-cells requires antigen identification and the formation of cell contact with the target cell; therefore, commencing the release of cytolytic enzymes into the immunological synapse to induce apoptosis in target cells.^{4, 7,}

8

IV) Regulatory T-cells (Treg)

Treg cells are defined as T-cells that are responsible for maintaining self-tolerance and prevention of autoimmune diseases, as well as down-modulation of the magnitude of an immune response by inhibiting/suppressing the deleterious effects of activated immune cells. Treg cells contribute in all cell-mediated immune responses by directly influencing Th1, Th2, Th17, cytotoxic T-cells and B-cell responses against self- and non-self-antigens. Treg cells are subdivided into natural cells that mature in thymus and comprise most of Treg cells in the circulation, and inducible Treg cells which differentiate in peripheral tissues from CD4⁺ cells in response to pathogens and cytokines. Even though

most of Treg cells population express the CD4 cell surface marker, suppressor T-cell regulatory activity was also reported with CD8⁺ T cells; however, most of the attention has focused on CD4⁺ Treg cells, especially naturally arising Treg cells that express the IL-2 receptor (CD25) and the transcription factor forkhead box p3 (Foxp3). Foxp3 is crucial for the development and function of Treg cells. The deficiency of Foxp3 is sufficient to cause autoimmune diseases in otherwise healthy animals. The suppressor role of Treg cells requires activation of TCR. Upon stimulation, Treg cells will perform their function through direct cell contact via inhibitory molecules such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), or through release of IL-10 and TGF- β . IL-10 can inhibit differentiation of Th1 and Th2 lymphocytes directly by decreasing the release of IL-2, TNF- α , and IL-5, and indirectly by down-regulating MHC and co-stimulatory molecules on APC; therefore, decreasing T-cell activation.^{7,9}

4.3 Associations between immune system aberrations and hypertensive pathophysiology

Manifestations of high blood pressure directly affect and are affected by the central and autonomic nervous systems inducing exaggerated sympathetic stimulation, the kidneys causing glomerular damage, and the cardiovascular system inducing heart failure and vascular dysfunction. The immune system contributes directly to these abnormalities by stimulating inflammatory processes in the nervous system, the renal system and, the cardiovascular system. The magnitude of the pro-inflammatory immune response may differ between different animal models of hypertension.¹⁰

Even though the underlying causes for high blood pressure in spontaneously hypertensive rats (SHR) have been investigated for decades, the fundamental etiologies remain undefined. It has been postulated that hypertension in SHR may result from impaired immune function.¹¹ This speculation is based on findings that the immune system is depressed in SHR. Takeichi *et al* have demonstrated that SHR has a lower number of T-cells in the peripheral blood as well as decreased numbers of immature T lymphocytes in the thymus as compared to WKY rats.¹² These researchers also demonstrated that the blastogenic response of T-cells isolated from SHR to phytohemagglutinin A and concanavalin A (a lectin isolated from red kidney beans, and a protein extracted from jack beans, respectively, used to stimulate T-cell proliferation *in vitro*) is depressed, suggesting T-cell dysfunction.¹³ Together, these findings show that in addition to having a low circulating T-cell number, T-cell function in SHR is depressed. Further studies by Takeichi *et al* have suggested the presence of natural thymocytotoxic antibodies in 1-month-old SHR. The authors concluded that these antibodies are specific for T-cells and may be the basis for depressed T-cell function.¹² To identify whether the decline in T-cell number and function is related to high blood pressure in SHR, Ba *et al* investigated the effect of implanting thymus grafts from normotensive rats into SHR. These investigators showed normalization of T-cell function in 24-week-old SHR by implantation of thymus grafts from 1-month-old WKY rats and this was combined with a significant decrease of arterial blood pressure. Nonetheless, blood pressure lowering effect was not sustained as it increased to its initial levels 5 weeks after implantation.¹⁴ SHR underwent thymus graft implantation at the neonatal age had long-lasting

normalization of blood pressure with arterial pressure of 125 mmHg at 32 weeks of age compared to 190 mmHg in untreated rats. These researchers concluded that the effect of thymus implants on SHR could be attributed to the compensatory effect of effector T-cells from thymus implants for the depressed T-cell function, or the effect of hormones released from thymus implants that stimulated differentiation and growth of T-cells in SHR. Other forms of impaired immune responsiveness in SHR have also been reported. For example, T-cells from SHR cannot work effectively with and stimulate B lymphocytes to produce antibodies. SHRs have also been reported to have depressed delayed-type hypersensitivity reaction and depressed allograft rejection time, indicating depressed cellular immune reaction.¹¹

Renal infarction induced by ligation of renal arteries in rats causes necrosis in these arteries, glomerular damage, and chronic elevation in blood pressure.¹¹ Sokabe and Grollman¹⁵ demonstrated that blood pressure could be lowered to its normal levels in these rats if the infarcted kidney was removed within a few days after the infarction. Nonetheless, postponing the removal of the infarcted kidney by more than 7 days resulted in full expansion of the hypertensive condition. These researchers suggested that the development of hypertension in these rats could be attributed to the release of some components from the infarcted tissue that impair the function of kidneys and results in sustained hypertension. The presence of antibodies against the renal tissue has been reported in animals with renal infarction.² Furthermore, cortisone or 6-mercaptopurine treatment in these animals mitigated the development of hypertension. Together these

findings indicated that an autoimmune condition causes the development and the maintenance of hypertension in rats with renal infarction. Additional findings supporting the role of immune mechanisms in inducing hypertension in animals with renal infarction were demonstrated by Okuda and Grollman who induced hypertension in healthy animals by transfer of lymph node cells from animals with hypertension induced by renal infarction.³ In another study, the removal of infarcted kidney 3 weeks after ligation of left renal artery failed to reverse the hypertensive state in rats. Immunosuppressive treatment with cyclophosphamide in these rats lowered blood pressure to that of control rats.¹⁶

The immune system has also been suggested to play a critical role in inducing and maintaining mineralocorticoid-salt-induced hypertension, a form of hypertension induced by concurrent treatment with salt-retaining hormones such as desoxycortisone (DOC) and substituting drinking water with a high sodium load (1% NaCl) solution.¹¹ Immunosuppressive treatment in Wistar rats with DOC-salt-induced hypertension decreased the incidence of arteriolar damage.¹ Another study has shown that transfer of spleen cells from rats with mineralocorticoid-salt hypertension induced hypertension in normotensive control rats.¹⁷ Raji *et al* demonstrated a significant decrease in the incidence of glomerular damage in complement-deficient mice compared to normal mice treated with DOC and exposed to high salt load. These findings indicate that complement system stimulation is critical in hypertensive glomerular injury.¹⁸

4.3.1 Role of T-cell dysfunction in the development of hypertension

A) Regulatory T-cells

Treg cells have been demonstrated to have a potent antihypertensive effect in different animal models of hypertension. Barhoami et al reported that infusion of angiotensin II in mice resulted in a significant increase in systolic blood pressure (43 mmHg), and caused a significant decrease in FOXP3+ T-cells in the renal cortex. Adoptive transfer of Treg cells ameliorated angiotensin II-induced hypertension. This antihypertensive effect of Treg cells was associated with decreased oxidative stress in the vasculature, decreased plasma concentrations of the inflammatory cytokines (IFN- γ , IL-6 and TNF- α), and decreased infiltration of macrophages in aortic adventitia.¹⁹ In another study, transfer of Treg cells was reported to mitigate angiotensin II-induced cardiac damage; however, it did not have a significant antihypertensive effect.²⁰ In the stroke-prone spontaneously hypertensive rats (SHRSP), a sub-strain of SHRs characterized by extreme high blood pressure levels and a high susceptibility to stroke, a significant decrease in the proportion of Tregs in the spleen preceded the development of hypertension compared to WKY rats was reported. Furthermore, treating SHRSP at 4-5 weeks of age with IL-2/anti IL-2 mAb complex increased the percentage of Tregs in the spleen, delayed the onset of hypertension, and mitigated the development of cardiac hypertrophy.²¹

IL-10, a cytokine produced by Treg cells, has been reported to be responsible for the anti-inflammatory/antihypertensive effects exerted by Treg cells. Chatterjee *et al*

reported that IL-10 knockout mice have significantly high blood pressure and high serum levels of TNF- α and IFN- γ .²² Transfer of Treg cells from normotensive mice into IL-10 null hypertensive mice decreased blood pressure, as well as oxidative stress as evidenced by a decrease in the activity of NADPH oxidase.²³ Collectively these findings indicate that Treg cells may have a critical role in maintaining normal blood pressure levels, at least in part, by producing the anti-inflammatory cytokine IL-10.

B) Effector T-cells

Angiotensin II infusion in rats has been reported to increase the expression of the Th1-produced pro-inflammatory cytokine (IFN- γ) and decrease the expression of the Th2 cytokine IL-4 in the spleen. These effects of Ang II on T-cell subsets were mitigated by treatment with the olmesartan, an angiotensin II receptor blocker.²⁴ Even though elevated IFN- γ levels have not been reported to be associated with hypertension, increases in Th1 subset proportion increases expression of TNF- α . Treatment with etanercept, a TNF- α antagonist, has been reported to prevent hypertension associated with angiotensin II administration.²⁵

Th17 cells, a subset of the pro-inflammatory helper T-cells characterized by release of IL-17, have been suggested to have a role in angiotensin II-induced hypertension and vascular damage. Madhur *et al* demonstrated that blood pressure increases at a similar rate in the first 4 weeks in IL-17 null mice receiving angiotensin II compared to control mice. However, blood pressure decreased significantly in IL-17 null mice 4 weeks after the initiation of angiotensin II infusion. Furthermore, IL-17 null mice

were protected against vascular dysfunction, oxidative stress, and infiltration of T-cells in the aorta. These findings suggest that IL-17 is implicated in blood pressure elevation and vascular damage in angiotensin II-induced hypertension.²⁶

4.4 Influence of anthocyanins on the immune system

Anthocyanins are flavonoids that constitute the largest class of water-soluble pigments present ubiquitously in plant kingdom providing the red, blue, and purple colors of many fruit and vegetables.²⁷ Due to their high abundance and relatively high dietary intake, the impact of anthocyanins on various human diseases has been widely examined. In particular, anthocyanins have anti-oxidant,²⁷ anti-hyperglycemic,²⁸ anti-hyperlipidemic,²⁹ and anti-inflammatory effects,³⁰ which are important in the prevention and treatment of various forms of chronic diseases. However, with the exception of one study that reported a suppressive effect of the anthocyanin, cyanidin-3-*O*-glucoside (C3G), on T-helper 2 cell cytokines (IL-4 and IL-13), while it increased the levels of the T-helper 1 cytokine (IL-2) using the EL-4 T-cell line,³¹ the direct effects of anthocyanins on the immune system including immune cell phenotypes and function have not been investigated.

4.5 Rationale

As previously mentioned, impaired immune function may contribute to hypertension in SHRs.¹¹ Observations that support impaired immune function in SHR include depressed mitogen-induced proliferation of T-cells from SHRs compared to WKY rats.¹³ Attempts to verify whether the decline in T number and functionality are related to high blood pressure in SHRs have shown that implantation of thymus grafts from the normotensive

WKY rats into neonatal SHRs resulted in restoration of T-cell function and suppressed the development of hypertension.¹⁴ IL-2 is a cytokine produced by T-cells in response to T-cell activation by antigens. It is a key factor for differentiation, proliferation and growth of effector T-cell subsets.³² It has been reported that injection of IL-2 prevented the development of hypertension in prehypertensive SHRs and mitigated hypertension in adult SHRs with established hypertension.³³ Katsuki *et al* reported that IL-2 and anti-IL-2 mAb mitigated the development of hypertension and cardiac hypertrophy, and induced Tregs in SHRSP.²¹ C3G has been reported to induce the release of IL-2 from T-cells *in vitro*.³¹ However, the effects of C3G on T-cell function and phenotypes remain undetermined.

4.6 Hypothesis and objectives

We hypothesize that commencing C3G treatment in prehypertensive SHRs at 5-weeks of age for 15 weeks will mitigate T-cell dysfunction and improve immune response. Therefore, the objectives of this study were as follows:

1. To compare immunological function in SHRs at 20-weeks of age compared to normotensive WKY rats by examining splenic T-cell phenotypes and function.
2. To evaluate whether C3G and HCT, a diuretic routinely used as a first line treatment in management of hypertension, have direct effects on T-cell dysfunction in SHR.

4.7 Methods

4.7.1 Animal Model and treatments

Spleen from SHRs and their age-matched controls, Wistar-Kyoto rats (WKY) used in the cardiac study reported in chapter 3 were used to examine the effects of genotype and treatments on the immune system. WKY and SHR rats were treated daily by oral gavage for a period of 15 weeks with vehicle (deionized water), C3G (10 mg/kg/day) and/or HCT (10 mg/kg/day). C3G and HCT were dissolved in deionized water. A total of 8 groups (8 rats per group) were examined. (1) WKY treated with vehicle, (2) WKY treated with C3G, (3) WKY treated with HCT, (4) WKY treated with C3G+HCT; (5) SHR treated with vehicle, (6) SHR treated with C3G, (7) SHR treated with HCT, and (8) SHR treated with C3G+HCT.

4.7.2 Tissue collection

At the end of the study period, all rats were weighed and anesthetized with ketamine/xylene injection. Spleens were excised aseptically and placed in a phosphate buffered saline solution (PBS). Weights of spleens were recorded. The remaining spleen tissue was processed immediately for T-cell phenotyping and *ex vivo* cytokine production.

4.7.3 Isolation of immune cells from the spleen

Single cell suspensions of spleens were prepared by pushing tissues through a 100µm cell strainer into Hank's buffered saline (10 mM HEPES, 4% (v/v) fetal bovine serum, and 1% antibiotic/antimycotic; pH 7.4). Erythrocytes from spleen cell suspensions were lysed

with ammonium chloride lysis buffer (155 mM NH₄Cl, 0.1 mM EDTA, 10 mM KHCO₃; pH 7.4). Cells were washed with Hank's buffered saline and suspended in PBS supplemented with 2% (v/v) fetal bovine serum, 23 mM sodium azide, and 1% antibiotic/antimycotic (pH 7.4). Cell concentration and viability analyses were performed using Nexcelom AutoT4 Plus hemocytometer and trypan blue dye, respectively.

4.7.4 Determination of cytokine concentration

Splenocytes (3 x 10⁶ cells from each animal) were incubated in 3 mL complete culture media (Roswell Park Memorial Institute 1640 media) supplemented with 10 mM HEPES, 5% (v/v) fetal bovine serum, 50 µM 2-mercaptoethanol, and 1% antibiotic/antimycotic (pH 7.4). To stimulate cytokine production, splenocytes were incubated for 72 hours with 5% CO₂ in either the presence or absence of the mitogen, concanavalin A (ConA; 0.25 µg/mL). After incubation for 72 hours, samples were centrifuged at 400 g for 5 minutes to pellet the cells. Supernatant fractions were collected and stored at -80°C for analysis. Concentrations (lower limit of detection in parentheses) of IL-2 (0.46 pg/ml), IL-10 (19.4 pg/ml), TNF-α (27.7 pg/ml), and IFN-γ (6.8 pg/ml) in the supernatant fractions were measured using a BD Cytometric Bead Array rat assay kit on a FACSCanto II flow cytometer and FCAP Array™ v3 software. All samples were analyzed in duplicate with CV <10% according to the BD Cytometric Bead Array Mouse/Rat Soluble Protein Buffer Kit Instruction Manual. In BD Cytometric Bead Array assay methods, soluble analytes are captured with antibody-coated beads of known size and fluorescence intensities.

4.7.5 Phenotypic determination of T lymphocytes

Monoclonal antibodies against rat CD3 (APC label, clone 1F4, isotype Ms IgM, κ and FITC label, clone 1F4, isotype Ms IgM, κ), CD4 (PE-CyTM7 label, clone OX-35, isotype Ms IgG2a, κ), CD8 (PerCP label, clone OX-8, isotype Ms IgG1, κ), CD62L (PE label, clone HRL1, isotype Ar Ham IgG2, λ 1), CD44 (FITC label, clone OX-49, isotype Ms IgG2a, κ) FOXP3 (PE label, clone FJK-16s, isotype IgG2a, κ) and CD25 (APC label, clone OX39, isotype Ms IgG1) were used to determine T lymphocyte subpopulations in the spleen immediately after cell isolation. Fluorescent antibodies were used in combination to determine the % of total T cells (CD3⁺), helper T-cells (CD3⁺/CD4⁺/CD8⁻), cytotoxic T-cells (CD3⁺/CD4⁻/CD8⁺), naïve T-cells (CD3⁺/CD62L^{hi}/CD44⁺), memory T-cells (CD3⁺/CD62L^{lo}/CD44⁺), naïve helper T-cells (CD3⁺/CD4⁺/CD8⁻/CD62L^{hi}/CD44⁺), memory helper T-cells (CD3⁺/CD4⁺/CD8⁻/CD62L^{lo}/CD44⁺), naïve cytotoxic T-cells (CD3⁺/CD4⁻/CD8⁺/CD62L^{hi}/CD44⁺), memory cytotoxic T-cells (CD3⁺/CD4⁻/CD8⁺/CD62L^{lo}/CD44⁺), and regulatory T-cells (CD3⁺/CD4⁺/CD8⁻/FOXP3⁺/CD25⁺). Mix 1 contained CD44-FITC, CD62L-PE, CD8-PerCP, CD4 -PE-CyTM7, and CD3-APC. Mix 2 contained CD3-FITC, FOXP3-PE, CD8-PerCP, CD4 -PE-CyTM7 and CD25-APC. The %CV for each sample between tubes was below 15% for CD3, CD3⁺CD4⁺CD8⁻ and CD3⁺CD4⁻CD8⁺. Isolated splenocytes (1×10^7 /mL) from each treatment group were incubated with fluorescent antibodies immediately for 30 minutes at 4°C in the dark. After the incubation period, cells were washed with PBS supplemented with 2% (v/v) fetal bovine serum, 23 mM sodium azide, and 1% antibiotic/antimycotic (pH 7.4) and centrifuged for 5 minutes at 400 g, 4°C. Cells were fixed with 1%

paraformaldehyde and stored at 4°C overnight. For intracellular staining for FOXP3, 1 ml of FOXP3 Fixation/Permeabilization working solution was added to samples. Samples were pulse vortexed and incubated in the dark at 4°C for 30 minutes. Cells were washed twice in 2 ml permeabilization buffer. Cells were resuspended in 100 µl permeabilization buffer and FOXP3 antibody was added directly. Samples were incubated at 4°C for 30 minutes. Cells were washed with 2 ml of permeabilization buffer. Cells were washed and re-suspended in PBS. Data were acquired on a FACSCanto II flow cytometer using the 488 nm and 633 nm lasers. **Figures 4.2 and 4.3** shows representative flow cytometry plots. Forward vs. side-scatter plots were used to gate on intact lymphoid cells. Ten thousand events were collected in list-mode format and analyzed using BD FACSDiva software (v8.0.1). Unstained cells were used to assess auto-fluorescence, isotype controls to assess background staining and single-color samples were employed to adjust color compensation.

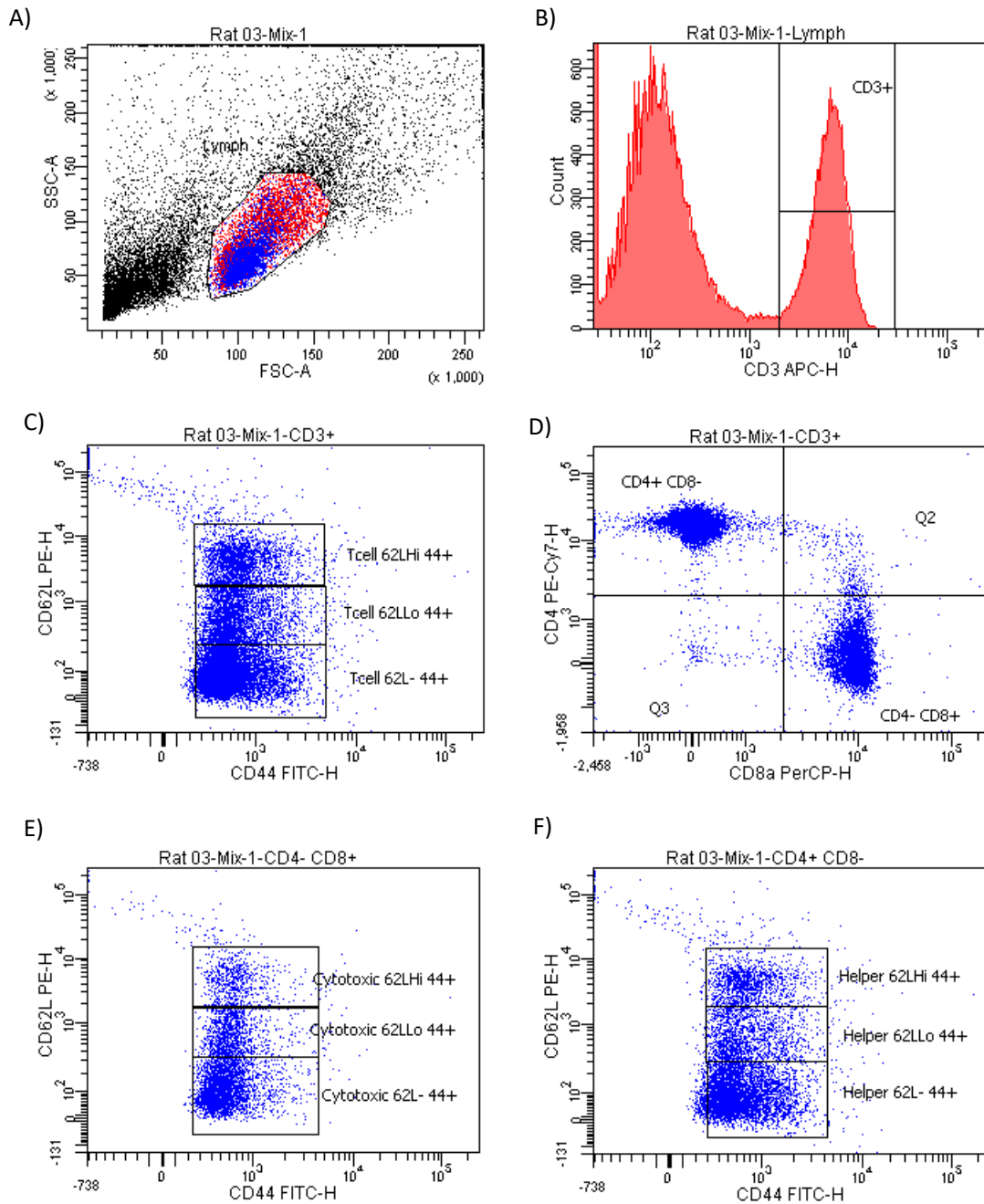


Figure 4.2: Representative flow cytometry plot for mix 1. Definition of viable lymphocytes (Lymph) [A]; Definition of CD3 binding after gating on Lymph [B]; Definition of CD62L and CD44 after gating on CD3+ Lymph [C]; Definition of CD4 and CD8 binding after gating on CD3+ Lymph [D]; Definition of CD62L and CD44 after

gating on CD3+CD4-CD8+ Lymph [E]; Definition of CD62L and CD44 after gating on CD3+CD4+CD8- Lymph [F].

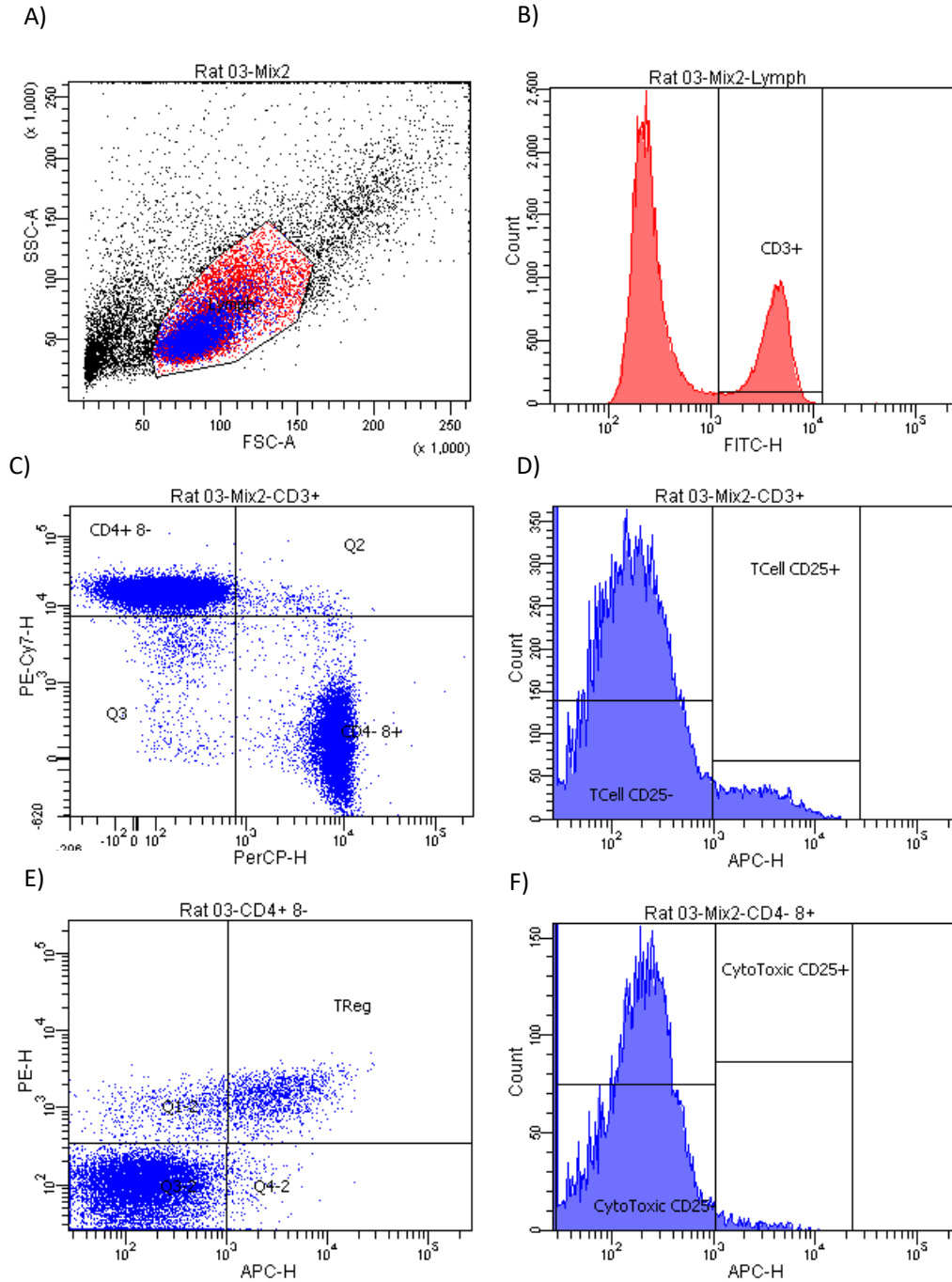


Figure 4.3: Representative flow cytometry plot for mix 2. Definition of viable lymphocytes (Lymph) [A]; Definition of CD3 binding after gating on Lymph [B];

Definition of CD4 and CD8 after gating on CD3⁺ Lymph [C]; Definition of CD25 binding after gating on CD3⁺ Lymph [D]; Definition of Foxp3 and CD25 after gating on CD3⁺CD4⁺CD8⁻ Lymph [E]; Definition of CD25 binding after gating on CD3⁺CD4⁻CD8⁺ Lymph [F].

4.7.6 Statistical Analyses

Three-way ANOVA was used to analyze the effect of C3G (factor 1), HCT (factor 2), genotype (factor 3), and their interaction effects for all variables measured. Type 3 fixed effects and corresponding p-values were calculated for C3G, HCT, genotype, and interaction effects. Least square means were calculated for each treatment group, genotype, and combination and expressed as mean $\pm$ SEM. Post-hoc testing was done to compare each treatment, genotype, and interactions. Multiple comparisons for post hoc testing were accounted for by controlling using the False Discovery Rate (FDR) with an FDR adjusted p-value of 0.05 considered statistically significant (Benjamini & Hochberg). All statistical analyses were conducted using SAS statistical software (version 9.4; SAS Institute Inc. Cary, NC, USA).

4.8 Results

4.8.1 Spleen weights

There were no significant interactions between genotype*C3G*HCT on spleen weights or spleen weights corrected for body weights. SHRs had 7% lower spleen weights compared to WKY rats, but spleen weights corrected for body weights did not differ between SHRs and WKY rats. There was no significant effect of C3G or HCT on spleen weights or spleen weights corrected for body weights (**Table 4.1**).

	Main effects									Interactions								
	Genotype			C3G			HCT			WKY				SHR				
	WKY n=32	SHR n=31	<i>P</i> - value	0 n=32	+ n=31	<i>P</i> - value	0 n=32	+ n=31	<i>P</i> - value	Water n=8	C3G n=8	HCT n=8	C+H n=8	Water n=8	C3G n=8	HCT n=8	C+H n=7	<i>P</i> -value
Spleen Weight (g)	0.745 ±0.01	0.690 ±0.01	0.003	0.728 ±0.01	0.706 ±0.01	0.21	0.733 ±0.01	0.701 ±0.01	0.07	0.779 ±0.02	0.734 ±0.02	0.722 ±0.02	0.733 ±0.02	0.720 ±0.02	0.690 ±0.02	0.692 ±0.02	0.658 ±0.03	G*C= 0.58 G*H= 0.93 C*H= 0.54 G*C*H=0.47
Spleen weight/bwt (mg/g bwt)	2.04 ±0.03	2.08 ±0.03	0.36	2.10 ±0.03	2.02 ±0.03	0.06	2.09 ±0.03	2.03 ±0.03	0.11	2.16 ±0.06	2.04 ±0.06	1.99 ±0.06	1.99 ±0.06	2.15 ±0.06	2.03 ±0.06	2.10 ±0.06	2.04 ±0.06	G*C= 0.70 G*H= 0.23 C*H= 0.28 G*C*H=0.70

Table 4.1: Effect of genotype, C3G (10 mg/kg/day), HCT (10 mg/kg/day), and their interactions on spleen weight. Values are LS means ± SEM. Means in the “interaction” section of a row with different letters are significantly different from each other based on LS means adjusted for multiple comparisons using the False Discovery Rate, $P \leq 0.05$. Abbreviations: C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; C+H, Cyanidin 3-*O*-glucoside + hydrochlorothiazide; G*C, interaction

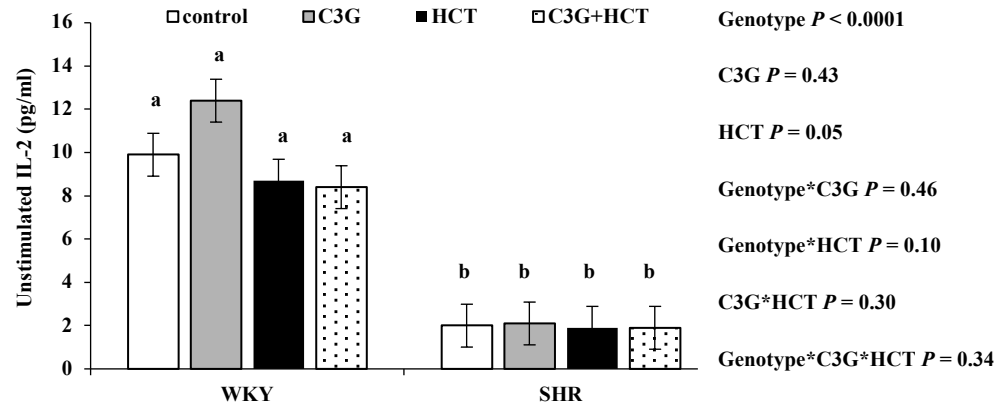
between genotype and Cyanidin 3-*O*-glucoside; G*H, interaction between genotype and hydrochlorothiazide; C*H, interaction between Cyanidin 3-*O*-glucoside and hydrochlorothiazide; G*C*H, interaction among genotype, Cyanidin 3-*O*-glucoside and hydrochlorothiazide; bwt, body weight.

4.8.2 *Ex vivo* cytokine production

4.8.2.1 *IL-2*

There were no significant interactions between genotype*C3G*HCT on IL-2 levels produced from unstimulated splenocytes. Unstimulated splenocytes from SHRs produced lower concentrations of IL-2 compared with WKY rats. There was no effect of C3G or HCT on IL-2 levels produced from unstimulated splenocytes (**Figure 4.4A**). There was a significant interaction between genotype*HCT treatment on the levels of IL-2 produced from ConA-stimulated splenocytes. There was no significant difference between different treatment groups in the concentration of IL-2 produced from ConA-stimulated splenocytes from WKY rats. Splenocytes from all SHR treatment groups produced lower levels of IL-2 in response to ConA compared to all WKY treatment groups. The concentration of IL-2 produced from ConA-stimulated splenocytes from SHRs treated with HCT alone or SHRs treated with C3G+HCT were lower than IL-2 concentrations produced from ConA-stimulated splenocytes from SHRs treated with C3G alone by 49 and 48%, respectively (**Figure 4.4B**).

A



B

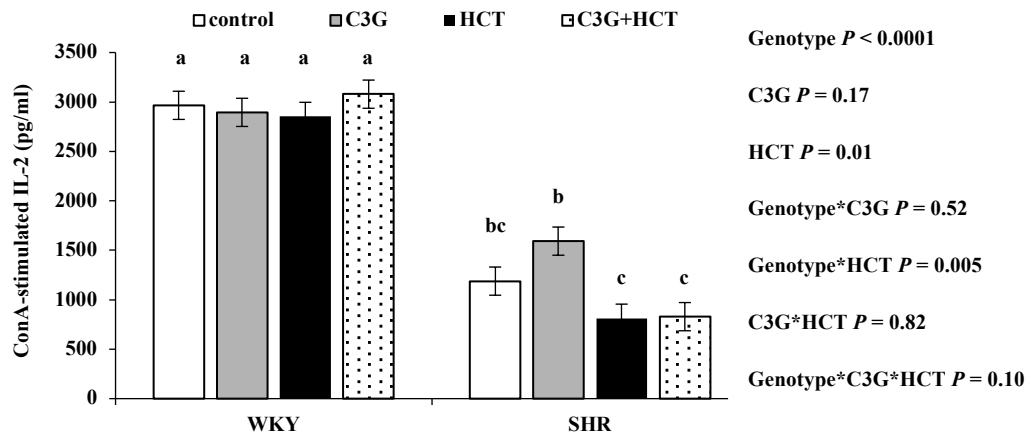


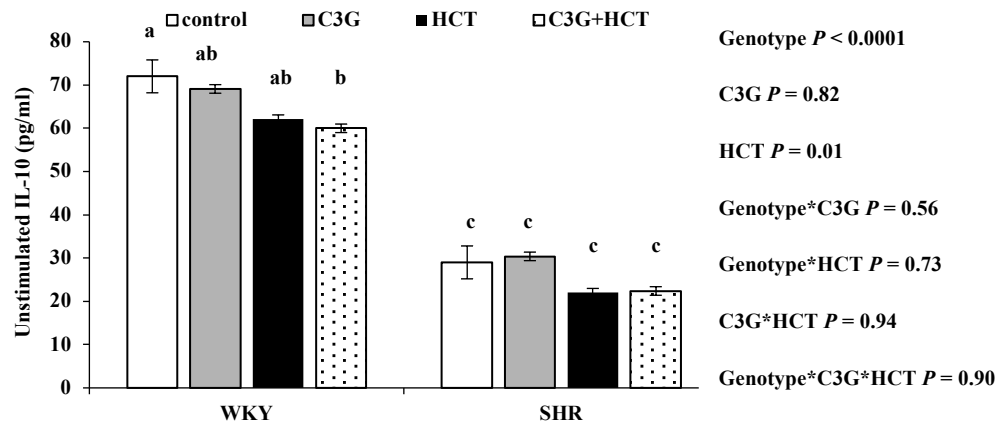
Figure 4.4: Effect of genotype, C3G (10 mg/kg/day), HCT (10 mg/kg/day), and their interactions on ex vivo IL-2 production by (A) unstimulated and (B) concanavalin A stimulated splenocytes. Values are LS means $\pm$ SEMs (For stimulated IL-2 samples $n = 8$ rats per group except SHR C3G+HCT $n = 6$ rats; unstimulated IL-2 samples $n = 7-8$

rats per group). Labeled means with different letters are significantly different from each other, $P \leq 0.05$. Abbreviations: C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

4.8.2.2 *IL-10*

There was no significant interaction between genotype*C3G*HCT on the concentrations of IL-10 produced from unstimulated and ConA-stimulated splenocytes. Unstimulated and ConA-stimulated splenocytes from WKY rats produced 61 and 54% higher levels of IL-10 compared with SHR, respectively. Unstimulated and ConA-stimulated splenocytes from rats treated with HCT produced 17 and 21% lower levels of IL-10 compared with control rats, respectively. There was no effect of C3G treatment on the levels of IL-10 produced from unstimulated and ConA-stimulated splenocytes (**Figures 4.5A and B**).

A



B

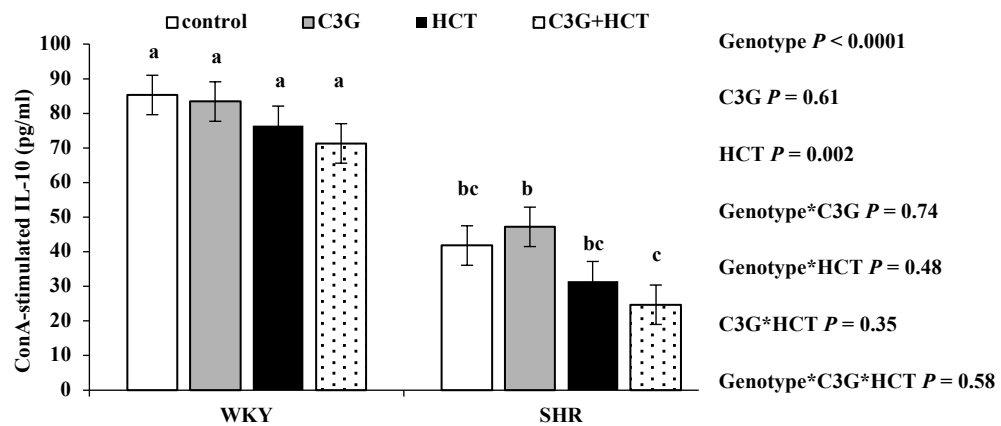


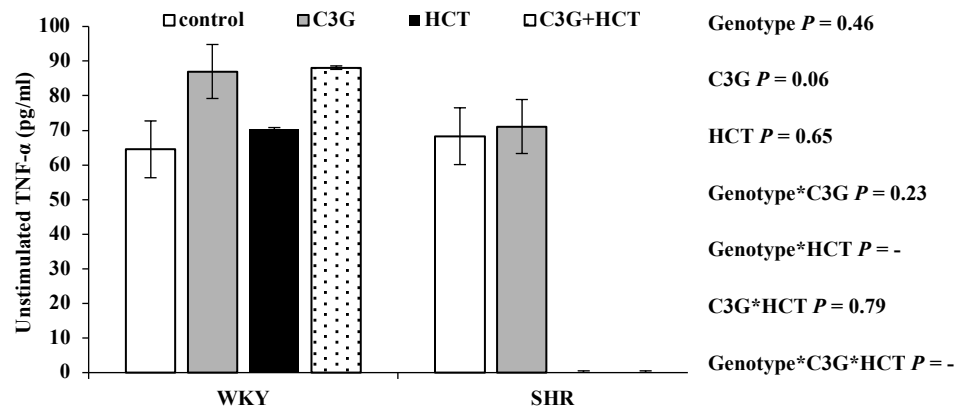
Figure 4.5: Effect of genotype, C3G (10 mg/kg/day), HCT (10 mg/kg/day), and their interactions on ex vivo IL-10 production by (A) unstimulated and (B) concanavalin A stimulated splenocytes. Values are LS means $\pm$ SEMs (For stimulated IL-10 samples $n = 8$ rats per group except SHR C3G+HCT $n = 6$ rats; unstimulated IL-10 samples $n = 8$ per

group, except SHR HCT samples n=6 and SHR C3G+HCT n=3). Labeled means with different letters are significantly different from each other, $P \leq 0.05$. Abbreviations: C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

4.8.2.3 *TNF- α and IFN- γ*

The levels of TNF- α produced from unstimulated splenocytes from SHR treated with HCT or C3G+HCT (**Figure 4.6A**), as well as the levels of IFN- γ produced from unstimulated splenocytes from all WKY rats and SHR were below detection levels. There was a significant interaction between genotype*HCT on the levels of TNF- α and IFN- γ produced from ConA-stimulated splenocytes. There was no significant difference among the different treatment groups in the concentrations of TNF- α and IFN- γ produced from ConA-stimulated splenocytes from WKY rats. ConA stimulated TNF- α concentrations from SHR control rats were 26-33% lower than all WKY groups. ConA stimulated IFN- γ concentrations from SHR control rats were 50-65% lower than all WKY groups. ConA-stimulated splenocytes from SHR treated with HCT alone and C3G+HCT produced 61 and 58% lower concentrations of TNF- α compared with control SHR, respectively. ConA-stimulated splenocytes from SHR treated with HCT alone or C3G+HCT produced 79 and 78% lower concentrations of IFN- γ compared with control SHR, respectively. Compared with control SHR, ConA-stimulated splenocytes from SHR treated with C3G produced higher concentrations of TNF- α and IFN- γ by 27 and 52%, respectively. Levels of TNF- α and IFN- γ produced from ConA-stimulated splenocytes from SHR treated with C3G were not different from the WKY groups (**Figures 4.6B and 4.7, respectively**).

A



B

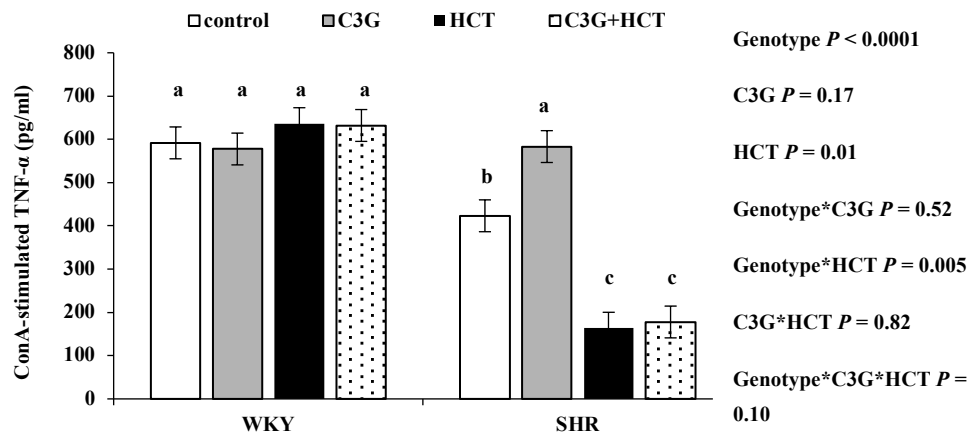


Figure 4.6: Effect of genotype, C3G (10 mg/kg/day), HCT (10 mg/kg/day), and their interactions on ex vivo TNF- α production by (A) unstimulated and (B) concanavalin A

stimulated splenocytes. Values are LS means $\pm$ SEMs (For stimulated TNF- α samples n = 8 rats per group except SHR C3G+HCT n = 6 rats; unstimulated TNF- α samples n=8 per group, except SR HCT and SHR C3G+HCT samples were below detection range. Labeled means with different letters are significantly different from each other, $P \leq 0.05$. Abbreviations: C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide: WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

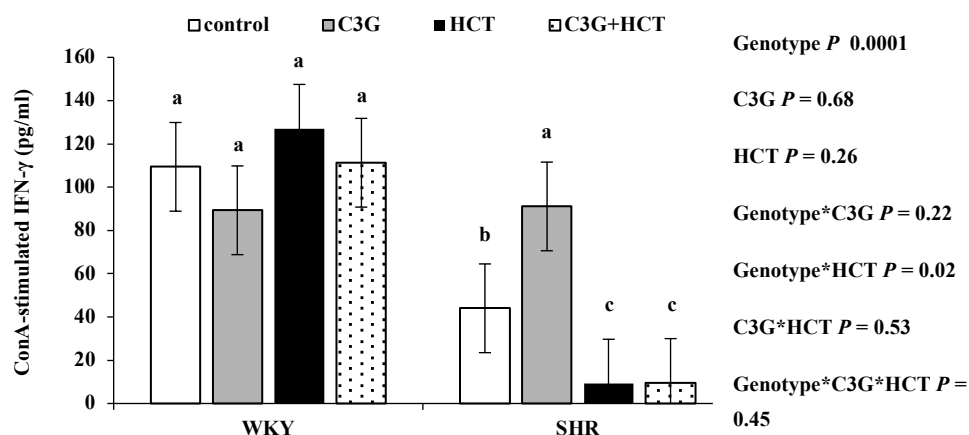


Figure 4.7: Effect of genotype, C3G (10 mg/kg/day), HCT (10 mg/kg/day), and their interactions on ex vivo IFN- γ production by concanavalin A-stimulated splenocytes. Values are LS means $\pm$ SEMs (For stimulated IFN- γ samples $n = 8$ rats per group except SHR C3G+HCT $n = 6$ rats; unstimulated IFN- γ were below detection range). Labeled means with different letters are significantly different from each other, $P \leq 0.05$. Abbreviations: C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats.

4.8.3 Immune cell phenotypes

Flow cytometry analysis showed that there was no significant interactions between genotype*C3G*HCT on the proportion of splenic T-cells ($CD3^+$ cells) and the proportion of helper ($CD3^+CD4^+CD8^-$) T-cells or cytotoxic ($CD3^+CD8^+CD4^-$) T-cells. SHRs had a 2% higher proportion of splenic T-cells compared to WKY rats. There was no significant effect of C3G or HCT treatment on the proportion of T-cells cells in the spleen (**Table 4.2**). There was no effect of genotype or treatments on the percentage of helper T-cells (**Table 4.2**). There was a significant interaction between genotype*HCT on the proportion of cytotoxic T-cells. SHR control rats had a higher proportion of cytotoxic T-cells compared to WKY rats, but there was no difference among treatments and their respective controls (**Table 4.2**). There was a significant interaction between C3G*HCT on the proportion of T-cells that were naïve helper T-cells ($CD3^+CD4^+CD8^-CD62L^{hi}CD44^+$). WKY rats and SHRs treated with HCT had a 31-54% higher proportion of T-cells that were naïve helper T-cells compared to all other groups (**Table 4.2**). There was a significant interaction between genotype*C3G*HCT on the proportion of T-cells that were memory helper T-cells ($CD3^+CD4^+CD8^-CD62L^{lo}CD44^+$). WKY rats treated with C3G or C3G+HCT had 32 and 41% lower proportions of T-cells that were memory helper T-cells compared to control WKY rats, respectively. There was no effect of HCT treatment on the proportion of T-cells that were memory helper T-cells in WKY rats compared to control WKY rats. Control SHR rats had a 27% lower proportion of T-cells that were memory helper T-cells compared to control WKY rats. SHRs treated with C3G, HCT, or C3G+HCT had 27-23% higher proportions of T-cells that were memory helper

T-cells compared to control SHR rats and were not significantly different from WKY controls (**Table 4.2**).

There was a significant interaction between genotype*C3G*HCT on the proportion of T-cells that were naïve cytotoxic T-cells ($CD3^+CD8^+CD4^-CD62L^{hi}CD44^+$). C3G or HCT treated-WKY rats had 48 and 52% higher proportions of T-cells that were naïve cytotoxic T-cells compared to control WKY rats, respectively. The proportion of T-cells that were naïve cytotoxic T-cells in SHR rats treated with C3G, HCT, or C3G+HCT were not statistically different from control SHR rats (**Table 4.2**). There was no significant interaction between genotype*C3G*HCT on the proportion of T-cells that were memory cytotoxic T-cells ($CD3^+CD8^+CD4^-CD62L^{lo}CD44^+$). There was no effect of genotype, but the proportions of T-cells that were memory cytotoxic T-cells were 12 and 10% higher in rats treated with C3G or HCT, respectively, compared with control rats (**Table 4.2**).

There was a significant interaction between genotype*C3G*HCT on the proportion of T-cells that were Tregs ($CD3^+CD4^+CD25^+FOXP3^+$). C3G-treated WKY rats had a 17% higher proportion of T-cells that were Tregs compared to control WKY rats. There was no difference in the proportion of T-cells that were Tregs between WKY rats treated with HCT or C3G+HCT and control WKY rats. All SHR groups had 31-46% lower proportions of T-cells that were Tregs compared to all WKY groups, but there was no significant difference among SHR rats treated with C3G, HCT, or C3G+HCT and control SHR rats (**Table 4.2**).

There were no significant interactions between genotype*C3G*HCT on the proportion of activated T-cells ($CD3^+CD25^+$). WKY rats had a 36% higher proportion of activated T-cells compared to SHRs. There was no effect of C3G or HCT on the proportion of activated T-cells (**Table 4.2**).

There were significant interactions between genotype*C3G, genotype*HCT, and C3G*HCT on the proportion of T-cells that were activated helper T-cells ($CD3^+CD4^+CD8^-CD25^+$). WKY rats treated with C3G had a 12% higher proportion of T-cells that were activated helper T-cells compared to control WKY rats. There was no effect of HCT or C3G+HCT treatments in WKY rats as compared with control WKY rats. All of the SHR groups had 25-40% lower proportions of T-cells that were activated helper T-cells compared to all of the WKY groups. There was no significant difference in the proportion of T-cells that were activated helper T-cells between control SHRs and SHRs treated with C3G, HCT, or C3G+HCT (**Table 4.2**).

There was a significant interaction between genotype*C3G*HCT on the proportion of T-cells that were activated cytotoxic T-cells ($CD3^+CD8^+CD4^-CD25^+$). WKY rats treated with C3G had a 25% lower proportion of T-cells that were activated cytotoxic T-cells compared to control WKY rats. The SHR rats all had 30-52% lower proportions of T-cells that were activated cytotoxic T-cells compared to the WKY rats. There was no effect of C3G or HCT treatment in SHRs. The combination of C3G and HCT resulted in a 35-41% increase in the proportion of T-cells that were activated cytotoxic T-cells in both WKY rats and SHRs (**Table 4.2**).

	Main effects									Interactions								
	Genotype			C3G			HCT			WKY				SHR				
	WKY n=32	SHR n=31	p-value	0 n=32	+ n=31	P-value	0 n=32	+ n=31	P-value	Water n=8	C3G n=8	HCT n=8	C+H n=8	Water n=8	C3G n=8	HCT n=8	C+H n=7	P-value
CD3 ⁺ cells (% of lymphocytes)	31.2 ± 0.5	33.3 ± 0.5	0.002	32.0 ± 0.5	32.5 ± 0.5	0.5	32.3 ± 0.5	32.2 ± 0.5	0.9	30.6 ± 0.9	31.1 ± 0.9	31.2 ± 0.9	31.9 ± 0.9	33.1 ± 0.9	34.4 ± 0.9	33.1 ± 0.9	32.6 ± 1.0	G* <i>C</i> = 0.9 G* <i>H</i> = 0.2 C* <i>H</i> = 0.6 G* <i>C</i> * <i>H</i> = 0.5
CD3 ⁺ CD4 ⁺ CD8 ⁺ cells (% of lymphocytes)	17.4 ± 0.3	17.6 ± 0.3	0.71	17.6 ± 0.3	17.4 ± 0.3	0.72	17.4 ± 0.3	17.7 ± 0.3	0.55	17.6 ± 0.6	17.1 ± 0.6	17.8 ± 0.6	17.4 ± 0.6	17.1 ± 0.6	17.9 ± 0.6	18.0 ± 0.6	17.5 ± 0.6	G* <i>C</i> = 0.5 G* <i>H</i> = 0.98 C* <i>H</i> = 0.5 G* <i>C</i> * <i>H</i> = 0.4
CD3 ⁺ CD8 ⁺ CD4 ⁺ cells (% of lymphocytes)	12.4 ± 0.3	14.5 ± 0.3	<0.0001	13.2 ± 0.3	13.8 ± 0.3	0.08	13.8 ± 0.3	13.2 ± 0.3	0.12	11.8 ^c ± 0.5	12.8 ^{b,c} ± 0.5	12.1 ^c ± 0.5	12.9 ^{b,c} ± 0.5	14.9 ^a ± 0.5	15.5 ^a ± 0.5	13.8 ^{a,b} ± 0.5	13.9 ^{a,b} ± 0.5	G* <i>C</i> = 0.5 G* <i>H</i> = 0.04 C* <i>H</i> = 0.6 G* <i>C</i> * <i>H</i> = 0.9
CD62LhiCD44 ⁺ (% of CD3 ⁺ CD4 ⁺ CD8 ⁺ cells)	18.7 ± 1.2	21.7 ± 1.2	0.07	23.1 ± 1.2	17.3 ± 1.2	0.001	17.5 ± 1.2	22.9 ± 1.2	0.002	15.0 ^b ± 2.3	19.4 ^b ± 2.3	28.0 ^a ± 2.3	13.0 ^b ± 2.3	19.0 ^b ± 2.3	17.4 ^b ± 2.3	31.2 ^a ± 2.3	20.0 ^b ± 2.5	G* <i>C</i> = 0.7 G* <i>H</i> = 0.2 C* <i>H</i> = <0.0001 G* <i>C</i> * <i>H</i> = 0.1
CD62LloCD44 ⁺ (% of CD3 ⁺ CD4 ⁺ CD8 ⁺ cells)	15.6 ± 0.4	18.6 ± 0.4	<0.0001	18.2 ± 0.4	16.0 ± 0.4	0.0003	16.7 ± 0.4	17.6 ± 0.4	0.12	20.0 ^a ± 0.8	13.3 ^b ± 0.8 ^c	18.0 ^a ± 0.8	12.0 ^c ± 0.8	14.2 ^b ± 0.8	20.0 ^a ± 0.8	21.1 ^a ± 0.8	20.0 ^a ± 0.8	G* <i>C</i> = <0.0001 G* <i>H</i> = <0.0001 C* <i>H</i> = 0.002 G* <i>C</i> * <i>H</i> = 0.005
CD62LhiCD44 ⁺ (% of CD3 ⁺ CD8 ⁺ CD4 ⁺ cells)	20.3 ± 1.3	19.9 ± 1.3	0.9	22.1 ± 1.3	18.1 ± 1.3	0.04	18.4 ± 1.3	21.8 ± 1.3	0.07	13.3 ^b ± 2.6	26.0 ^a ± 2.6	27.2 ^a ± 2.6	15.0 ^b ± 2.6	22.0 ^{a,b} ± 2.6	13.0 ^b ± 2.0	27.0 ^a ± 2.6	19.0 ^{a,b} ± 2.8	G* <i>C</i> = 0.03 G* <i>H</i> = 0.3 C* <i>H</i> = 0.003 G* <i>C</i> * <i>H</i> = 0.001
CD62LloCD44 ⁺ (% of CD3 ⁺ CD8 ⁺ CD4 ⁺ cells)	25.8 ± 0.6	25.0 ± 0.7	0.4	23.8 ± 0.6	27.1 ± 0.7	0.001	24.1 ± 0.6	26.7 ± 0.7	0.01	22.1 ^b ± 1.3	26.1 ^{a,b} ± 1.3	27.4 ^a ± 1.3	28.0 ^a ± 1.3	23.0 ^b ± 1.3	25.4 ^{a,b} ± 1.3	23.0 ^b ± 1.3	29.0 ^a ± 1.4	G* <i>C</i> = 0.2 G* <i>H</i> = 0.4 C* <i>H</i> = 0.9 G* <i>C</i> * <i>H</i> = 0.06
CD25 ⁺ FOXP3 ⁺ cells (% of CD3 ⁺ CD4 ⁺ CD8 ⁺ cells)	12.4 ± 0.4	8.2 ± 0.4	<0.001	10.3 ± 0.4	10.2 ± 0.4	0.7	10.6 ± 0.4	9.9 ± 0.4	0.01	12.0 ^b ± 0.4	14.4 ^a ± 0.4	12.0 ^b ± 0.4	11.1 ^b ± 0.4	8.3 ^{c,d} ± 0.4	7.8 ^d ± 0.4	9.1 ^c ± 0.4	7.6 ^c ± 0.4	G* <i>C</i> = 0.002 G* <i>H</i> = 0.001 C* <i>H</i> = 0.0001 G* <i>C</i> * <i>H</i> = 0.03
CD25 ⁺ cells (% of CD3 ⁺ CD4 ⁺ CD8 ⁺ cells)	14.5 ± 0.2	9.9 ± 0.2	<0.0001	12.3 ± 0.2	12.2 ± 0.2	0.6	12.5 ± 0.2	11.9 ± 0.2	0.04	14.3 ^b ± 0.4	16.2 ^a ± 0.4	14.3 ^b ± 0.4	13.3 ^b ± 0.4	9.9 ^{c,d} ± 0.4	9.8 ^{c,d} ± 0.4	10.7 ^c ± 0.4	9.3 ^d ± 0.4	G* <i>C</i> = 0.04 G* <i>H</i> = 0.01 C* <i>H</i> = 0.001 G* <i>C</i> * <i>H</i> = 0.2
CD25 ⁺ cells (% of CD3 ⁺ CD8 ⁺ CD4 ⁺ cells)	3.9 ± 0.1	2.2 ± 0.1	<0.0001	2.8 ± 0.1	3.2 ± 0.1	<0.0001	2.7 ± 0.1	3.3 ± 0.1	<0.0001	4.0 ^b ± 0.1	3.0 ^c ± 0.1	3.6 ^b ± 0.1	5.0 ^a ± 0.1	1.9 ^c ± 0.1	2.1 ^c ± 0.1	1.8 ^c ± 0.1	2.9 ^d ± 0.1	G* <i>C</i> = 0.01 G* <i>H</i> = 0.01 C* <i>H</i> = 0.001 G* <i>C</i> * <i>H</i> = 0.003
CD25 ⁺ cells (% of CD3 ⁺ cells)	11.1 ± 0.1	7.0 ± 0.1	<0.0001	9.1 ± 0.1	9.0 ± 0.1	0.8	9.0 ± 0.1	9.1 ± 0.1	0.7	11.2 ^a ± 0.2	11.2 ^a ± 0.2	11.0 ^a ± 0.2	11.0 ^a ± 0.2	6.8 ^b ± 0.2	6.9 ^b ± 0.2	7.4 ^b ± 0.2	7.1 ^b ± 0.2	G* <i>C</i> = 0.6 G* <i>H</i> = 0.7 C* <i>H</i> = 0.5 G* <i>C</i> * <i>H</i> = 0.7

Table 4.2: Effect of genotype, C3G (10 mg/kg/day), HCT (10 mg/kg/day), and their interactions on T-lymphocyte phenotypes. Values are LS means $\pm$ SEMs. Means in the “interaction” section of a row with different letters are significantly different from each other based on LS means adjusted for multiple comparisons using the False Discovery Rate, $P \leq 0.05$. Abbreviations: C3G, Cyanidin 3-*O*-glucoside; HCT, hydrochlorothiazide; WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats; C+H, Cyanidin 3-*O*-glucoside + hydrochlorothiazide; G*C, interaction between genotype and Cyanidin 3-*O*-glucoside; G*H, interaction between genotype and hydrochlorothiazide; C*H, interaction between Cyanidin 3-*O*-glucoside and hydrochlorothiazide; G*C*H, interaction among genotype, Cyanidin 3-*O*-glucoside and hydrochlorothiazide.

4.9 Discussion

The present study demonstrates that spleen weights corrected for body weight were not different between adult SHR and WKY rats. These findings are consistent with another study that showed spleen weights were not different between adult WKY rats and SHR despite splenic weights being markedly higher in young WKY rats (1-3 weeks).³⁴ However, the volume density of the white splenic pulp, which consists mainly of lymphocytes, was significantly lower in adult SHR compared to WKY rats despite no difference in spleen weight. These previous findings imply that immune cell numbers in SHR are depressed, which might explain the impaired immune response.³⁴ Nevertheless, the findings in the present study do not support the hypothesis that low T-cell numbers account for the immunodeficiency seen in SHR, as the proportion of T-cells was not lower in SHR.

Blastogenesis in response to ConA is known to depend on T-cell subsets. Previous studies have reported a defective proliferative response of splenocytes from SHR with ConA stimulation.¹³ We examined T-cell function in 20-week-old SHR and WKY rats using cytokine release in response to ConA challenge as an indicator of T-cell function. Our findings show that splenocytes of SHR produced lower concentrations of all cytokines measured compared to WKY rats, which supports lower T-cell function in SHR. Impaired cytokine production by splenocytes from SHR could be due to a lower proportion of helper T-cells or impairment in T-cell receptors/cytokine receptor-mediated signaling.³⁵ However, there was no effect of genotype on the proportion of helper T-cells, which points to impairment in T-cell receptor/cytokine receptor-mediated signaling as the likely cause.

In the present study we show, for the first time, that HCT inhibits TNF- α and IFN- γ release by splenocytes from SHR rats at a dose of 10 mg/kg/day, whereas it did not affect cytokine release in splenocytes from WKY rats. This difference in response to HCT treatment between WKY rats and SHR rats may be related to genetic differences between the two genotypes. The use of HCT in hypertensive patients has been implicated in different forms of immunological disorders such as cutaneous T-cell lymphomas and immunological-hypersensitivity-induced pericardial effusion.³⁶ However, a review of the literature failed to show a mechanistic explanation of the effects of HCT on ConA-stimulated cytokine release observed in this study.

Naïve helper T-cells are mature lymphocytes that unlike memory and activated T-cells have not encountered their cognate antigens. Upon activation naïve helper T-cells will predominantly produce IL-2, a principal mediator of T-cell proliferation and differentiation into activated effector cells.³⁵ Low IL-2 levels produced by splenocytes from SHR rats upon stimulation imply poor proliferation and differentiation of naïve helper T-cells into activated effector T-cells. This is supported by our findings of no difference in the proportion of naïve helper T-cells between control WKY rats and SHR rats, but a lower proportion of memory helper T-cells in control SHR compared to control WKY rats.

Activation of immune cells results in the initiation of an immune response during which cells undergo phenotypic changes and become activated. CD25 is the α chain for IL-2 receptors and is considered as one of the surface markers of activated T-cells.¹¹ Therefore, cells that express CD25 can proliferate in response to IL-2. We demonstrated that SHR rats have lower proportions of splenic T-cells that express CD25 compared with WKY rats with no effect of C3G or HCT. The lower proportion of activated T-cells along with lower concentrations of IL-2

produced in response to ConA stimulation suggest that T-cells from SHRs have lower proliferative response compared to WKY rats. Earlier studies have shown that splenocytes from SHRs have a depressed proliferative response to ConA and phytohemagglutinin.^{34, 38} Furthermore, the addition of IL-2 to splenocytes from SHRs in culture failed to improve the proliferative response of cells. Prolactin is a hormone that has been shown to upregulate IL-2 receptor expression.³⁹ Purcell et al have reported that the responsiveness of splenocytes from SHRs to IL-2 *ex vivo* can be improved by addition of prolactin to cell culture.³⁴ These findings suggest that impaired proliferative response of splenocytes from SHRs is the result of decreased expression of IL-2 receptors.³⁴

Stimulation of the sympathetic nervous system is a critical mediator in the development of hypertension.⁴⁰ Increased sympathetic innervation of the spleen in SHRs has been reported.⁴¹ Furthermore, beta-adrenergic receptor activation has been shown to decrease IL-2 receptor expression.⁴² The effects of sympathetic nervous system on the immune system are thought to be the result of increased cAMP levels in lymphocytes due to beta-adrenergic receptor activation.⁴³ Accordingly, it is likely that the depressed immune function in SHRs observed in this study is due, at least in part, to increased sympathetic innervation to the spleen which in turn decreases IL-2 receptor expression.

Previous studies have shown that Tregs prevent blood pressure elevation and vascular dysfunction induced by Ang-II.¹⁹ Adoptive transfer of regulatory T-cells ameliorated Ang-II-induced cardiac hypertrophy and fibrosis in mice independently of blood pressure levels. Furthermore, a decrease in the proportion of regulatory T-cells in spleens of stroke-prone SHRs (SHRSP) has also been hypothesized to play a role in the pathogenesis of hypertension and cardiac hypertrophy.²¹ The decline in the proportion of Tregs in SHRSP was attributed to enhanced

sympathetic innervation to the spleen as splenic sympathetic denervation in prehypertensive SHRSP ameliorated the decline in Tregs and hypertension.²¹ The present findings show that at 20 weeks of age, SHRs have a lower proportion of Tregs compared with WKY rats. Treatment with C3G or HCT in SHRs did not ameliorate the decline in the proportion of Tregs. Therefore, these findings indicate that our previously reported cardio-protective effects of C3G and HCT are not related to the proportion of Tregs. Therefore, it is still unclear based on our study whether there is a link between impaired immune function and the development of hypertension and end-organ damage in SHRs.

4.10 Conclusions

In conclusion, this study supports the previously reported findings of impaired T-cell function in SHRs. SHRs had low cytokine production in response to mitogenic challenge compared to WKY rats. While there was no effect of C3G on cytokine production in both WKY rats and SHRs, HCT treatment resulted in a further decrease in cytokine production in SHRs. This finding is of importance in patients who use HCT to manage hypertension and needs to be investigated in future studies. Low concentrations of IL-2 produced by splenocytes from SHRs upon mitogenic stimulation along with low proportion of activated T-cells in the spleen indicate that T-cells from SHRs have impaired proliferative response. Our findings also show that SHRs have a lower proportion of Tregs compared to WKY rats with no effect of C3G or HCT. Although cardioprotective effects of Tregs have been reported, our findings indicate that our previously reported cardioprotective effects of C3G and HCT in SHRs are independent of Tregs. It is still unclear whether/how impaired immune function in SHRs leads to hypertension and end-organ damage (**Figure 4.8**). These areas and the role of nutraceuticals in altering immune function should be further investigated in future studies.

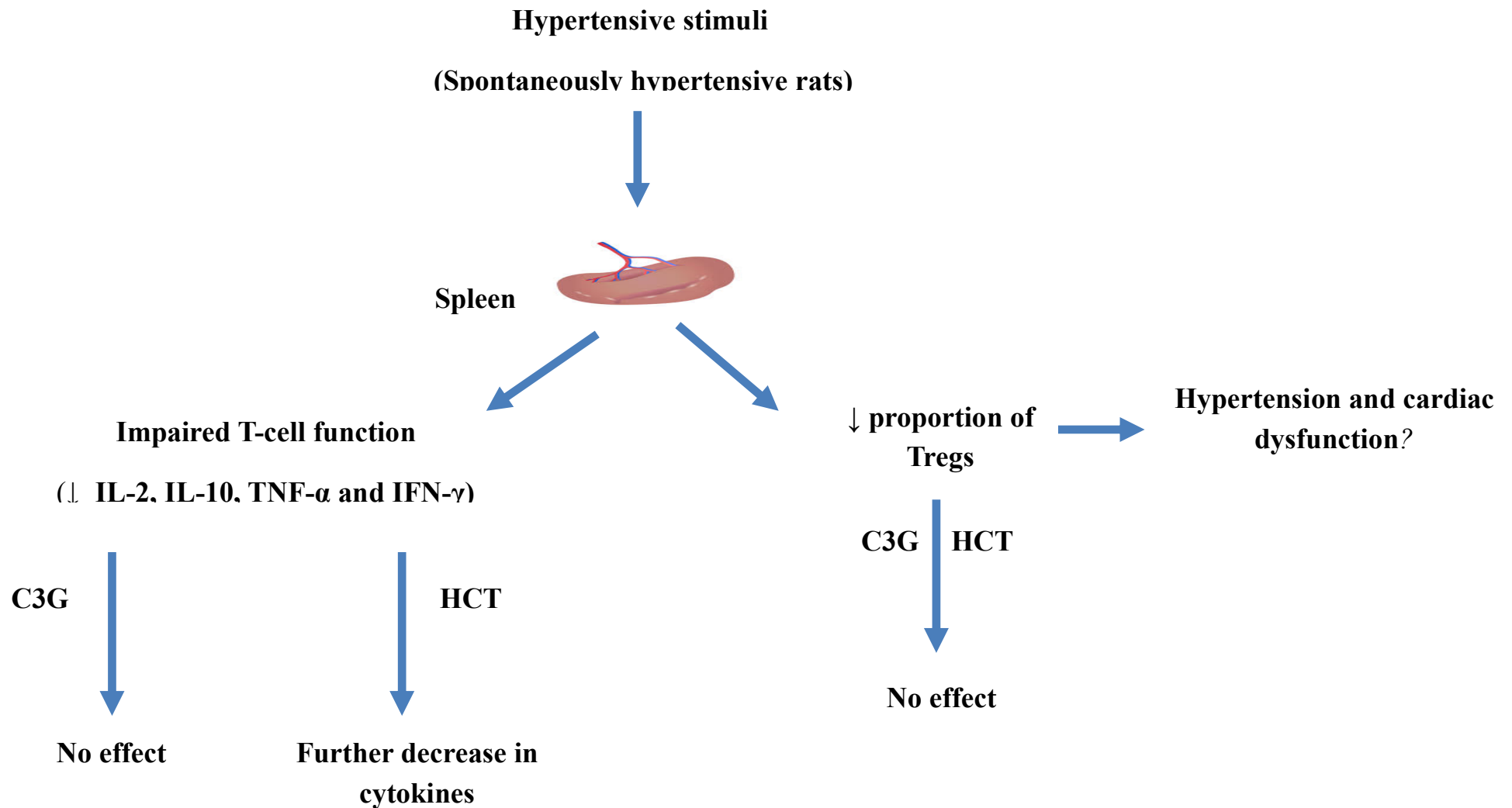


Figure 4.8: Effects of cyanidin 3-*O*-glucoside (C3G) and hydrochlorothiazide (HCT) on T-lymphocytes in spontaneously hypertensive rats.

4.10 Literature cited

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Chapter 5

General discussion, significance of the thesis, limitations and future directions

5.1 General discussion

Several *in vitro* and *in vivo* animal studies have shown that anthocyanins have beneficial effects on cardiovascular disease (CVD) risk markers.¹ Epidemiological studies as well as meta-analyses of randomized controlled trials (RCTs) have also demonstrated that a high consumption of anthocyanins in the form of anthocyanin-rich whole foods, purified anthocyanin mixtures, or anthocyanin-rich extracts can reduce the risk of CVD in individuals at high risk.²⁻⁵ To the best of our knowledge, none of the clinical trials that assessed the CVD-related effects of anthocyanins used interventions containing individual purified anthocyanins (e.g. purified cyanidin 3-*O*-glucoside (C3G)). One of the first objectives of this thesis was to systematically review RCTs assessing the effects of purified anthocyanin mixtures and anthocyanin-rich extracts on CVD risk factors. The findings of our systematic review add to the current scientific evidence regarding the cardiovascular effects of anthocyanins suggesting that purified anthocyanin mixtures (320 mg/day) have positive impacts on different markers of CVD, particularly in subjects with dyslipidemia; however, there is limited evidence to support the efficacy of purified anthocyanins in patients with hypertension (Chapter 1) (**Figure 5.1**). Anthocyanin interventions used in all the RCTs included in our systematic review contained a mixture of 17 different anthocyanins with C3G as one of the most abundant anthocyanins contained in these anthocyanin mixtures.

To address research gaps identified in our systematic review, we examined whether C3G is beneficial against hypertension and its related cardiac aberrations *in vitro* in adult rat cardiomyocytes and cardiac fibroblasts (CF) exposed to endothelin 1 (ET1), and *in vivo* alone or in combination with the diuretic hydrochlorothiazide (HCT) in spontaneously hypertensive rats (SHRs) (Chapter 3). The findings of the current study show that C3G can prevent ET1-induced cardiomyocyte death and hypertrophy and cardiac fibroblast activation. Treatment with C3G alone

or in combination with HCT was also effective in ameliorating the development of cardiac hypertrophy and diastolic dysfunction in SHR. However, significant blood pressure lowering effects were only observed with HCT treatment indicating that the cardioprotective effects of C3G were blood pressure-independent (**Figure 5.1**). HCT treatment caused a significant increase in plasma glucose levels in SHR. Nevertheless, SHR treated with a combination of C3G and HCT had lower plasma glucose levels compared with SHR treated with HCT alone, suggesting that C3G has antidiabetic effects that may have contributed to the mitigation of HCT-induced hyperglycemia. The levels of oxidative stress and inflammatory markers in the heart tissue, as well as the activity of the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA2a) in the heart tissue were not affected in 20-week-old SHR indicating that cardiac structural and functional aberrations are not attributed to these contributors at this time point in SHR. Therefore, more mechanistic studies are needed to explain the mechanisms underlying the cardioprotective effects of C3G.

To examine whether the observed effects of C3G and HCT are related to immune mechanisms, we next focused on characterizing immune system dysfunction in SHR compared with WKY rats and investigated whether C3G alone or in combination with HCT can affect T-cell function in SHR (**Figure 5.1**). Our results demonstrate that the function of T-cells from SHR is depressed compared to WKY rats as evidenced by lower concentrations of cytokines released in response to ConA stimulation. Lower levels of IL-2 produced from splenocytes from SHR in response to mitogenic stimulation along with lower proportion of activated T-cells in the spleen indicate depressed proliferation of T-cells from SHR. While there was no effect of C3G treatment on cytokine release in WKY rats and SHR, HCT treatment inhibited the release of cytokines in SHR suggesting that HCT may have adverse effects on immune system function. Concomitant to this, we found that SHR have lower proportion of Tregs compared to WKY rats with no effect of

different treatments. Although Tregs have been reported to play a crucial role in preventing hypertension-induced cardiac hypertrophy, our findings suggest that the protective effects of C3G and HCT on cardiac structure and function are independent on Tregs (Chapter 4).

In summary, despite the reported beneficial effects of purified anthocyanin mixtures in subjects at high risk for CVD, there is limited evidence surrounding the potential of purified anthocyanins in subjects with hypertension. Our findings show that C3G has the potential to mitigate cardiac abnormalities in SHR_s independently of any effects on blood pressure. The observed cardioprotective effects of C3G and HCT are not mediated through Tregs. Future studies are needed to explore the potential of C3G in subjects with established hypertension and to identify the mechanisms underlying its cardioprotective effects observed in this study.

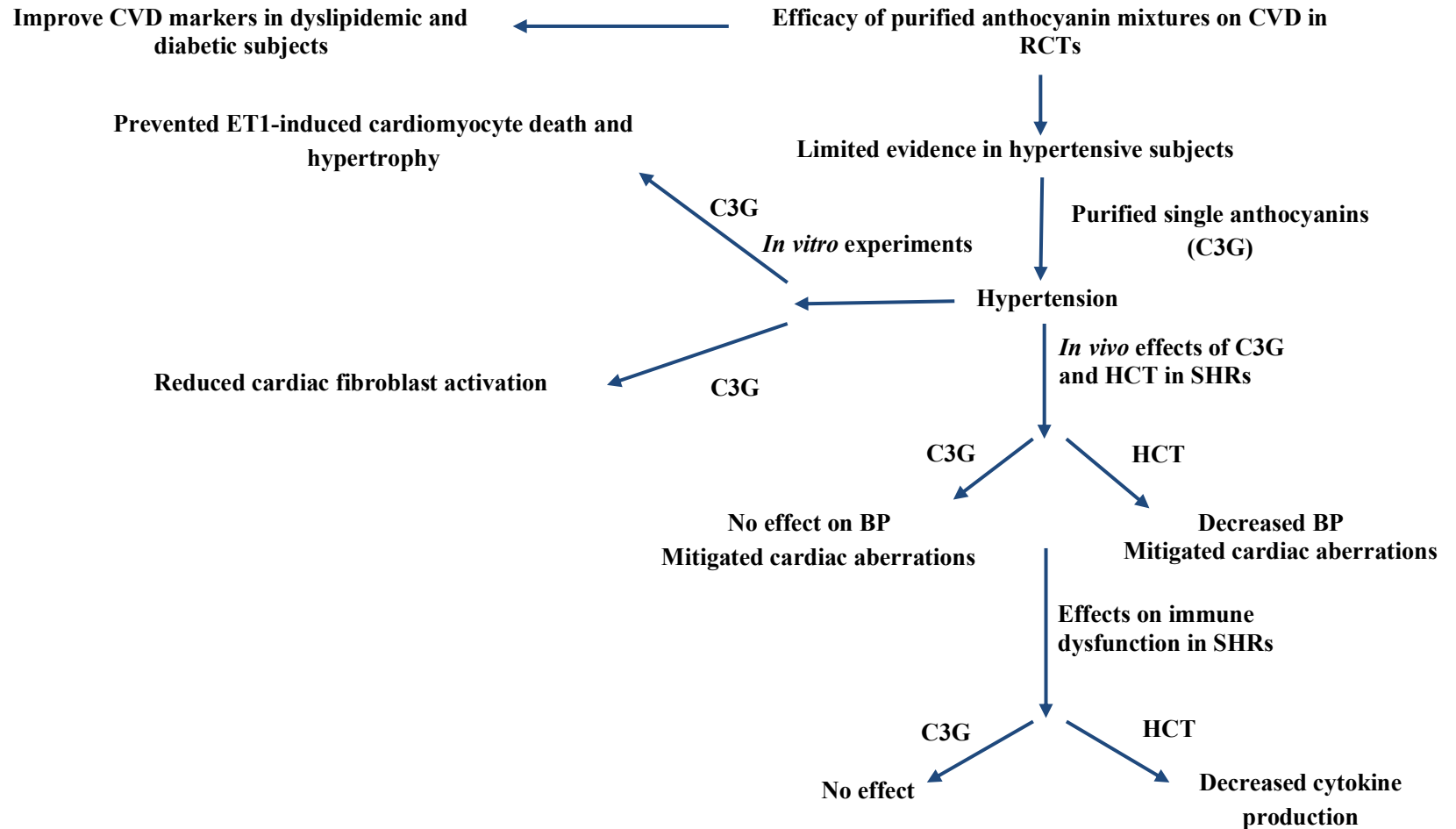


Figure 5.1: Summary of systematic review, *in vitro* and *in vivo* findings. BP, blood pressure; C3G, Cyanidin 3-*O*-glucoside; CVD, cardiovascular disease; ET1, endothelin 1; HCT, hydrochlorothiazide; RCTs, randomized controlled trials; SHRs, spontaneously hypertensive rats.

5.2 Significance of the thesis

The studies outlined in this thesis present several novel findings and add to the contemporary knowledge in the area of CVD and the potential of anthocyanins as CVD preventative nutraceuticals. This is the first study to compare the antihypertensive and the cardioprotective effects of a standard blood pressure lowering medication to those of a purified anthocyanin, helping us to better characterize the efficacy and potency of C3G in hypertensive heart disease. Furthermore, comparing the effects of C3G to those of a conventional antihypertensive medication is essential to understand potential synergistic or antagonistic effects when used in combination. Despite the lack of antihypertensive effects with C3G in SHR, its effectiveness in preventing HCT-induced hyperglycemia and ameliorating cardiac hypertrophy and diastolic dysfunction will hopefully stimulate further research to assess the potential of C3G in other animal models of diastolic heart failure.

5.3 Limitations

The studies outlined in this thesis include a number of limitations. First, using anthocyanins in a mixture may significantly affect their metabolism and biological activities. All RCTs included in our systematic review used interventions containing purified anthocyanin mixtures, whereas in our experimental approach we used a single purified anthocyanin (C3G). Therefore, it is hard to accurately correlate the findings reported in RCTs included in our systematic review to those obtained from our *in vivo* study. Second, the *in vivo* study did not test different doses of C3G to determine an optimal effective dose that is effective in ameliorating hypertension as well as hypertensive heart disease. Third, even though experiments described in this thesis confirmed that SHR have depressed immune system, it is still unclear whether the immune dysfunction in SHR is a cause or a consequence of hypertension and its related cardiac aberrations.

5.4 Future directions

Since the cardioprotective effects of C3G in 20-week-old SHRs were independent of blood pressure, and other contributors to cardiac dysfunction such as inflammation and SERCA2a activity were not involved at this stage of hypertension, other studies are required to understand the mechanisms behind the cardioprotective effects of C3G. For example, it would be valuable to investigate whether the protective effect of C3G on diastolic heart function is attributed to its effects on other calcium handling proteins such as sodium-calcium exchanger and troponin I. It is also important to investigate whether C3G can reach and accumulate in the heart tissue in its native form or it needs to be metabolized to exert its observed cardioprotective effects. To date, no study has examined whether C3G is effective in ameliorating hypertension and hypertension-related cardiac complications and immune system aberrations in humans. More testing is needed to know whether the findings of our animal trial can be translated to humans.

5.5 Literature cited

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